Development of gas chromatography/mass spectrometry analysis of urinary acylcarnitines: application to metabolism studies

Thesis

How to cite:

For guidance on citations see FAQs.

© 1991 The Author

Version: Version of Record
Stephen Lowes B.Sc.

DEVELOPMENT OF GAS CHROMATOGRAPHY/MASS SPECTROMETRY
ANALYSIS OF URINARY ACYLCARNITINES: APPLICATION TO
METABOLISM STUDIES IN HUMANS

Submitted for the Award of
Doctor of Philosophy

Discipline of Analytical Chemistry

July 1991

Author's number: M7033914
Date of submission: 22nd July 1991
Date of award: 14th October 1991
# CONTENTS

<table>
<thead>
<tr>
<th></th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>3</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>5</td>
</tr>
</tbody>
</table>

## CHAPTER 1: INTRODUCTION

1.1 THE PHARMACOLOGY OF CARNITINE 10

1.1.1 History and Metabolic Function 10

1.1.2 Impaired Fatty Acid Oxidation 19

1.2 DIAGNOSIS OF INBORN ERRORS OF FATTY ACID OXIDATION 24

1.3 NEW APPROACHES TO THE ANALYSIS OF PHYSIOLOGICAL ACYL-CARNITINES 37

1.3.1 Derivatization for GC and GC/MS 37

## CHAPTER 2: DEVELOPMENT OF A METHOD BASED ON GAS CHROMATOGRAPHY: RESULTS AND DISCUSSION 42

2.1 INTRODUCTION 43

2.2 SYNTHESIS OF ACYL-CARNITINES 45

2.3 SYNTHESIS OF STANDARD \( \gamma \)-BUTYROLACTONE COMPOUNDS (3) 54

2.4 DERIVATIZATION CONDITIONS 60
2.4.1 Effect of Heat on Octanoylcarnitine Hydrochloride

2.4.2 Development of a Controlled Derivatization

2.4.2(a) Reaction in N,N-Dimethylformamide

2.4.2(b) Reactions in Acetonitrile

2.4.3 Application of Derivatization to Physiological Quantities of Standard Acylcarnitines

2.4.4 On-Column Derivatization

2.5 HANDLING THE BIOLOGICAL MATRIX

2.5.1 Why Extract?

2.5.2 Solvent Extraction

2.5.3 Ion-Exchange Purification

2.5.4 Use of Bond Elut CERTIFY Columns

2.6 GC AND GC/MS: CONDITIONS AND TECHNIQUES APPLIED TO ANALYSIS OF ACYLCARNITINE DERIVATIVES

2.6.1 Vaporizing Injection GC

2.6.1.1 Split Injection

2.6.1.2 Splitless Injection

2.6.2 Cold On-column Injection GC

2.6.3 GC/MS Investigations
CHAPTER 3: APPLICATION OF THE ACYL-CARNITINE DERIVATIZATION:

3.1 INTRODUCTION

3.2 INVESTIGATION OF DEFECTIVE LIPID AND BRANCHED-CHAIN AMINO ACID METABOLISM

3.2.1 Medium-chain Acyl-CoA Dehydrogenase Deficiency (MCADD)

3.2.2 Propionic Acidemia

3.2.3 Isovaleric Acidemia

3.2.4 Other Applications and General Observations

3.3 INVESTIGATIONS OF THE METABOLISM OF SOME EXOGENOUS COMPOUNDS

3.3.1 Acylcarnitine Determination of Urine Samples from Patients Administered Phenylpropionic Acid (PPA)

3.3.2 Acylcarnitine Determination of Urine Samples from Patients Administered Valproic Acid

CHAPTER 4: CAPILLARY ZONE ELECTROPHORESIS AND ITS APPLICATION TO ACYL-CARNITINE ANALYSIS

4.1 INTRODUCTION TO CZE TECHNIQUES

4.2 CZE THEORY AND METHODOLOGY

4.2.1 Sample Injection
I confirm that I am willing that my thesis be made available to readers and maybe photocopied, subject to the discretion of the Librarian.
ABSTRACT

The metabolism of fatty acids in humans is recognized as an important source of energy. It is especially vital to newborn infants when metabolic pathways are often stressed as the early stages of life demand fundamental changes and developments of body processes. Defective fatty acid oxidation pathways can rapidly lead to a life-threatening situation. It has been suggested that such disorders may be responsible for a proportion of "cot death" or sudden infant death syndrome (SIDS) cases.

Recently, it has been found that the levels of acylcarnitines in body fluids and tissue are potential indicators of fatty acid metabolism status. This is due to carnitine esters being produced and excreted in an attempt to alleviate the toxic accumulation of incompletely metabolized acyl units in the mitochondrion. However, clinical studies have been limited by a lack of convenient, unambiguous, sensitive and affordable analytical techniques for the measurement of physiological acylcarnitines. The nature of carnitine and its acyl esters presents analysis problems. They are involatile, zwitterionic compounds which makes them unsuitable for direct gas chromatography (GC) and combined gas chromatography/mass spectrometry (GC/MS). The latter of these is the favoured technique for normal urinary organic acid assays and is ideal in terms of selectivity and sensitivity. The work reported here details the development of a simple unambiguous and novel derivatization procedure in which acylcarnitines are cyclized to give volatile lactones, amenable to GC and GC/MS. The technique was subsequently applied to acylcarnitines extracted from urine. As such, the method has been used to identify acylcarnitine metabolites in the urine of children with diseases of fatty acid oxidation and amino acid catabolism. Investigations of the metabolism of exogenous 3-phenylpropionic and valproic acids was also conducted.
The preliminary results from the application of capillary zone electrophoresis methods to acylcarnitine analysis are also reported.
ACKNOWLEDGEMENTS

This work was supported by the Analytical Chemistry Trust of the Royal Society of Chemistry with an SAC Studentship. Grateful acknowledgement is also expressed to the Foundation for the Study of Infant Deaths for research grants for the purchase of gas chromatograph and capillary zone electrophoresis equipment (Project 110). An equipment grant from the British Mass Spectrometry Society (BMSS) allowed the purchase of a GC on-column injector. Thanks are also expressed to BMSS for the three travel grants which enabled me to attend international mass spectrometry conferences during the course of this project.

I am indebted to Dr Malcolm E Rose (The Open University) for the valuable supervision and guidance he has consistently provided throughout my graduate and postgraduate studies. I would also like to thank Dr Rodney J Pollitt (Neonatal Screening Laboratory, Sheffield Children’s Hospital) for useful discussion and the provision of clinical samples. Similarly, gratitude is expressed to Dr Graham A Mills (Southampton General Hospital now Portsmouth Polytechnic) and Dr Priscille Divry (Hospital de Lyon, France) for clinical samples and valuable discussions and to Dr Alan T Hewson (Sheffield City Polytechnic) for useful discussions. Finnigan MAT and Hewlett Packard are also thanked for the use of GC/MS instrumentation.

I would like to acknowledge the following members of staff of the Open University for their help, Jenny Burrage for taking my original manuscript and converting it into legible form, Gordon Howell for recording $^1$H NMR and $^{13}$C spectra, Graham Jeffs, Alan Leslie, Jim Gibbs, Pravin Patel, Brandon Cook and Judy Challis for helping with the running of the laboratories and maintenance of instruments, and Dr Robert J Morrow for some preliminary results referenced in this thesis. Thank you all.
My final acknowledgement goes to my parents whose unwavering support and encouragement over my educational years has been so vital. I thank them deeply for the opportunities they have helped open for me.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcCoA</td>
<td>Acetylcoenzyme A</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>Carnitine acyltransferase</td>
</tr>
<tr>
<td>CFFAB</td>
<td>Continuous flow fast atom bombardment</td>
</tr>
<tr>
<td>CI</td>
<td>Chemical ionization</td>
</tr>
<tr>
<td>CIMS</td>
<td>Chemical ionization mass spectrometry</td>
</tr>
<tr>
<td>CNL</td>
<td>Constant neutral loss</td>
</tr>
<tr>
<td>CPT</td>
<td>Carnitine palmitoyl transferase</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CZE</td>
<td>Capillary zone electrophoresis</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNS</td>
<td>5-Dimethylamino-1-naphthalenesulfonic acid</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>EI</td>
<td>Electron ionization</td>
</tr>
<tr>
<td>EIMS</td>
<td>Electron ionization mass spectrometry</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>ETF</td>
<td>Electron transfer flavoprotein</td>
</tr>
<tr>
<td>ETF-DH</td>
<td>Electron transfer flavoprotein dehydrogenase</td>
</tr>
<tr>
<td>FAB</td>
<td>Fast atom bombardment</td>
</tr>
<tr>
<td>FABMS</td>
<td>Fast atom bombardment mass spectrometry</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detector</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-transform infrared (Spectroscopy)</td>
</tr>
<tr>
<td>GAII</td>
<td>Glutaric aciduria type II</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared (spectroscopy)</td>
</tr>
<tr>
<td>α-KG</td>
<td>α-Ketoglutarate</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LCAD</td>
<td>Long-chain acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>LCADD</td>
<td>Long-chain acyl-CoA dehydrogenase deficiency</td>
</tr>
<tr>
<td>MADD</td>
<td>Multiple acylcoenzyme A dehydrogenase deficiency</td>
</tr>
<tr>
<td>MCAD</td>
<td>Medium-chain acylcoenzyme A dehydrogenase</td>
</tr>
<tr>
<td>MCADD</td>
<td>Medium-chain acyl-CoA dehydrogenase deficiency</td>
</tr>
<tr>
<td>MECC</td>
<td>Micellar electrokinetic capillary chromatography</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PKU</td>
<td>Phenylketonuria</td>
</tr>
<tr>
<td>PPA</td>
<td>3-Phenypropionic acid</td>
</tr>
<tr>
<td>RR-MAD</td>
<td>Riboflavin responsive multiple acyl-CoA dehydrogenation deficiency</td>
</tr>
<tr>
<td>Rf</td>
<td>Retardation factor</td>
</tr>
<tr>
<td>SCAD</td>
<td>Short-chain acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>SCADD</td>
<td>Short-chain acyl-CoA dehydrogenase deficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SIDS</td>
<td>Sudden infant death syndrome</td>
</tr>
<tr>
<td>SIM</td>
<td>Selected ion monitoring</td>
</tr>
<tr>
<td>SUDS</td>
<td>Sudden unexplained death syndrome</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion current</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>TMA</td>
<td>Trimethylammonium acid</td>
</tr>
<tr>
<td>TML</td>
<td>Trimethyllysine</td>
</tr>
</tbody>
</table>
TMS : Trimethylsilyl
TSP : Thermospray
UV  : Ultraviolet
WCOT: Wall-coated open tubular
CHAPTER 1

INTRODUCTION
\[
\begin{align*}
& \text{OH} \\
& + (\text{CH}_3)_3\text{NCH}_2\text{CHCH}_2\text{COO}^- \\
& 1
\end{align*}
\]

\[
\begin{align*}
& \text{OCR} \\
& + (\text{CH}_3)_3\text{NCH}_2\text{CHCH}_2\text{COO}^- \\
& 2
\end{align*}
\]
1.1 THE PHARMACOLOGY OF CARNITINE

1.1.1 History and Metabolic Function

In 1905, Gulewitsh and Krimberg assigned the empirical formula $C_7H_{15}NO_3$ to a compound isolated from meat extract. Carnitine (L-3-hydroxy-4-N-trimethylaminobutanoic acid; (1)) had been discovered, but it was not until 1927 that the correct structural formulae was proven. For almost forty years after its discovery, carnitine was regarded solely as a constituent of vertebrate muscle. It required the identification of carnitine in insects to precipitate an interest which would lead to the ongoing interest in the pharmacological role of this compound throughout nature, and in particular, in man.

Carter et al., in 1951, identified carnitine as the dietary factor named vitamin B₇, which was regarded as essential for the growth of the mealworm, Tenebrio molitor. The requirement for carnitine by the mealworms to sustain growth was developed into a sensitive assay, "The Tenebrio Test." The test was applied to an extensive range of biological material. It is interesting to note that this was the first reported method used to determine carnitine levels in human urine and blood, an assay closely associated with the objectives of the project reported here.

By 1954, hundreds of biological samples had been subjected to the Tenebrio Test. The results indicated that carnitine was universally distributed throughout nature with few exceptions. The quantities determined varied considerably between samples, with some (notably mammalian muscle tissue) presenting relatively large amounts. Subsequent analyses have found carnitine concentrations in mammalian tissue to vary between 0.1 and a few millimoles per litre. The highest concentrations are found in heart and skeletal muscle. When carnitine is found in plants, lower concentrations around the micromole per litre level are encountered.

In 1957, Fraenkel and Friedman reasoned that a substance of apparently universal occurrence and of assumed important functional significance to the organisms
Scheme 1. Biosynthesis of carnitine
which contain it, would have been described as a vitamin earlier if it were not synthesized by those organisms. Therefore, there began an interest in elucidating the endogenous biosynthesis pathway in mammals. Early investigations\(^9\) demonstrated that the quaternary ammonium methyl groups were derived from methionine, but precursors for the four-carbon chain and the nitrogen moiety remained unknown. Conversion of \(\gamma\)-butyrobetaine to carnitine was reported\(^10\) but it was not until the discovery that lysine served as a precursor\(^11\) that an acceptable biosynthetic pathway could be postulated and then experimentally confirmed.\(^12\) In animals, the amino acid becomes available as a peptide residue in certain lysine-rich proteins. It is methylated by S-adenosylmethionine and a protein methylase before proteolysis liberates \(e\)-N-trimethyllysine (TML), Scheme 1. Through a further three enzyme-dependent steps, TML is oxidatively converted to \(\gamma\)-butyrobetaine via \(\beta\)-hydroxy-\(e\)-N-trimethyllysine and \(\gamma\)-butyrobetaine aldehyde. The final hydroxylation step to carnitine, requiring cytosolic hydroxylase, is restricted to liver, brain and human kidney tissue.\(^13\) In humans the liver is the principle site for carnitine synthesis. Tissues which lack the hydroxylase enzyme can export the \(\gamma\)-butyrobetaine precursor, via blood circulation, to the hydroxylating tissue, but rely on the return of newly synthesized product or dietary intake for their carnitine supply. In nutritional terms, there is also a role for four additional micronutrients, vitamin C, niacin, vitamin B-6 and iron, as cofactors required by the various enzymes involved in the biosynthesis. Thus, deficiencies of these nutrients along with lysine and methionine are all known to result in reduced plasma and/or tissue levels of carnitine.\(^14,15\)

In 1955, Fritz\(^16\) began to establish the primary role of carnitine in man. He demonstrated that adding carnitine to liver slices and homogenates facilitated the oxidation of long-chain fatty acids. In the same year, Friedman and Fraenkel\(^17\) reported the reversible enzymatic acetylation of carnitine by acetyl coenzyme-A (AcCoA) to form the acylcarnitine, acetylcarnitine, \((2, R = CH_3)\) as given in
Scheme 2. Enzymic acetylation of carnitine
Scheme 2. After showing that isolated rat mitochondria from a variety of tissues would catalyze the same reversible reaction (Scheme 2), Bremer hypothesized that because the inner mitochondrial membrane is impermeable to coenzyme-A (CoA) and AcCoA, carnitine transports acetyl groups (as acetylcarnitine) across the membrane. This requires the intervention of an acetylcarnitine-CoA-acetyltransferase enzyme. Bremer also correctly postulated that because added carnitine stimulates palmitate (hexadecanoate) oxidation, carnitine also transports other acyl groups. The work of Fritz on long-chain fatty acid oxidation supported Bremer's theory. That carnitine promotes long-chain fatty acid oxidation by translocating activated long-chain fatty acids into the mitochondrial matrix (the site of β-oxidation), was accepted almost immediately. However, in the case of acetyl and other short-chain acid metabolism, the formation of carnitine esters (2) is now regarded of more relevance in removing acyl groups from the mitochondrial matrix.

Oxidation of fatty acids is an essential energy supply for the cell, especially when glucose availability is low. Mitochondrial β-oxidation is the only carnitine-dependent pathway for the oxidation of long-chain fatty acids, and it is the main metabolic route for the oxidation of all fatty acids. Extramitochondrial pathways are available for oxidation of fatty acids and consist of microsomal ω- and ω-1-oxidation and peroxisomal β-oxidation. Peroxisomal β-oxidation is important for very long-chain fatty acids which are poor substrates for the mitochondrial pathway and may represent 35% of total palmitic acid oxidation. (Whilst peroxisomal β-oxidation does not require carnitine to transport fatty acids into the organelle, evidence does suggest that carnitine is involved in removal of chain shortened products out of peroxisomes.) Microsomal ω- and ω-1-oxidation, under normal conditions, will only metabolize 4 to 5% of the fatty acids. As the main metabolic route for fatty acid oxidation, mitochondrial β-oxidation and its reliance on the carnitine transport shuttle system has attracted most attention over the past 25 years.
Free fatty acids (FFA's) in plasma enter the cell by a diffusion process after binding to a lipoprotein receptor on the cell membrane. Once in the cell, the FFA's are activated by combination with CoA to form highly polar thiol esters, soluble in the aqueous phase of the cell. The fatty acyl group is then subject to the carnitine transport shuttle system, (Figure 1). The CoA esters pass through the outer mitochondrial membrane to the inter-membrane space. Here they are converted to fatty acylcarnitines, 2, facilitated by the enzyme, carnitine acyl transferase I (CAT I). Three separate CAT I enzymes exist with chain length specificities which overlap. Recent evidence suggests that at least one of the CAT I enzymes resides in the outer mitochondrial membrane, as in Figure 1, rather than the conventional assumption that all CATs are enzymes of the inner membrane. The carnitine esters formed are transported through the inner mitochondrial membrane, a process catalyzed by the trans-membrane protein, acylcarnitine translocase. Once within the mitochondrial matrix, the acyl groups are re-equilibrated with CoA by carnitine acyltransferase II (CAT II), situated on the matrix side of the inner mitochondrial membrane. Analogous to the CAT I enzymes, three chain length specific CAT II enzymes are available. The free carnitine formed in the mitochondria may pass back to the cytosol to repeat its transport role.

In both CAT I and CAT II cases, the identified, chain length specific, enzymes are: carnitine acetyl transferase, utilizing free fatty acyl groups of two or three carbon atoms in length; carnitine octanoyl transferase, utilizing free fatty acyl groups of six to ten carbon atoms in length; and carnitine palmitoyl transferase, utilizing free fatty acyl groups of fourteen to sixteen carbon atoms in length. Equimolar amounts of the respective CAT I and CAT II enzymes are present in the mitochondrial membrane.

Once present in the mitochondrion, the acyl-CoA species may be progressively catabolized to acetyl-CoA by the β-oxidation process (Scheme 3) generating cellular ATP directly through the citric acid cycle or through ketogenesis. Each cycle
Figure 1. Carnitine transport shuttle system
involves four enzymic reactions, the first stage being a dehydrogenation process. Three different fatty acyl-CoA dehydrogenase enzymes have been identified with different chain length specificities and overlapping activities: short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD). The dehydrogenase process involves an enzymatic complex consisting of the relevant acyl-CoA dehydrogenase and two electron carriers, electron transfer flavoprotein (ETF) and electron transfer flavoprotein dehydrogenase (ETF-DH), all having flavin adenine dinucleotide (FAD) as coenzymes, as shown in Scheme 3.

The transporting role of carnitine described above is the major function of this compound in humans. The naturally occurring isomer is the L- or (-) isomer, with an absolute configuration found to be R(-)-carnitine. In a healthy, 70 kg, human adult, the total body pool of carnitine is approximately 100 mmol. Muscle, incapable of complete carnitine synthesis, accounts for 98%, with only 1.5% of the carnitine in the liver and kidneys. The concentration in muscle is dependent on blood transport from synthesizing tissue such as the liver and kidneys and the absorption of exogenous dietary carnitine. Carnitine concentrations in skeletal and cardiac muscle are reported to be twenty to forty times higher than in plasma. This concentration gradient is unfavourable for transport from blood to muscle tissue and necessitates a special transport mechanism thought to be based on an exchange of γ-butyrobetaine and/or acylcarnitines for carnitine.

The carnitine dietary requirements of a healthy adult are small. Strict vegetarian diets may contain less than 10% as much carnitine as a typical omnivorous diet in the developed nations. Nevertheless, studies of healthy well-nourished individuals in the United States eating vegetarian diets for prolonged periods, found they had normal plasma carnitine concentrations. The levels were similar to those of individuals consuming typical omnivorous diets. It is generally assumed that healthy vegetarians consuming diets with adequate lysine, methionine and
Scheme 3. Mitochondrial β-oxidation:
DH = dehydrogenase, ETF = electron transfer flavoprotein,
FAD = flavin adenine dinucleotide,
NAD = nicotinamide-adenine dinucleotide
micronutrients, required for carnitine biosynthesis, will maintain a normal carnitine status.

Carnitine is not metabolized in humans although the degradation of the unnatural \( S(\tau) \)-carnitine enantiomer to trimethylaminoacetone has been observed in rats and mice.\(^{41}\) The only known route for removal of carnitine from the body is urinary excretion. The human kidney filters carnitine at a similar rate to creatinine. Renal clearance parallels, to an extent, the plasma concentrations of carnitine. A fall in the plasma concentration will usually be reflected in a fall in urinary carnitine. Over 90% of the filtered carnitine is reabsorbed by the renal tubules,\(^{42}\) a similar degree of conservation as that for the circulating amino acids.

A comprehensive review of the biochemical aspects of carnitine and the carnitine acyltransferase enzymes has been presented by Bremer.\(^{27}\)

1.1.2 Impaired Fatty Acid Oxidation

The consequence of defective fatty acid metabolism can be dramatic, especially when the cell is dependent on this energy source during periods of low glucose availability. These consequences initially affect the energy supply to vital tissue by reducing ketone body formation (hepatic hypoketogenesis). The diminished formation of acetyl-CoA will also impair glucogenesis, explaining the observation of severe hypoglycaemia in affected patients during crisis conditions. Onset of the condition is usually attributed to low carbohydrate supply resulting from incorrect diet, prolonged fasting, increased caloric requirements or vomiting. An increase in urinary dicarboxylic acid excretions (due to elevated microsomal \( \omega \)-oxidation) has led to the term 'dicarboxylic aciduria'. These non-ketotic episodes of hypoglycaemia are often accompanied by excretion of \( \alpha \)-acyl-glycine conjugates.

Seven inborn errors of fatty acid oxidation have been described to date:

1. carnitine deficiency;
CAT deficiency, particularly relevant is carnitine palmitoyl transferase (CPT), required for transport of the long-chain fatty acids into the mitochondrion;

long-chain acyl-CoA dehydrogenase deficiency (LCADD);

medium-chain acyl-CoA dehydrogenase deficiency (MCADD);

short-chain acyl-CoA dehydrogenase deficiency (SCADD);

riboflavin responsive defects of β-oxidation or riboflavin responsive multiple acyl-CoA dehydrogenation deficiency (RR-MADD);

multiple acyl-CoA dehydrogenation deficiency (MADD) or glutaric aciduria type II (GA II).

Figure 2 summarizes these disorders.

The primary role of carnitine, transport of long-chain fatty acids, is of direct relevance to carnitine deficiency disorders (i.e. Figure 2 [1]). However, over the past decade, additional functions of carnitine in humans have been identified. They are important in the mechanism of some of the other defective fatty acid oxidation diseases. These additional roles also rely on the ability to form acylcarnitines (2) by ester bond formation through the hydroxy function of carnitine.

It is now firmly established that carnitine is capable of removing incompletely metabolized acyl groups from the mitochondrion. The process is essentially the reverse of the transport of long-chain acyl groups into the mitochondrion but is also applicable to medium- and short-chain metabolites. A transfer of the CoA-bound acyl group to carnitine will result in an acylcarnitine, capable of passing out of the cell for urinary excretion or redistribution into other tissues for utilization. This is an important mitochondrial detoxification process in disorders (Figure 2, [3] to [7]) which present a metabolic block to complete catabolism of the fatty acyl group,
Figure 2. Impaired fatty acid oxidation disorders (simplified scheme of localization of defects); numbers in brackets refer to those in text.
because incompletely metabolized acyl-CoA compounds are potentially toxic to the cell.

The formation of acylcarnitines also serves as a mechanism to maintain a balance of free to esterified CoA, believed to be important for cell function. A more rapid renal clearance of acylcarnitines than of free carnitine supports the protective role of carnitine against metabolic acidosis. In states of elevated mitochondrial acyl-CoA concentrations, carnitine levels may be depleted to an extent that the ability to transport long-chain fatty acids across the inner mitochondrial membrane is reduced. This represents a life threatening situation whereby energy production is curtailed. Such carnitine depletion is not restricted to fatty acid catabolism. The metabolism of branched-chain amino acids involves a mitochondrial process and appropriate disorders may utilize carnitine in its detoxification role. Therefore, the analysis of urinary and plasma acylcarnitines constitutes a potentially excellent biochemical indication of many metabolic disorders.

Impaired fatty acid oxidation and carnitine deficiency is likely to have consequences early in life. In the newborn, a life-dependent switch from carbohydrate to fatty acid oxidation for energy production takes place. The foetus and neonate are unable to biosynthesize carnitine due to low activity of cytosolic hydroxylase. Activity of this enzyme, in infants of less than three months of age, is under 12% of adult activity. In utero, foetal carnitine levels are dependent on maternal carnitine status and placental transfer. Neonatal carnitine levels are dependent on exogenous dietary supplies. Studies have been conducted into breast milk carnitine content and its variation over the postpartum period. Whilst it is interesting to note that the carnitine content of human breast milk increases over the first two to three days of suckling, the results are presently inconclusive since carnitine levels required for optimal fatty acid oxidation remain unknown. The clinical symptoms of carnitine insufficiency are recognized, however.
Separate carnitine deficiencies may be classified as follows:

a) **Carnitine deficient myopathy.** Characterized by extreme muscle weakness, exercise intolerance and increased lipid storage in skeletal muscle.\(^5\) The defect may be due to decreased synthesis of \(\gamma\)-butyrobetaine in the muscle or defective uptake mechanism from the blood supply.

b) **Systemic carnitine deficiency.** This syndrome is characterized by recurrent hepatic encephalopathy, lipid storage in liver and muscles and low plasma and tissue carnitine levels. Poor reabsorption by the renal tubules, resulting in a carnitine leak to the urine, seems to be the main pathogenic defect.

c) **Secondary carnitine deficiency.** This deficiency is invariably associated with genetic defects of the mitochondrial \(\beta\)-oxidation. The condition arises from excessive esterification with overproduced or underutilized acyl groups and subsequent urinary excretion. Such genetic disorders have been associated with Reye's syndrome and sudden infant death syndrome (SIDS). Medium-chain acyl-CoA dehydrogenase deficiency (MCADD) may be a contributive factor in up to 10% of SIDS cases\(^5\) (although current views suggest a lower percentage). Administration of L-carnitine and/or fasting, results in increased excretion of urinary acylcarnitines which are disease specific. These disorders have the potential for rapidly reducing carnitine concentrations essential for mitochondrial \(\beta\)-oxidation, hence the connection with cases of sudden unexpected deaths.

The effects of carnitine deficiency may be reversible with exogenous L-carnitine administration. There are many reported cases to substantiate the supplementation of carnitine, however, the degree of improvement is very variable. Further
investigations into the causes of carnitine deficiencies and the diagnosis of \( \beta \)-oxidation defects is likely to lead to advances in therapeutic treatment. Due to the metabolic role of acylcarnitines, the analysis of these compounds in biological samples will be important to the investigations. Unfortunately, none of the analytical techniques reported to date are ideal for clinical application. There remains an urgent need for a simple, precise and unambiguous method of analysing urine (and other biological fluids) for acylcarnitines. It is envisaged that an ideal method would be employed by a number of clinical screening laboratories. Therefore, the development of a routine procedure has to account for the equipment, workload, expertise and financial status of such a laboratory.

1.2 DIAGNOSIS OF INBORN ERRORS OF FATTY ACID OXIDATION

The clinical symptoms of carnitine deficiency are often indicative of fatty acid oxidation defects. However, precise diagnosis requires biochemical investigation of abnormal metabolites and identification of enzyme defects. Enzymatic characterization of fatty acid oxidation disorders has been reviewed. The analysis of diagnostic metabolites in biological samples was the objective of this project.

Many of the advances which have furthered the understanding and diagnosis of these diseases, have arisen from identifying and interpreting urinary organic acid profiles, especially dicarboxylic acids and acylglycine metabolites. Attention has also been given to the measurement of free and total (free plus esterified) carnitine in plasma. Recently, however, the separation and identification of individual acylcarnitines in biological fluids, has become of major interest.

The determination of carnitine and its esters presents a number of problems to the analyst. These highly polar, zwitterionic compounds, are non-volatile and therefore-
not directly amenable to gas chromatography (GC) techniques. The analyte does not contain any strong chromophoric function, thus limiting the sensitivity of high performance liquid chromatography (HPLC) with UV detection. In the case of neonatal urine, suitable analytical methods need to handle analyte levels of nmol ml⁻¹, with low sample availability (typically 1 ml). A selective method is required because biological mixtures present a very complex matrix. Analysis of acylcarnitines in biological samples has been reviewed recently.⁵⁸

Early investigations of carnitine determination included a number of chemical methods.⁸ Such techniques usually utilized a solvent extraction of the biological material followed by chromatographic separation of carnitine. Once separated, the carnitine level could be estimated by any one of a number of methods. Liberation of trimethylamine from the reaction of carnitine with KOH and Zn dust at 120°C and subsequent colorimetric analysis has been reported.⁵⁹ The conversion of carnitine to crotonobetaine (4-N-trimethylammoniobut-2-enoate) and KMnO₄ titration of the product has been used,⁶⁰ as has a gravimetric method, based on the formation of carnitine reineckate,⁶¹ (carnitine tetra(thiocyanato) diamminochromate (III)). These early carnitine determinations were complex, difficult to apply to physiological quantities, inaccurate, not applicable to carnitine esters, and open to interference from other quaternary amines.⁶²

A number of enzymic methods exploit the recognition of acylcarnitines by carnitine acetyltransferase in the presence of CoA. Unreacted CoA may be reacted with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB)⁶³ or coupled with other enzymic reactions, the products of which are determined spectrophotometrically.⁵⁶ Good sensitivity in such determinations may be obtained with radioisotopes but the techniques tend to be complicated and expensive.⁶⁴-⁶⁷ Many of the enzymatic methods are only appropriate to free and total carnitine determinations. Attempts to identify and quantify individual acylcarnitines using enzymatic reactions have achieved only limited success.⁶⁸ Enzyme inhibition,⁶⁹,⁷⁰ carnitine-affected equilibria and
restricted acylcarnitine range make the techniques unsuitable for many investigations.

L-carnitine administration to patients with certain organic acidurias may result in relatively large urinary acylcarnitine levels (mmol ml⁻¹) as accumulating acyl groups in the mitochondria are removed. Such concentrations are amenable to proton nuclear magnetic resonance spectroscopy (¹H NMR). The method has been successfully applied to cases of propionic acidemia and methylmalonic acidemia. No sample pretreatment was required and the procedure allowed simultaneous measurement of all urinary metabolites including organic acids, glycine conjugates and acylcarnitines. Whilst the method is promising in terms of these advantages, the insensitivity to compounds below the millimolar range severely limits the technique. There are problems regarding carnitine administration on ethical and therapeutic grounds to patients with ill-defined disorders. Also, there is the possibility that the sudden presence of an unnaturally high concentration of carnitine may perturb the metabolic processes under test, leading to ambiguous and/or erroneous conclusions. These disadvantages, along with the requirement for complex and expensive 400 MHz NMR instruments, precludes the routine use of NMR techniques in the clinical laboratory. Separation of carnitine esters, present in biological samples, has been achieved by paper chromatography, thin-layer silica chromatography and ion-exchange chromatography. The lack of quantitative and structural information severely limits the application of these techniques, however. Analysis by desorption chemical ionization mass spectrometry after TLC separation has been reported, as has combined TLC/MS. The non-routine nature of such analyses is a significant disadvantage for clinical applications.

GC and gas chromatography/mass spectrometry (GC/MS) are routinely used in the clinical laboratory. The application of these techniques to investigations of inborn errors of metabolism include screening of organic acid profiles and analysis of derivatized acylglycines. Acylcarnitines are not directly amenable to GC
\[
\text{Me}_3\text{NCH}_2\text{CHCH}_2\text{COO}^- + \text{H}_2\text{O} \rightarrow \text{Me}_3\text{NCH}_2\text{CHCH}_2\text{COO}^- + \text{RCOOH}
\]

Scheme 4
techniques because of their involatile nature but derivatization to volatile analogues seems to have been poorly investigated. An assay for carnitine itself, based on the conversion of carnitine into a volatile butyrolactone compound has been applied to tissue extract. In the case of biological fluids and acylcarnitines, however, GC-based procedures have relied on hydrolysis of the acylcarnitines and examination of the liberated fatty acids (Scheme 4). The procedure is lengthy and difficult to apply routinely and it is also possible that the fatty acid analytes originate from acyl-containing species other than acylcarnitines.

The involatility of the analytes may suggest the application of HPLC. However, conventional HPLC detectors lack sensitivity and selectivity towards acylcarnitines. The known, naturally occurring acylcarnitines in biological fluids are neither fluorophoric nor electrophoric to any useful degree and the carboxyl group is only weakly chromophoric (\( \lambda_{\text{max}} = 210 \text{ nm} \)). Transesterification with CoA and carnitine acetyltransferase has enabled HPLC resolution of the generated CoA esters. The method was applicable only to short-chain acylcarnitines for the diagnosis of acidemias when the accumulating acid(s) contained five or fewer carbon atoms.

Chemical derivatization is a common approach to gaining increased sensitivity in HPLC and it has been applied to both carnitine and acylcarnitine determinations. Precolumn and post column derivatization techniques have been employed. All reported derivatizations for HPLC analysis of carnitine compounds involve reactions with the carboxyl function. However, carnitine, acylcarnitines and other four-carbon trimethylammonium acids (TMA's; e.g. betaine) are unreactive towards common carboxyl-O-alkylating reagents. This is thought to be due to a folded structural conformation of these compounds in solution, permitting strong ionic interaction of the cationic quaternary ammonium group with the carboxyl function. The resulting close proximity of the two ends of the molecule thereby inhibits the approach of electrophiles to the acid group. Homologous two-,
five- and six-carbon TMA-carboxylates are readily derivatized, presumably because these molecules do not have the same folded conformation.83

Formation of 4'-bromophenacyl ester derivatives are among the most useful reported for carboxylates with no other functionality. Two methods have been reported for forming UV absorbing, bromophenacyl derivatives of carnitine and its esters. The reaction with 2,4'-dibromoacetophenone (Scheme 5) has been the most widely applied. The reaction usually occurs in the presence of 5-10% of 18-crown-6 crown ether catalyst and potassium ions.89 The derivatization is performed after isolation of carnitine and/or acylcarnitines from the biological matrix, usually by ion-exchange extraction. Subsequent analysis by reverse-phase HPLC has enabled quantitative determination of physiological carnitine and acylcarnitines in urine and plasma.90,91

In contrast, Minkler et al,83 found the use of 2,4'-dibromoacetophenone inappropriate for carnitine determinations, especially at low concentrations. High temperatures and extended reaction times were said to be necessary for appreciable derivatization but this was accompanied by analyte decomposition. The use of a more reactive alkylating reagent suited to derivatization at lower sample concentration and at lower temperature was investigated.

4'-Bromophenacyl trifluoromethanesulphonate (4'-bromophenacyltriflate), synthesized from 4'-bromo-2-diazoacetophenone and trifluoromethanesulphonic acid,92 was used by Minkler and coworkers83-85 to derivatize carnitine and acylcarnitines (Scheme 6) for subsequent reverse-phase HPLC analysis. The reaction was performed in the presence of N,N-diisopropylethylamine, used to neutralize any residual acid present in the sample. This sterically hindered base was only slowly alkylated by the derivatization reagent under the mild conditions employed. Ten minutes at 25 °C was sufficient to convert approximately 95% of the carnitine present to the 4-bromophenacyl ester derivative.83 The same
2,4′-Dibromoacetophenone +

\[ \text{2,4′-Dibromoacetophenone} \]

\[ \text{18-Crown-6 Ether} \]

\[ \text{K}^+ \]

\[ \text{Acylcarnitine 4′-Bromophenacyl Ester} \]

\[ \text{Acylcarnitine 4′-Bromophenacyl Ester} + \text{KBr} \]

\[ \text{Scheme 5} \]
4'-Bromophenacyl Trifluoromethanesulphate

\[
\text{Acylcarnitine 4'-Bromophenacyl Ester}
\]
derivatization procedure has been used to determine urinary acylcarnitine profiles from patients having propionic acidemia, isovaleric acidemia and medium-chain acyl-CoA dehydrogenase deficiency. The investigations required selective extraction procedures to isolate carnitine and its esters from the urine matrix. This is essential since 4-bromophenacyl trifluoromethanesulphonate reacts readily with other urinary components and consequently leads to interpretation difficulties. The procedure allows sensitive HPLC analysis of acylcarnitines and has even been used to determine these metabolites in normal human urine samples. Despite this, the technique is currently not as popular as derivatization with 2,4'-dibromoacetophenone, possible due to the synthetic steps required to prepare a usable reagent, although once formed it is claimed to be stable for more than 1 year when stored desiccated at room temperature.

Reverse-phase HPLC with postcolumn derivatization has largely been restricted to a group of Japanese workers. The derivatization apparatus and procedure has been collectively termed a carboxylic acid analyser and is equally applicable to organic acids as well as carnitine and acylcarnitines. The carboxylate group is reacted with 2-nitrophenylhydrazine enabling specific colorimetric determination of acetylcarnitine and glutarylcarnitine.

Whilst further development of some of these HPLC methods appears to be worthwhile there is one fundamental disadvantage of any stand-alone chromatographic technique. The inability of HPLC (and GC) to provide any structural information limits the application. Such knowledge is essential if diagnostic interpretation of uncharacterized disorders are to be made. The requirement for structural information and a sensitive/selective detector suggests coupling of chromatography to mass spectrometry.

Mass spectrometry is widely utilized in the clinical chemistry laboratory. The value of unambiguous structural information makes it an attractive technique in its own
right or as a chromatographic detector. The major obstacle to acylcarnitine analysis by conventional mass spectrometry is once again the involatility and thermal lability of the analyte. Application has largely awaited advances in mild ionization techniques.

The technique of desorption chemical ionization mass spectrometry was one of the earliest applications of MS methods to intact acylcarnitine analysis. It consisted of the pyrolysis of the sample on a tungsten filament in a chemical ionization source. As the acylcarnitine analyte is heated on the filament, some evaporation of the molecules occurs but because of the thermal lability of these compounds, mostly decomposition products are released. In either case, the processes happen in the presence of reactant gas ions, such as NH$_4^+$ when ammonia is used. This results in ionization of the evaporated molecules and pyrolysis products, as in other chemical ionization techniques. Mass analysis of the ions produced gave characteristic mass spectra for individual acylcarnitines. By comparing such spectra from standard acylcarnitine samples with those obtained from the analysis of isolated fractions of a clinical urine, octanoylcarnitine was identified in a case of MCADD. No further applications have been reported, probably because of (i) poor sensitivity, (ii) the inability to couple to a chromatographic method and (iii) transient spectra are obtained which are not compatible with MS/MS (see later).

The 1980's saw the advent of fast atom bombardment (FAB) as a mild method of producing ions from involatile and thermally labile compounds for mass spectrometric analysis. It soon became apparent that the ionic nature of carnitine and acylcarnitines made them excellent analytes for FAB techniques. Since the first application of FAB to the identification of physiological acylcarnitines, it has been used extensively to analyse for these compounds in biological samples. Early studies however, were hindered by suppression effects of Na$^+$, K$^+$ and urea in urine, reducing the abundance of ions from the acylcarnitines. Methylation of raw urine (3M- HCl/MeOH, 80 °C, 15 min) overcame the sensitivity problem and
allows detection of acylcarnitines as their methyl esters at concentrations down to 50 nmol/ml.96

Despite the popularity of the method there exists a degree of uncertainty in the specificity of FAB analysis of acylcarnitine methyl esters. This is because a given methyl ester has the same elemental composition and mass as the free acid of the next higher homologue. For example, the predominant ions [M]+ for the methyl ester of isovaleryl carnitine and [M + H]+ for hexanoylcarnitine (both m/z = 260) are not distinguished by conventional FABMS. Incomplete methylation of the sample could therefore lead to erroneous conclusions. The formation of isotopically labelled methyl esters has recently overcome this disadvantage.97 Using perdeuteriomethanol, [2H3 - Me] methyl esters of the acylcarnitines are made which give an increase of 17 daltons to the molecular mass (instead of 14: one methylene unit), forming derivatives which are distinct in mass from other homologues. Subsequent isotopic dilution experiments have enabled quantitative acylcarnitine profiling of normal and disease-state urines using FABMS.97 Whilst FAB has the ability to produce a good yield of intact acylcarnitine ions, this is not sufficient for clinical interpretation, especially when mixtures are analysed. More structural information is required to confirm that an ion of interest is an acylcarnitine and to distinguish between isomers. High resolution MS has been used to confirm the molecular formulae of the ions corresponding to the acylcarnitines97 but this method cannot distinguish isomers and the accuracy of the quantitative assay is variable for different acyl chain lengths. The favoured technique is to combine FAB with tandem mass spectrometry (FABMS/MS). In such an experiment, the intact acylcarnitine ions produced by FAB, are separated according to mass then allowed or encouraged to fragment within the mass spectrometer before separation and detection of the resultant ions. This may be performed on a double-sector instrument using linked scanning,95,98,99 or a triple quadrupole system105 or a hybrid instrument.100,101 In each case, the objective is to gain information on how...
suspected acylcarnitine ions fragment, confirming or otherwise the presence of the carnitine esters. MS/MS can be applied to mixtures since it effectively performs a separation based on the mass of the analyte(s). Another advantage is the speed of analysis associated with MS/MS experiments, seconds compared with, say, a 30 minute GC/MS analysis.

Typically, a normal FAB spectrum from a clinical urine sample may show an ion of m/z value corresponding to an intact and known acylcarnitine ion. This information would be combined with data from a constant neutral loss scan (CNL), for example, searching for losses of 59 daltons, i.e. loss of Me₃N. If this procedure indicated that the suspected acylcarnitine ion observed in the normal FAB analysis did loose 59 daltons, this would support the identification of a carnitine ester. Further confirmation would require another experiment, a daughter ion scan, performed on the identified molecular ion and/or any other ion which lost 59 daltons and hence was observed in the CNL scan. Alternatively, or additionally, a parent ion scan on an ion such as that occurring at m/z 85, due to \( +CH_2-CH=CHOOH \) and common to all acylcarnitines, may be obtained. For reliable identification the process of performing those separate experiments may take longer than one might expect for an MS/MS investigation. The technique is also dependent on the yield of daughter ions from the parent molecular ion and in the case of acylcarnitines this usually requires high energy collisional activation with expensive and complex hybrid sector instruments. This is certainly so for the differentiation of isomeric acylcarnitines as KeV collision energies are required to cause cleavage of the acyl chain. The difficulties associated with isomeric acylcarnitines are compounded when a mixture of isomers is encountered, for example if octanoylcarnitine and valproylcarnitine occur in the same sample. Therefore, for complete characterization workers have sometimes resorted to the complementary use of thermospray LC/MS or hydrolysis followed by GC/MS of the liberated acids.
The advances made in HPLC analysis of carnitine esters has led to the use of mass spectrometry as a detection system for reverse-phase HPLC separations. Thermospray (TSP) is currently the most popular LC/MS interface and capable of handling the high salt concentrations of eluent from reverse-phase columns. Successful analysis of acylcarnitines in biological fluids has been achieved using TSP/MS.\textsuperscript{103,104-106} The technique has proven particularly useful in distinguishing isomeric acylcarnitines, which is not possible by conventional FABMS and is difficult by FABMS/MS techniques.

Recently, a continuous flow FAB interface (CFFAB) has been used in conjunction with microbore reverse-phase HPLC columns.\textsuperscript{107} The separation achieved with these short columns made necessary CFFABMS/MS for full analytical interpretation; requiring a complicated instrument set-up.

LC/MS approaches to acylcarnitine determinations are still proving themselves at the physiological levels present in urine and plasma. As with MS/MS methods the necessary equipment is not common in hospital screening laboratories and is unlikely to enjoy widespread and routine clinical application.

None of the methods discussed is yet ideal for routine clinical analysis of acylcarnitines. Advances and further application of reverse-phase HPLC and FABMS/MS should continue to generate valuable data. It is believed however, that there is a role for an alternative, simple, unambiguous method of analysing individual acylcarnitines. In terms of technique, GC/MS and its associated capabilities of unrivalled separation efficiency with excellent structural specificity and sensitivity, is most likely to provide a definitive analysis at realistic cost. Any such method could be readily integrated into existing GC/MS screening procedures for urinary organic acids. Addressing the requirement for a suitable derivatization procedure is paramount to the successful application of GC/MS in this area.
1.3 NEW APPROACHES TO THE ANALYSIS OF PHYSIOLOGICAL ACYL-CARNITINES.

1.3.1 Derivatization for GC and GC/MS

A suitable derivatization procedure for acylcarnitine analysis by GC and GC/MS must remove the zwitterionic, non-volatile nature of the analyte molecule. This is fundamental to the application of GC techniques, but also of diagnostic importance is the maintenance of molecular integrity in the formed derivative. As mentioned, a disadvantage of acylcarnitine hydrolysis and GC/MS of the liberated acids, is the uncertainty of the examined analytes' origin. The development of a simple and novel derivatization procedure in which acylcarnitines are cyclized to give volatile γ-butyrolactones, 3, (Scheme 7), is presented here. Importantly, the derivatives retain a structural 'memory' of their origin, that is, the fatty acyl unit remains bound to a diagnostic residue of carnitine. 108

The formation of γ-butyrolactone compounds from carnitine and acylcarnitines was first reported by Hvistendahl et al.94 The heated septum inlet system of a mass spectrometer was identified as the site of a pyrolysis reaction. Carnitine and acylcarnitine HCl salts were reported to eliminate trimethylamine and cyclize to the corresponding lactone. The GC assay for carnitine alone, reported by Lewis et al.80 was also based on the formation of 4-butyrolactone derivative by the action of NaOH and NaBH4. One of the main objectives of this project was to develop such a cyclization reaction into a useful derivatization process for GC and GC/MS analysis of a wide range of acylcarnitines.

Structurally, the acylcarnitine molecules are ideally arranged for the intramolecular cyclization shown in, Scheme 7. Analytical evidence (Section 2.1.1) and work of others on derivatization of carnitine and its esters to HPLC amenable analytes (Section 1.3), suggests that these compounds exist in solution as a folded structure with the carboxyl tail of the molecule close to the quaternary ammonium head.
Scheme 7
Nucleophilic attack of the carboxyl group on the methylene carbon attached to the nitrogen, eliminates trimethylamine and forms a γ-butyrolactone. Considerable evidence exists to suggest that heat alone may induce this reaction. In addition to the work of Hvistendahl et al.,\textsuperscript{94} analysis of acylcarnitines by desorption chemical ionization\textsuperscript{74} (pyrolysis in a chemical ionization source) identified trimethylamine as the major ion product with additional ions indicative of the lactone fragmentation (c.f. Section 2.4.2).

Refinement and control of the cyclization reaction was the starting point for the development of the derivatization. The lability of acylcarnitines towards Hofmann elimination (Scheme 8) and hydrolysis (Scheme 4) is known and needed to be respected. For this reason, strongly basic and/or aqueous reaction conditions were avoided. Also, careful control of heat was required to avoid decomposition of the formed derivative. This made necessary a bench reaction rather than one carried out \textit{in situ} or on-column. An on-column reaction would not be appropriate anyway since cold on-column injection is generally regarded as the optimal technique to obtain precise quantification and good sensitivity when using GC/MS.

The successful conversion of acylcarnitines to the corresponding lactones (Scheme 7) has enabled application of capillary GC and GC/MS.\textsuperscript{108} The lactones do not possess the highly polar, zwitterionic nature of the parent compounds and are suitably volatile for use of the previously inappropriate analytical techniques of GC and GC/MS. Initial investigations with a model acylcarnitine standard were used to identify appropriate derivatization conditions. The model compound continued to be useful in many subsequent studies as a suitable internal standard. Once the proposed derivatization had been validated with naturally occurring acylcarnitine standards, spiked urine experiments were conducted.

The complex nature of a biological matrix places great demands on the selectivity of any analytical procedure for clinical investigations. It was found necessary to
Scheme 8
extract the acylcarnitines from urine prior to derivatization and analysis. The most suitable technique employed ion-exchange resins and enabled selective isolation of the carnitine compounds.

The developed analytical method has been applied to many clinical urine samples. Results from the study of urine from patients with defective fatty acid and amino acid metabolism are reported. Unambiguous identification of endogenous acylcarnitines in such samples was observed using GC/MS. Similarly, carnitine ester metabolites were identified in urine from children undergoing certain therapeutic treatments. In this role, the technique enabled investigation of the metabolism of two acidic drugs; 3-phenylpropionic acid and 3-propylvaleric acid.
CHAPTER 2

DEVELOPMENT OF A METHOD BASED ON GAS CHROMATOGRAPHY

RESULTS AND DISCUSSION
2.1 INTRODUCTION

A limited range of acylcarnitines is commercially available: acetyl-, octanoyl- and palmitoylcarnitine, all in the form of their HCl salts. A larger range of these carnitine esters would be required for confirmatory purposes, if a developed analytical method were to be applied to clinical investigations. Therefore, it was thought important to be able to synthesise a range of acylcarnitines (2) of varying R group. These should include acylcarnitines previously identified and/or suspected in the metabolic disorders of interest. For example, hexanoyl- and octanoylcarnitine in cases of medium-chain acyl-CoA dehydrogenase deficiency and isovaleryl carnitine in cases of isovaleric acidemia. Identification of such metabolites in biological fluids would be characteristic of the respective diseases.

Also of initial interest was the synthesis of a "model acylcarnitine" suitable for testing the derivatization reaction (Scheme 7). Monitoring the cyclization of a naturally occurring, acylcarnitine standard such as octanoylcarnitine presents some analytical problems. The ideal technique would be to use GC and GC/MS to detect any conversion to the volatile lactone product. However, there exists the possibility of the cyclization occurring in the hot injector zone of the GC or even the GC column or the hot ion source of the mass spectrometer. That is, the analytical procedure itself may interfere with the process under observation. To prevent such interference, a chromatographic method was needed that did not involve heating of the analytes. As a convenient and rapid method of monitoring reactions, TLC is well established in synthesis work. However, most naturally occurring acylcarnitines, lacking any significant chromophoric properties, would require the use of developing reagents to monitor any reaction by TLC. Rather than investigate the viability of developing reagents, it was decided to synthesise an unnatural acylcarnitine containing a UV absorbing functionality to facilitate ease of detection. Such a compound, whilst being easy to detect must behave in the same way as the natural acylcarnitines of primary interest, under the analysis conditions. For this
reason, 4-phenylbutanoylcarnitine \([2, R = \text{C}_3\text{H}_6\text{C}_6\text{H}_5]\) was synthesised, in which, the UV active phenyl group was sufficiently remote from the cyclization site so as not to affect the derivatization process.

The successful cyclization of this model acylcarnitine would result in a 4-phenylbutanoyl-containing \(\gamma\)-butyrolactone compound \([3, R = \text{C}_3\text{H}_6\text{C}_6\text{H}_5]\). The independent synthesis of this acyloxylactone from a non-carnitine origin, would be useful in checking for the successful conversion from acylcarnitine to volatile lactone (Scheme 7). The infrared (IR), mass, NMR and UV spectra of this product \([3, R = \text{C}_3\text{H}_6\text{C}_6\text{H}_5]\), along with its TLC behaviour, could be used to confirm, or otherwise, the successful development of appropriate derivatization conditions. This was regarded as a more effective strategy than alternatively subjecting natural acylcarnitines to various reaction conditions and attempting to analyse the products.

The identification of appropriate derivatization conditions was necessary before application to acylcarnitines in biological fluids could be attempted. In addition, coping with the biological matrix would be essential to biochemical investigations. Extraction of the acylcarnitines from urine or blood prior to analysis has been necessary for other analytical approaches. The likelihood of interference by the biological matrix on the derivatization step, suggested that extraction of the analyte would also be required in this study.

Successful extraction and derivatization of naturally occurring acylcarnitines from urine would enable the application of GC and GC/MS analyses. In order to gain the full benefits of selectivity, sensitivity and structural identification from these techniques, attention must be given to optimization of operating conditions and parameters. Correct choice of capillary column, injection technique, gas flows and temperature programming are fundamental to achieving suitable GC resolution of the sample components. This may require detailed attention when similar compounds (e.g. isomers) are being chromatographed. The mass spectral data
derived from an analyte are equally, if not more, dependent on operating parameters. Electron ionization (EI) and chemical ionization (CI) MS techniques are capable of producing completely different but often complementary analytical information. Utilizing the versatility of these techniques was regarded as important to realizing the objectives of the project.

2.2 SYNTHESIS OF ACYLCARNITINES (2)

Various syntheses of acylcarnitines have been reported\textsuperscript{10,11} but all are based on the condensation of carnitine hydrochloride with an appropriate acid chloride, as shown in Scheme 9. The methods of Ziegler et al.\textsuperscript{111} and variations on the procedures were employed exclusively in the studies reported here. All syntheses were performed with D,L-carnitine hydrochloride. The formation of racemic acylcarnitines, as their HCl salts, was regarded as suitable for the purposes required. It should be noted, however, that only L-carnitine occurs naturally.

Experience of the synthesis procedure was initially gained with known acylcarnitines and particularly those which could be analytically checked against authentic commercial samples. Acetyl-, octanoyl- and palmitoylcarmitine were prepared and analysed on this basis. The synthesis techniques were then applied to the preparation of 4-phenylbutanoylcarnitine (2, \(R = (\text{CH}_2)_3\text{C}_6\text{H}_5\)), hexanoylcarnitine (2, \(R = (\text{CH}_2)_4\text{CH}_3\)) and 3-phenylpropionylcarnitine (2, \(R = (\text{CH}_2)_2\text{C}_6\text{H}_5\)). Ziegler et al.\textsuperscript{111} describe three synthetic methods for acylcarnitines of differing chain length, from acetylcarnitine to palmitoylcarnitine. Depending on the carnitine ester required, one of the following methods of preparation was used. The phenyl-containing acylcarnitines were prepared by Method C.

Method A: Acetylcarnitine was formed from the reaction of acetyl chloride with carnitine hydrochloride in acetic acid (Section 6.3.1). The product was obtained as a white crystalline solid in good yield after recrystallization from propan-2-ol.
\[
\begin{align*}
\text{(CH}_3\text{)}_3\text{NCH}_2\text{CHCH}_2\text{COO}^- \quad + \quad \text{RCOCl} & \quad \longrightarrow \quad \text{(CH}_3\text{)}_3\text{NCH}_2\text{CHCH}_2\text{COO}^- \quad + \quad \text{IICl} \\
\text{Scheme 9}
\end{align*}
\]
Method B: Acylcarnitines with an acyl chain up to six carbon atoms in length were also prepared in the absence of a conventional reaction solvent (Section 6.3.2). The appropriate acid chloride was prepared in situ by the action of freshly distilled thionyl chloride on the corresponding free acid. Carnitine hydrochloride, being soluble in the acid/thionyl chloride mixture, was added to form the appropriate acylcarnitine. The product was purified by recrystallization from propan-2-ol.

Method C: The solubility of carnitine hydrochloride in the fatty acid/thionyl chloride mixture decreases with increased chain length of the acid. When acids of carbon atom chain lengths greater than six are encountered, carnitine is insoluble in the reaction mixture and hence it becomes necessary to use an appropriate solvent. In accordance with previous workers,111 trichloroacetic acid was used as the solvent for such reactions. This procedure was used to synthesise acylcarnitines with seven to sixteen carbon atoms in the acyl chain. Other than the use of a solvent, the preparation of these carnitine esters was as for Method B above (Section 6.3.3).

Care was required to ensure the preparations were performed in a dry atmosphere. Reactions under dry nitrogen were investigated but apparatus connected to CaCl₂ drying tubes proved adequate. Higher yields of acylcarnitines were obtained when freshly distilled thionyl chloride was used. Trichloroacetic acid was recrystallized from ethanol-free chloroform prior to use as the carnitine solvent.

The yields obtained for the syntheses varied from poor (28% for 3-phenylpropionylcarnitine), to good (81% for palmitoylcarnitine). Since sufficient product was usually obtained for the analytical purposes required, investigations into the reason(s) for the varying yields were not undertaken.
After reaction of the relevant acid chloride with carnitine, the crude acylcarnitine product was precipitated, with unreacted carnitine, by the addition of dry ether (Na-dried) to the reaction mixture (see for example, Section 6.3.2). This procedure sometimes caused problems. It was found that the ether should be introduced dropwise until precipitation had started to avoid the product forming as an oil. If an oil was produced it was difficult to obtain the pure acylcarnitine in an appreciable yield.

The recrystallization of the acylcarnitine product from hot propan-2-ol also required careful attention. Excessive heating of the alcohol/carnitine ester mixture could cause 1-methylethyl (isopropyl) esterification of the carnitine, acid function. This was observed in the case of the 3-phenylpropionylcarnitine preparation (Section 6.3.3). The positive-ion FABMS spectrum showed a peak at 42 daltons above that for the expected intact acylcarnitine ion. That is, the ion at $m/z$ 336 was attributed to $[M + \text{CH(CH}_3\text{)}_2]^+$ whilst the base peak in the spectrum was observed at $m/z$ 294, $[M + \text{H}]^+$, where:

\[
\begin{align*}
M &= \text{Me}_3\text{NCH}_2\text{CHCH}_2\text{COO}^- \\
&\quad + \text{OCOCH}_2\text{CH}_2\text{Ph}
\end{align*}
\]

That is, $M$ represents the acylcarnitine zwitterion. It is proposed that esterification of the free carboxyl of $M$, by the action of propan-2-ol, gave the $m/z$ 336 ion. This was supported by the $^1$H NMR spectrum, in which the integral ratios were not consistent with pure 3-phenylpropionylcarnitine and a doublet occurred at 1.2 ppm that could be assigned to the methyl protons of the isopropyl ester group.
The IR spectra (nujol mull) of the synthesised acylcarnitines were predictably similar. They all showed two carbonyl stretching bands, although in the case of 3-phenylpropionylcarnitine and hexanoylcamidne these were present as one unresolved, broad absorption band at 1730 cm\(^{-1}\). In the other cases stretching bands at 1710-1720 (acid) and 1730-1740 cm\(^{-1}\) (ester) were observed (see Appendix, IR 2). This compared with the single carbonyl stretching band of carnitine itself which occurred in the IR spectrum at 1720 cm\(^{-1}\) (acid), as detailed in the Appendix (see IR 1).

Similarities were also observed in the 90 MHz \(^1\)H NMR spectra of the synthesised acylcarnitines. All of them gave a complex multiplet signal at 5.4-5.5 ppm due to the single methine proton of the carnitine unit and a singlet at 3.1 ppm due to the nine methyl protons of the quaternary ammonium group. C-2 protons (\(\alpha\) to the carboxyl group) gave a broad doublet at ~2.7 ppm and C-4 protons gave a distorted doublet at ~3.6 ppm. All of these chemical shift values matched those reported by Millington et al.\(^{95}\) Other signals for protons of the acyl chain were consistent with the proposed structures (see Appendix, \(^1\)H NMR 2).

Occasionally some unreacted carnitine was identified as a contaminant of the synthesised acylcarnitine by the presence of a multiplet at 4.2 ppm. This signal is due to a methine proton in an \(\alpha\)-position to the hydroxy function of carnitine as opposed to the ester function of an acylcarnitine. The \(^1\)H NMR spectrum of synthesised 3-phenylpropionylcarnitine was complicated by contamination with the isopropyl ester of the required acylcarnitine and unreacted carnitine (Section 6.3.3). Integral ratios which were inconsistent with the pure acylcarnitine, and 'foreign' signals at 4.2 ppm (multiplet due to carnitine methine proton) and 1.2 ppm (doublet due to methyl protons of the isopropyl ester functionality) were evident in this spectrum.
The chemical shift values of the $^{13}$C NMR spectra for the acylcarnitines proved interesting, in that they did not fit the open-chain structure as well as might have been expected. This observation was also noted for free carnitine. Taking free carnitine as an example, TABLE 1 summarizes the $^{13}$C NMR signals observed and the predicted chemical shift values of the open-chain structure. Also included in TABLE 1 are the hypothetical, chemical shift values predicted for the folded structural conformation shown, where the ionic interaction between the carboxyl and quaternary ammonium groups, effectively constitutes a bond. It may be concluded from TABLE 1, that the observed signals were more consistent with the predicted signals for a folded carnitine structure than for an open-chain confirmation (e.g. structure 1). Minkler et al. have previously suggested acylcarnitines, in solution, have a folded structure and suggested it was the close proximity of the carboxyl group to the quaternary ammonium group, which made esterification of the former difficult in derivatization attempts. The results presented here, support the proposed structural configuration and indicate that acylcarnitines, in solution, are ideally arranged to facilitate the cyclization reaction (Scheme 7), required for the purposes of this project.

FABMS was a particularly useful technique for analysing the synthesised acylcarnitines, without derivatization. The relative molecular mass was invariably easy to assign through a prominent [M + H]$^+$ ion, where M represents the intact acylcarnitine zwitterion, as described earlier. The positive-ion FAB spectra obtained corresponded with those of others. Postulated structures for the major ions observed and proposed fragmentation pathways are summarized in Scheme 10. In common with other workers, an ion at $m/z$ [M + H + 14]$^+$ was observed with all synthesised acylcarnitines and was attributed to intermolecular methylation of the carnitine carboxyl function. Free carnitine has been reported to undergo extensive intermolecular methylation in secondary ion mass spectrometry (SIMS) and a similar process is predicted for acylcarnitines in glycerol under
<table>
<thead>
<tr>
<th>Carbon Nuclei</th>
<th>Observed Chemical Shifts /ppm</th>
<th>Predicted Chemical Shifts /ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CARNITINE (in D&lt;sub&gt;2&lt;/sub&gt;O)</td>
<td>CARNITINE (Open-chain Structure)</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;H</td>
<td>176</td>
<td>51</td>
</tr>
<tr>
<td>C2</td>
<td>43</td>
<td>76</td>
</tr>
<tr>
<td>C3</td>
<td>72</td>
<td>50</td>
</tr>
<tr>
<td>C4</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;N</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 1:** <sup>13</sup>Carbon NMR Data For Free Carnitine

Folded structural conformation showing ionic interaction between the anionic carboxyl group and the cationic quaternary ammonium group.
Scheme 10
Scheme 11
FAB conditions. Cluster ions, corresponding to \([2(M + H) - H]^+\), were observed in some spectra but the abundance of these ions was dependent on the concentration of acylcarnitine in the glycerol matrix. The relative abundances of the major FAB ions also varied to some extent with sample loading and was therefore also assumed to be dependent on the concentration in glycerol.

An ion at \(m/z\) 162 was observed in all acylcarnitine positive-ion FAB spectra and was assigned the structure of free carnitine. This ion probably occurs as a combination of rearrangement accompanying fragmentation of the acylcarnitine and contamination of the sample with unreacted carnitine from synthesis.

The negative-ion FAB spectra of the acylcarnitines also indicated the molecular mass with ions at \(m/z\) \([M + Cl]^-\) and \([M + H + 2Cl]^-\). Other common fragments in the negative-ion spectra are summarized in Scheme 11. FABMS was also used to confirm the identity of six synthesised acylcarnitines provided as a gift from the laboratory of Dr Graham A Mills (Southampton General Hospital). Samples of propionylcarnitine (2, \(R = C_2H_5\)), 2-methylbutyrylcarnitine (2, \(R = CH(CH_3)CH_2CH_3\)), valerylcarnitine (2, \(R = (CH_2)_3CH_3\)), isovalerylcarnitine (2, \(R = CH_2CH(CH_3)_2\)), heptanoylcarnitine (2, \(R = (CH_2)_5CH_3\)), and valproylcarnitine (2, \(R = CH(CH_2CH_2CH_3)_2\)) were provided. In accordance with previous FABMS analyses of synthesised acylcarnitines, the positive-ion and negative-ion spectra of the above compounds supported the proposed structures.

2.3 SYNTHESIS OF STANDARD \(\gamma\)-BUTYROLACTONE COMPOUNDS (3)

The synthesis of acyl-containing lactones, 3, was important to the development of the derivatization. They provided an unambiguous check of successful, or otherwise, acylcarnitine cyclizations. Initially, only 4-phenylbutanoyl-containing lactone (3, \(R = (CH_2)_3C_6H_5\)) was prepared, to help monitor the cyclization of the model acylcarnitine (2, \(R = (CH_2)_3C_6H_5\)), as in Scheme 7. As the project
progressed however, a requirement for standards to match derivatized natural acylcarnitines arose. The availability of these compounds was also helpful when optimizing analytical conditions, especially GC and GC/MS for examination of derivatized acylcarnitines. Analysis of authentic samples of acyl-containing lactones allowed the chromatographic characteristics and mass spectral fragmentations of these expected acylcarnitine derivatives to be elucidated.

The preparation of the standard lactone compounds was based on the synthesis of β-hydroxy-γ-butyrolactone (4) followed by reaction with the corresponding acid chloride (Scheme 12).

Several synthetic pathways have been used to prepare compound (4).

The simplicity of the method reported by Henrot et al., starting from malic acid and shown in Scheme 13, favoured its application (Section 6.3.4).

Malic acid (8) was acylated with acetyl chloride to produce the cyclic anhydride (7). The action of methanol, under neutral conditions, on (7) formed the diester-acid compound (6). The selectivity of the reaction is postulated to arise from attack of the methanol nucleophile only on the more hindered carbonyl group. That there was selectivity to this process was supported by the quantitative yield of (6). The proposed explanation for this reaction is the formation of a second five-membered ring in the intermediate species, prior to opening to the diester (6), as in Scheme 14 (where the nucleophile shown is MeO⁻ for simplicity). A loose association with the acetyl group of (7) may also guide the in-coming nucleophile to the hindered carbonyl function. Analytical ¹H NMR and IR spectra recorded for both (7) and (6) were consistent with the proposed structures and in good agreement with the data reported by others. The selective reduction of the ester groups of (6), initially proved difficult. Care had to be taken to ensure the 2-methylpropan-2-ol (tBuOH) solvent was dry. Success was only achieved when the reaction time quoted in the literature, 2hr, was increased to 20 hr. This effectively formed the
Scheme 12.

\[
\text{4} + \text{RCOCl} \rightarrow \text{3}
\]
Scheme 13

\[
\begin{align*}
\text{HO}_2\text{C}\text{CH(OH)CH}_2\text{CO}_2\text{H} & \xrightarrow{\text{AcCl}} \text{AcO}\text{C}_2\text{H}_2\text{O}_2\text{C}_2\text{H}_2\text{O} \\
\text{HO}_2\text{C}\text{CH(OH)CH}_2\text{OH} & \xrightarrow{\text{Cyclization}} \text{HO}_2\text{C}\text{CH(OH)}\text{CH}_2\text{OAc} \\
& \xrightarrow{\text{NaBH}_4} \text{HO}_2\text{C}\text{CH(OH)CH}_2\text{CO}_2\text{Me} \\
\end{align*}
\]
Scheme 14
dihydroxy-acid compound (5), which spontaneously cyclized to the β-hydroxy-γ-lactone (4). There was no evidence to suggest that over-reduction had taken place, i.e. NaBH₄ did not open the formed lactone to a triol compound (i.e., butan-1,2,4-triol). The crude lactone product obtained was purified by silica gel column chromatography.

The resulting pure sample of (4) was analysed by IR and ¹H NMR spectroscopy. The IR spectrum showed a typical OH stretching band in the region 3700-3100 cm⁻¹ and a carbonyl stretch at 1770 cm⁻¹. All absorbance signals, in the IR range, were also consistent with data published for an alternative method of synthesis.¹⁴ The ¹H NMR spectrum also supported the structure of the hydroxylactone and was consistent with data published for compound (4).

Four standard acyl-containing γ-butyrolactone compounds were synthesised and all were formed from the reaction of (4) with the corresponding acid chloride as in Scheme 12 (Section 6.3.5). For instance, an authentic sample of the UV absorbing, acyloxy lactone compound [3, R = (CH₂)₃C₆H₅], was prepared using 4-phenylbutanoyl chloride. The acid chloride was formed in situ by the action of thionyl chloride with 4-phenylbutanoic acid. It was thought that the condensation of the hydroxylactone (4) with the acid chloride would be aided by the presence of a base such as triethylamine, but this proved unnecessary. Monitoring the reaction by TLC was difficult. Whilst the acyloxy lactone could be visualized on a silica TLC plate, under UV radiation, the R⁰ value was almost identical to that of phenylbutanoic acid. The origin of the interfering acid was either unreacted starting material or decomposition of the lactone product (see Section 2.4.1). The separation of the two components on silica was minimal in all solvent systems investigated and this predictably complicated column chromatography purification. The problem was overcome by washing the crude product with saturated NaHCO₃ solution which removed the acid. It was then possible to isolate the required product, on a column of silica gel, removing any unreacted starting materials.
The IR spectrum of the model derivative (3, R = (CH$_2$)$_3$C$_6$H$_5$), showed carbonyl stretching bands of the lactone at 1790 cm$^{-1}$ and that of the ester function at 1740 cm$^{-1}$. This characteristic pattern in the carbonyl region was found to be typical of the acyloxylactones and subsequently proved useful during the development of the acylcarnitine derivatization reaction (Section 2.4). Similarly, particular features of the $^1$H NMR spectrum of this compound became recognized as indicative of lactone formation. A very diagnostic signal pattern at 5.4 ppm due to one proton, was used in the development stages as a simple check for acylcarnitine cyclization. The signal occurred as a distinctive multiplet, (see Appendix, $^1$H NMR 3) and was assigned to the single methine proton of the lactone ring. The splitting pattern arises from coupling with the two pairs of non-equivalent methylene protons of the ring. Theoretically, this should result in four doublets and indeed, using a 90 MHz instrument, the signal was not fully resolved but appeared as a distorted octet.

Synthesis of the acetyl-, octanoyl- and palmitoyl-containing lactones was similar to that described for the model derivative above. In the case of the palmitoyloxylactone, the final product was a solid. Purification was achieved by recrystallization from diethyl ether rather than the column chromatography procedure used for the 4-phenylbutanoyl-, acetyl- and octanoyl-containing lactones. Satisfactory analytical data were obtained for all the $\gamma$-butyrolactone compounds prepared, using $^{13}$C and $^1$H NMR, IR and EI mass spectroscopy. The two carbonyl absorption bands in the IR spectrum and the ring, methine proton, multiplet signal in the $^1$H NMR spectrum, were common to all the lactone compounds synthesised.

2.4 DERIVATIZATION CONDITIONS

2.4.1 Effect Of Heat On Octanoylcarnitine Hydrochloride

Prior to the systematic investigations with the aromatic model acylcarnitine, a separate and simple investigation was conducted into the possibility of heat causing
the cyclization of acylcarnitines. An experiment was performed (Section 6.3.6) whereby an ethanolic solution of octanoylcarnitine hydrochloride was gradually heated in the septum inlet of a mass spectrometer. Any volatalized species formed in the inlet passed into the ion source of the mass spectrometer where the gaseous molecules were subject to electron ionization (EI). Subsequently, EI mass spectra were recorded for compounds originating from the ethanolic acylcarnitine mixture. The objective was to identify ions diagnostic of liberated trimethylamine (\(m/z\) 59) and possibly of any octanoyloxylactone (\(m/z\) 228) formed by heating. The experiment was similar to the work of others using desorption chemical ionization\(^7\) when the CI mass spectrum of trimethylamine was noted during the analysis of acylcarnitines.

On injecting a solution of octanoylcarnitine HCl in ethanol into the septum inlet, only the mass spectrum of the solvent was evident. The majority of the ethanol was allowed to evaporate before gradually heating the inlet. As the temperature slowly increased, residual ethanol continued to evaporate. No other significant ions were noted until an inlet temperature of 160° C was reached. At this stage mass spectra characteristic of octanoic acid were recorded, indicating decomposition of the acylcarnitine.

No evidence was obtained to suggest that cyclization had occurred. The experiment was stopped on observation of decomposition products.

It is speculated that active sites on the walls of the inlet chamber may have catalysed the ester pyrolysis process (Scheme 15). Esters are thermally quite stable, however, when heated to more than 400°C they are known to decompose into a carboxylic acid and an alkene.\(^{116}\) Evidence exists that the pyrolysis may occur at lower temperatures when the produced alkene bond forms in conjugation with a carbonyl function,\(^{117}\) as would be the case for an acylcarnitine (see Scheme 15).
Scheme 15
Therefore, it is reasonable to propose that an ester pyrolysis reaction on the walls of the inlet chamber, at 160°C, resulted in the detected octanoic acid.

Over the temperature range applied, small amounts of trimethylamine may have been released and gone undetected. Summed spectra at a temperature below that which induced decomposition may have produced evidence of cyclization. The presence of the acylcarnitine as the HCl salt may also have prevented lactone formation. The reaction requires a deprotonated carboxyl group to effect the necessary nucleophilic attack. Whilst the experiment was unsuccessful in identifying lactone formation, it indicated a temperature limit over which decomposition may be likely. This was taken into account in subsequent derivatization attempts.

2.4.2 Development of a Controlled Derivatization

The availability of fully characterized 4-phenylbutanoylcarnitine and the 4-phenylbutanoyloxy-γ-butyrolactone (3, R = C₃H₆C₆H₅) was beneficial to development of the derivatization. These model compounds enabled the convenient monitoring of reactions by TLC and confirmation of derivatization products by IR, NMR, GC and MS techniques. At the simplest level, they provided valuable information on physical state, solubility, stability and suitability of various work-up procedures. When interpreting analytical data, familiarity with the behaviour of the model compounds allowed rapid checks on reaction products. The first successful conversion of an acylcarnitine into a volatile acyl-containing γ-butyrolactone was observed and confirmed using these compounds. Initial investigations were concerned with identifying a suitable solvent for the reaction. ¹³C NMR data on carnitine and acylcarnitines suggested that they have a folded structural confirmation in solution (Section 2.2). This is likely to favour the cyclization reaction (Scheme 7). The same information is not available for acylcarnitines in the solid state. There exists the possibility of intermolecular ion associations in the solid. For instance,
the carboxyl group of one acylcarnitine molecule may interact electrostatically with the quaternary ammonium group of another. Such interactions would probably inhibit intramolecular cyclization since the carboxylate function of the carnitine backbone would no longer be in the vicinity of the required electrophilic site. This may be a further reason for the unsuccessful cyclization of octanoylcamitine in the heated septum inlet of the mass spectrometer (Section 2.4.1).

Considering the mechanism of the required reaction (Scheme 7, i.e. nucleophilic attack of the carboxylate group, in which charge is reduced in the transition state), a non-nucleophilic, aprotic solvent was thought appropriate. Heating acylcarnitines in an alcohol solvent has already been shown to cause esterification of the carboxyl group (Section 2.2). Other properties which were considered important in solvent choice were: availability, toxicity, volatility and ease of drying. Four solvents were investigated for application to cyclization of acylcarnitines; tetrahydrofuran (THF), ethylacetate (EtOAc), N,N-dimethylformamide (DMF) and acetonitrile (CH₃CN).

Experiments employing THF and EtOAc did not produce evidence of cyclization. The negative results may be due to the inability of these media to dissolve the highly polar carnitine compounds. Heterogenous reactions were attempted with varying reaction times and temperatures (up to reflux). The use of ultrasound is reported to be applicable to some heterogenous reactions with dramatic improvements in product yield and reaction time. However, simple experiments employing an ultrasound source indicated that the technique did not facilitate the proposed derivatization of acylcarnitines (Section 6.7).

2.4.2(a) Reactions in N,N-Dimethylformamide (Section 6.3.8)

Dry DMF was a hopeful candidate as a suitable reaction solvent from an early stage in the project. The relatively high boiling point (bp. 153 °C) provided a large reaction temperature range, and its ability to dissolve acylcarnitines was considered advantageous. Previous work (Section 2.4.1) suggested the reflux temperature of
DMF may induce decomposition of carnitine esters. This was recognized in the design of each experiment and reaction temperatures were not allowed to exceed 130 °C. The concentration of acylcarnitine (4-phenylbutanoylcarnitine) in reaction mixtures was also kept low. High concentrations may lead to intermolecular reactions and polymer formation. Acylcarnitine levels between 0.1 and 5% were subject to varying reaction conditions.

Simply heating a 0.1% solution of 4-phenylbutanoyl-carnitine in DMF at 100 °C overnight resulted in a reaction product which eluted on a TLC plate to the same extent as the 4-phenylbutanoyloxy-γ-butyrolactone. TLC analysis of the reaction mixture was complicated by the streaking effect of DMF on the silica plates used. This effect was minimized by heating the spotted TLC plates in an oven to evaporate the reaction solvent, prior to chromatography. The lactone product was heavily contaminated with unidentified by-products and 4-phenylbutanoic acid. At this stage, the free acid could not be distinguished as originating from ester hydrolysis of the acylcarnitine or from over-reaction of the lactone. The reaction mixture assumed a dark brown colour within 3 hours of initiating heating. The formation of by-products detracted from the suitability of DMF as a solvent. Adjusting reaction time and temperature did not improve the derivatization yield to a useful degree.

The addition of base to the reaction mixture was tried, in an attempt to improve the derivatization yield. The intention was to neutralize the acid from the HCl salt of the acylcarnitine. A free carboxylate group is required if an acylcarnitine molecule is to cyclize to the corresponding γ-butyrolactone as in Scheme 7. Any successful cyclizations will generate trimethylamine (NMe₃) in addition to the lactone. Whilst NMe₃ is a gas, any amine entrained in the reaction mixture may contribute to the neutralization process.
The application of inorganic bases was investigated with reactions in DMF. Sodium hydroxide, potassium hydroxide and calcium hydroxide were utilized in separate experiments. Excess base was avoided in all cases because of the known lability of quaternary ammonium salts to undergo Hofmann elimination (Scheme 8). Excess base may also cause ester hydrolysis (Scheme 4) and therefore quantities equimolar to the acylcarnitine were not exceeded. None of the bases were soluble in DMF. Results of these investigations indicated no improvement over previous attempts. In any event, DMF is not regarded as a good solvent for GC because of its poor GC chromatography characteristics (i.e. unsuitable tailing solvent peak with non-polar GC columns).

2.4.2(b) Reactions in Acetonitrile (Section 6.3.9)

The model acylcarnitine, and the naturally occurring acylcarnitines available, were all soluble in CH₃CN. This agrees with the work of others when CH₃CN was used as a solvent for the preparation of acylcarnitine derivatives for HPLC analysis.⁸³

A refluxing solution of 4-phenylbutanoylcarnitine (0.1%) produced the required lactone derivative, detected by TLC, within 20 hrs. Further examination of reaction products was simpler than for reaction in DMF because CH₃CN was easily removed (bp. 80 °C). ¹H NMR and IR analysis confirmed the presence of the 4-phenylbutanoyloxy-γ-butyrolactone (3, R = (CH₂)₃C₆H₅). Acetonitrile solutions remained colourless throughout the reflux period, unlike heated DMF mixtures. This indication of fewer side-reactions in CH₃CN was supported by analytical data on the respective products. The main contaminants of the required derivative were unreacted acylcarnitine and some free acid (i.e. 4-phenylbutanoic acid).

The addition of inorganic bases, as with DMF reactions, was unsuccessful in affecting the yield of derivative in CH₃CN mixtures. However, on the basis of relative GC peak areas, the use of a non-nucleophilic organic base, triethylamine,
produced a significant improvement in acylcarnitine cyclization. A solution of 4-phenylbutanoylcarnitine as its HCl salt in CH3CN, refluxed overnight with a molar equivalent of triethylamine, gave a good yield of the corresponding lactone derivative. The improvement was repeated in the derivatization of acetyl-, octanoyl- and palmitoylcarnitine. Products were confirmed by comparison of analytical data (1H NMR, IR, EI-MS) with that of synthesised lactone standards (Sections 2.3, 6.3.5).

The success of triethylamine may be attributed to its solubility in CH3CN, unique amongst the bases tried. Interaction of base with HCl in the same phase (i.e. CH3CN) appears to encourage the formation of the free carboxylate anion necessary for cyclization. Triethylamine hydrochloride, the by-product from the process, is insoluble in CH3CN and therefore probably takes no further part in the derivatization. Determinations of the yield of each derivative using GC with standard solution of the respective acyl-containing lactone, were performed after modification and improvement of the method so far described.

2.4.3 Application Of Derivatization To Physiological Quantities Of Standard Acylcarnitines

When less than 1 mg of any individual acylcarnitine was cyclized, by the method above, the corresponding lactone product was not detectable. A technique for analysing physiological levels of acylcarnitines with low urine sample availability (typically 1 ml) should detect µg quantities. The problem of free acid production also needed to be addressed. This reaction side-product was regarded as potential analyte, lost because of unsuitable reaction conditions. Heating a solution of standard octanoyl lactone (4, R = (CH2)6CH3) in CH3CN was found to produce octanoic acid within 30 mins. This confirmed the potential of the derivatization conditions to cause pyrolysis of the required lactone compounds (Scheme 16). Alternatively, the free acid may originate directly from the acylcarnitine (Scheme...
Scheme 16
Figure 3. The ReactiVial
15). In either case these reactions reduce the amount of derivative analyte and may limit the sensitivity of the method. Efforts were thus directed towards modifying the existing reaction to meet the requirements of clinical analysis.

Lower reaction temperatures with varying reaction times did not improve γ-butyrolactone yields. Higher reaction temperatures (i.e. > 80 °C) demanded a sealed reaction vessel. The ReactiVial (Figure 3) is designed for such applications. After introducing the reaction mixture, the vial is sealed with a teflon lined septum (teflon side down). Air sensitive reagents may be injected through the septum or samples withdrawn without opening the apparatus. Heat can be applied to the ReactiVial from a waterbath, sandbath or a reaction block. The latter consists of a block of aluminium drilled with holes slightly larger than the outside diameter of the ReactiVial. The aluminium block is placed in a heating unit which thermostatically maintains the aluminium, ReactiVial and reaction mixture at a constant temperature from ambient to 200 °C. Early attempts at derivatizing acylcarnitines in ReactiVials were based on the successful reactions achieved previously with conventional apparatus (i.e. round bottomed flask, condenser, and drying tube). Milligram quantities of the acylcarnitines in CH₃CN with Et₃N, heated in the sealed vessel at 80 °C overnight, produced only small amounts of the corresponding acid. However, no lactone derivatives were detected by IR or ¹H NMR spectroscopy.

Further investigations with smaller quantities of acylcarnitines and sensitive capillary GC analysis of the products, identified the ReactiVial as an excellent reaction vessel. Providing a total acylcarnitine content of around 1 mg was not exceeded, good derivatization yields could be achieved using a 1ml ReactiVial (see later). The cyclization of quantities of acylcarnitines greater than 1 mg however, was best achieved with the open apparatus system used in initial investigations. In such a system, the trimethylamine, liberated on cyclization, may escape from the apparatus. This was not so with a sealed vessel and it was noticed, when opening ReactiVials in which derivatization had been successful, that a strong pungent smell
of NMe3 was released. The 1 ml ReactiVial also limits the amount of CH3CN solvent used in the derivatization process. Concentrations above 1 mg ml⁻¹ of acylcarnitine may inhibit the required cyclization reaction. Intermolecular electrostatic interactions between the carboxylate and quaternary ammonium functions of the acylcarnitine molecules in concentrated solutions, is likely to suppress the required intramolecular reaction (Scheme 7). Effectively, the volume of the reaction vessel dictates the total amount of acylcarnitine which may be cyclized. A 1 ml ReactiVial was found suitable for all clinical urine applications. Total acylcarnitine content of such samples rarely exceeds 0.5 mg ml⁻¹ and therefore good derivatization yields could be expected when 1 ml or less of the urine was analysed.

After trying a number of alternative bases to triethylamine, a more hindered, tertiary amine, N,N-diisopropylethylamine, was found to improve slightly the yield of derivatives. Other workers have also found this base appropriate for neutralizing the dissociated HCl (i.e. when acylcarnitines are present as their HCl salts). If it is assumed that the base is only performing a neutralizing function then an equimolar amount, with respect to the total acylcarnitines present, should represent optimum conditions. Experimentally, it was discovered that varying the amount of base between 50 and 150% equimolar had no detrimental effect on the reaction. This lack of dependence on the amount of base (probably a result of the cyclization generating its own base, trimethylamine) is obviously important to the analysis of unknown quantities of acylcarnitines, and supported further development of the analytical method.

Higher temperatures over shorter reaction times (compared to those used previously) worked well with ReactiVial reactions. Temperatures above 140 °C however, often resulted in ruptured septa and loss of sample, due to the high internal pressures generated. Over many experiments the optimum conditions were found to be 125 °C for 35 minutes.
<table>
<thead>
<tr>
<th>Acylcarnitine (AC)</th>
<th>Quantity of AC Derivatized /µmoles</th>
<th>FID Response for Derivatized AC /area</th>
<th>Quantity of Standard Acyloxylactone /µmoles</th>
<th>FID Response for Standard /area</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcarnitine</td>
<td>0.29</td>
<td>10060</td>
<td>0.79</td>
<td>34050</td>
<td>80</td>
</tr>
<tr>
<td>Octanoylcarnitine</td>
<td>0.22</td>
<td>18955</td>
<td>0.51</td>
<td>59892</td>
<td>73</td>
</tr>
<tr>
<td>Palmitoylcarnitine</td>
<td>0.16</td>
<td>26382</td>
<td>0.29</td>
<td>53131</td>
<td>90</td>
</tr>
</tbody>
</table>

**TABLE 2** Determined derivatization yields for three common acylcarnitines

e.g. Octanoylcarnitine: Amount of derivative detected = \((0.51/59892) \times 18955 = 0.16 \mu\text{moles}\)

\[
\text{% Yield} = \frac{0.16}{0.22} \times 100 \approx 73\%
\]
Derivatization yields were determined by capillary GC using cold on-column injection (Section 6.3.10). Yields for the conversion of acetylcarnitine, octanoylcarnitine and palmitoylcarnitine into the corresponding acyloxylactones were evaluated by comparing the FID response for the derivatives with the response for known quantities of authentic lactone standards. Table 2 summarizes the results obtained. The experiment gave an indication of derivatization yields. The acceptable yields (all in excess of 70%) encouraged further development and application of the method. Results did not account for acylcarnitine impurities and detector response over the μmole range was assumed to be linear. A full quantitative evaluation is envisaged for future work (Chapter 5).

Whilst there appears to be scope for improving the cyclization yields (particularly the octanoyl-carnitine derivatization), yields approaching 100% may not be necessary. A high yield is beneficial in preserving the sensitivity of the method, but if a lower yield provides a detectable signal, reproducibility of the method becomes more important for quantitative analysis. Providing a signal can be monitored, a reproducible yield of the derivative allows quantification with standard samples. Besides, further investigation with standard solutions of acylcarnitines was deemed to have limited usefulness. When it comes to analysing these compounds in the presence of a biological matrix, the effect of other urinary components on the derivatization was expected to be detrimental and unlikely to be assessed by utilizing standard solutions. The effects of the urine matrix required attention before meaningful quantitative experiments could be conducted (Section 2.5).

The ReactiVial reaction conditions were found suitable for the full range of acylcarnitines available. Capillary GC analysis of a mixture of 11 derivatized acylcarnitines produced a chromatogram, shown in Figure 4, with 11 fully resolved peaks. Palmitoylcarnitine was not included in the mixture as such a hydrophobic acylcarnitine was not expected to be found in urine samples. However, this long-chain acylcarnitine was derivatized and the acyloxylactone product analysed by GC.
Figure 4. GC chromatogram of a standard mixture of 11 derivatized acylcarnitines

1. $R = \text{CH}_3$
2. $R = \text{CH}_2\text{CH}_3$
3. $R = \text{CH}($CH$_3)$CH$_2$CH$_3$
4. $R = \text{CH}_2\text{CH}($CH$_3)$$_2$
5. $R = ($CH$_2$)$_3$CH$_3$
6. $R = ($CH$_2$)$_4$CH$_3$
7. $R = \text{CH}($CH$_2$CH$_2$CH$_3$)$_2$
8. $R = ($CH$_2$)$_5$CH$_3$
9. $R = ($CH$_2$)$_6$CH$_3$
10. $R = ($CH$_2$)$_2$Ph
11. $R = ($CH$_2$)$_3$Ph
and GC/MS, in a separate mixture of carnitine esters, demonstrating the application of the technique to a wide range of acylcarnitine analytes. The identity of each peak was confirmed by separate derivatizations of individual acylcarnitines and GC/MS, using electron ionization (EI) and chemical ionization (CI). In three cases (acetyl-, octanoyl- and 4-phenylbutanoyl-containing) the cyclized acylcarnitines were also compared with the independently synthesised lactones (Section 2.3), confirming that the required derivatization had occurred.

Operating parameters for the GC and GC/MS (Section 2.5) were such that lactone derivatives of isomeric compounds (e.g. valerylcamitine/isovalerylcamitine and valproylcamitine/octanoylcamitine) were sufficiently separated by the chromatography, to distinguish each in terms of retention time. The EI mass spectra for isomeric derivatives were difficult to differentiate, and therefore the chromatographic separation was essential for unambiguous identification. The ability to distinguish isomers easily was an important advantage of the GC/MS method over alternative HPLC/MS and MS/MS techniques.

2.4.4 On-Column Derivatization

The early application of GC to the analysis of mixtures from attempted derivatization reactions suggested that the hot injector port could cause the cyclization. Before this was appreciated, false interpretations of the yield of a number of "on-the-bench" derivatizations were made. Investigations showed that comparable peaks to early bench derivatizations could be achieved by the direct injection of acylcarnitine/CH₃CN solutions using an appropriate injector temperature and glass port liner.

Above a temperature of 130 °C cyclization in the injector was found possible. Increasing the injector temperature to 200 °C produced the optimum lactone signal for octanoylcarnitine. Reproducibility however, was poor and the efficiency of the cyclization diminished as involatile residues built up on the walls of the glass liner.
after repeated injections. Periodic cleaning of the liner and replacement of its silanized glass wool packing helped, but cyclization yield from one cleaning procedure to another was very variable. Despite the very poor reproducibility of the method, it did find application as a quick check procedure in the development of ion-exchange extraction of acylcarnitines from various matrices (Section 2.5.3).

The experiments conducted into on-column derivatization confirmed earlier suspicions that the lack of control over the cyclization is a problem. Whilst such a technique would simplify analysis, a pre-column derivatization chamber which could cope with a build up of involatile material and perform reproducible cyclizations is difficult to envisage. Even if such apparatus could be developed it is unlikely that it would be applicable to physiological levels of acylcarnitines without prior isolation from the biological matrix. In addition, it is generally thought that for sensitive and quantitative GC analyses, the best injection method is the cold on-column technique. Clearly, this cold injector is not compatible with effecting a thermal reaction.

2.5 HANDLING THE BIOLOGICAL MATRIX

2.5.1 Why Extract?

Since the beginning of the project it was known that the successful development of a GC/MS method for acylcarnitine analysis would involve dealing with complex biological matrices. Major urine components include high concentration of urea, potassium and sodium salts. Organic acids are also encountered in urine, especially from patients with disorders of fatty acid metabolism (Section 1.1.2). Some existing techniques, notably FABMS and FABMS/MS, require minimum sample preparation, whilst others employ a variety of isolation procedures including solvent extraction, ion pair precipitation, paper chromatography, ion-exchange purification and purification on small silica columns.
The development of any derivatization must account for all components present in
the reaction mixture. This includes constituents of the matrix if the required analyte
has not been isolated. It should be remembered that even if matrix components do
not react with the derivatizing reagent, or under the derivatization conditions, they
may act to suppress (or less often, facilitate) the required reaction. The cyclization
reaction developed in this project was expected to be prone to suppression and
interference effects of matrices such as urine. It has already been shown with
standards that the derivatization required careful control of conditions to optimize
yields. Assuming acylcarnitines could be derivatized in the presence of the urine
matrix, analysis by GC and GC/MS would be complicated by volatile urinary
compounds interfering with qualitative and quantitative evaluations. Urine samples
from patients with β-oxidation disorders would contain levels of organic acids, the
signals from which would swamp any responses for derivatized acylcarnitines,
when analysed by GC and GC/MS. Conventional cold on-column injection
techniques could not be applied either. The build up of involatile material at the
head of the column would rapidly reduce the efficiency of modern capillary GC
columns.

A requirement for some form of sample purification was confirmed by freeze
drying an aliquot of urine spiked with octanoylcarnitine and then attempting to
derivatize the acylcarnitine as previous (Section 6.3.11). GC analysis was not able
to detect octanoyl-containing γ-butyrolactone (3, R = C7H15), above the high
background. As expected, numerous peaks were observed in the resultant
chromatogram, including many broad peaks typical of semi-volatile components.
At this stage, it was not determined whether the derivatization had failed or the
acyloxy lactone peak was swamped by other dominant urine components eluting
from the GC column. Irrespective of this however, the procedure was wholly
unsatisfactory for unambiguous analysis and so efforts were made to extract
acylcarnitines from the urine matrix.
### 2.5.2 Solvent Extraction

Extraction of urinary acylcarnitines has been achieved using various water immiscible solvents. The medium-chain length carnitine esters can be extracted with a chloroform/methanol mixture or butanol, but ion-exchange chromatography is required for short-chain acylcarnitines, such as acetylcarnitine and propionylcarnitine. Both solvent extraction procedures were applied to spiked urine samples. After evaporation of the solvent the oily residue was subject to the developed derivatization conditions (Section 6.3.12). Subsequent GC analysis failed to identify the derivatized acylcarnitines. Either the purification procedures were unsuccessful in extracting the carnitine esters or the techniques were not specific and additional urinary compounds contaminated the acylcarnitine fraction and prevented cyclization.

Whilst the results were obtained before the derivatization conditions had been optimized, they did indicate that alternative purification procedures should be investigated. Other workers analysing urinary acylcarnitines by techniques such as HPLC, HPLC/MS, and 1H NMR spectroscopy favour ion-exchange resins for extraction of the analyte prior to analysis.

### 2.5.3 Ion-Exchange Purification

The ionic nature of acylcarnitines makes them amenable to ion-exchange techniques. Under acidic conditions with the carboxyl group protonated, carnitine and its esters are cationic species. As such, they will pass through a column of anionic-exchange resin but may bind to a suitable cationic-exchange resin. Acylcarnitines bound to a cationic resin may be eluted by displacement with another cation of greater affinity for the resin. That is, by the use of appropriate ion-exchange resins, elution procedures and fraction collection, urinary acylcarnitines can be purified. In practice it is not feasible to isolate completely the acylcarnitines.
from all other matrix components. That is, absolute selectivity to acylcarnitines was not achieved. This is because a suitable extraction method needs to be applicable to a range of acylcarnitines across which there is some variation in affinity for a resin. Within this affinity range there are some urine components (e.g. some amino acids and dipeptides) that have the same ion-exchange elution characteristics as acylcarnitines and hence that are also extracted. The intention was to develop a sample pretreatment which would purify the acylcarnitines to a degree which would enable (i) successful derivatization to the cyclic analogues, and (ii) a relatively "clean" chromatogram to be obtained (i.e. peaks due to cyclized acylcarnitines should not be obscured by other urinary components).

The simplest ion-exchange method investigated was the use of a small column of anionic resin (Section 6.3.13). Two ionic forms of the resin were tried; chloride and formate. The resin was expected to bind most of the anionic species in urine including the organic acids. Under neutral conditions the acylcarnitines were expected to act as zwitterionic, overall neutral, species and pass through the column unretained. The folded structure of acylcarnitines in solution (Section 2.2), might be expected to prevent association of the carboxyl group with the fixed cationic function of the resin. Strong acidic conditions ensure a free passage for carnitine and its esters as cations, but they also protonate the organic acids preventing their binding and reducing the selectivity of the purification process.

Attempts to recover acylcarnitines from water were successful and derivatization to GC amenable derivatives was observed, indicating good recoveries of around 80% with the resin in its formate form. Acceptable purification of spiked urine samples with only an anionic resin was not achieved however. Much of the urine matrix passed through the purification process resulting in a complex GC chromatogram and fouling of the head of the capillary column when on-column injection was used. Peaks were assigned to octanoyl- and palmitoyl-containing lactones but these represented low recoveries (<20%) and were not confirmed by GC/MS. Similar
results were obtained with the chloride form resin although in these attempts even less of the derivatives were detected when analysing the spiked urine sample. Chloride ions have a higher selectivity for the anionic resin used and therefore it is less likely that the acylcarnitines would displace the resin counterions and thus be retained. More likely, the reduced specificity of the clean-up procedure allows more urine components to contaminate the acylcarnitine fraction and subsequently suppress the derivatization.

Cationic-exchange resins were expected to provide a more selective extraction procedure. Acylcarnitines may be bound to the resin and anionic, neutral and weakly bound cationic species washed from the column. The acylcarnitines are then eluted by equilibrating the column with cations of relatively high affinity for the resin, displacing the carnitine esters. The modified methods of others were applied to spiked urine samples and aqueous solutions of acylcarnitines (Section 6.3.14). After extraction, water was removed by lyophilization before derivatization and GC analysis.

The use of cationic resin in the pyridinium form, eluting with pyridinium acetate (Section 6.3.15(i)) was unsuccessful in determining acylcarnitines in spiked urine samples. Several attempts were made at this purification procedure examining the various column fractions, after lyophilization, with the hot-injection port GC method described (Section 2.4.4). Acylcarnitines could not be detected in any fraction. It was proposed that the extraction was still not sufficiently specific to the acylcarnitines and/or they had not been eluted from the column.

Results from the extraction of the aqueous solutions using ammonium hydroxide (0.5M) to elute the acylcarnitines off the resin in its pyridinium form were more positive (Section 6.3.15 (ii)). Analysis of the anionic/neutral washings did not detect any unretained acylcarnitines (as required), but elution with NH4OH did reveal acetylcarnitine and octanoylcarnitine content in the first two 5 ml fractions.
Figure 5. Acylcarnitine extraction and derivatization procedure for clinical urine samples
Estimates of 60% recovery in the first cationic fraction and a further 10-15% in the second were made using split injection GC (Section 6.3.22).

The success with aqueous solutions could not be repeated with spiked urine samples (Section 6.3.15). Lactone derivatives were detected but the estimated recoveries were only around 20%. In these samples the analysis of the anionic/neutral washing was impractical because of the many urinary components complicating the examination. Therefore the possibility of acylcarnitine complexing with other species, reducing the cationic nature and preventing binding, was not discounted.

At this stage in the development, an extraction procedure for urinary acylcarnitines was reported by Millington and co-workers. It involved the combination of anionic- and cationic-exchange purification steps. The process was applied to spiked urine samples with only the mesh size of the resins varying from the published method (Section 6.3.16). It has also been found that the use of the cationic resin in the formate form produces similar results. An aliquot of urine was first passed through an anionic resin, the acylcarnitines being unretained. Acidification of the anionic column effluent ensured that the carboxyl groups of any carnitine compounds were fully protonated before binding to a small column of cationic resin. Acylcarnitines were eluted with ammonium hydroxide solution (1M in 20% aqueous ethanol). Water and base needed to be removed as soon as possible because of the lability of acylcarnitines towards hydrolysis and Hoffman elimination under aqueous base conditions. Freeze-drying was found to be the most expedient method of removing both. This method, when combined with the derivatization procedure and GC or GC/MS analysis (Figure 5), was the most successful applied to date.

After the derivatization step the CH₃CN solvent was removed by evaporation in a stream of nitrogen and the residue triturated with ethyl acetate (EtOAc). The EtOAc
was then filtered prior to GC analysis. This procedure avoided the small amounts of unreacted acylcarnitines or other insoluble material being injected onto the GC column. However, care had to be taken when evaporating the CH₃CN. Prolonged evaporation resulted in loss of the lower boiling point derivatives notably, acetyl- and propionyl-containing acyloxy lactones. This effect was minimized by only evaporating to a damp residue before reconstituting in EtOAc.

Recoveries of acetyl carnitine and octanoylcarnitine from water and urine, using the double ion-exchange method above, were estimated by GC analysis of the resulting derivatives (Section 6.3.17). The FID responses for the extracted and derivatized acylcarnitines were compared with those for a matrix-free sample of the corresponding standard acylcarnitines which was not subject to ion-exchange purification but was derivatized under identical conditions. The results (Table 3) indicate that good recoveries may be obtained for acylcarnitines in water. They compare favourably with the results of others (60% recovery for octanoylcarnitine)¹⁰⁵ using alternative ion-exchange purification procedures. However, the results suggest poor recovery efficiencies for acylcarnitines from urine. Since urine is currently the most important matrix for acylcarnitine evaluation, this area required further investigation.

Recovery efficiencies were determined for a range of carnitine esters added to four infant control urines (Section 6.3.18). The control samples were obtained from infants without disorders of fatty acid oxidation and were expected to be typical of "normal urine" from patients of the age range which would be encountered with disease-state urines (Chapter 3). Results from these urines, spiked with a range of acylcarnitines (0.2 mg of each acylcarnitine per ml of urine) are summarized in Table 4.

Recovery figures were based on the average extraction across the acylcarnitine range investigated. For a given urine sample the percentage recovery for different
<table>
<thead>
<tr>
<th>Matrix</th>
<th>Acylcarnitine</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>WATER</td>
<td>Acetylcarnitine</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>Octanoylcarnitine</td>
<td>85</td>
</tr>
<tr>
<td>URINE</td>
<td>Acetylcarnitine</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Octanoylcarnitine</td>
<td>38</td>
</tr>
</tbody>
</table>

**TABLE 3**: Apparent Extraction Efficiencies for the Ion-Exchange Purification Procedure
<table>
<thead>
<tr>
<th>Urine Sample</th>
<th>Acylcarnitine (% Recovery)*</th>
<th>Average % Recovery ((\bar{x} \pm \sigma n^{-1}))</th>
<th>Ratio FID Responses; Acylcarnitine/4-phenylbutanoylcarnitine</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Acetyl (30)</td>
<td>31 ± 1</td>
<td>Acetyl; 0.6</td>
</tr>
<tr>
<td></td>
<td>Isovaleryl (32)</td>
<td></td>
<td>Isovaleryl; 1.4</td>
</tr>
<tr>
<td></td>
<td>Octanoyl (31)</td>
<td></td>
<td>Octanoyl; 1.3</td>
</tr>
<tr>
<td></td>
<td>4-Phenylbutanoyl (30)</td>
<td></td>
<td>4-Phenylbutanoyl; 1</td>
</tr>
<tr>
<td>C2</td>
<td>Acetyl (56)</td>
<td>57 ± 1</td>
<td>Acetyl; 0.6</td>
</tr>
<tr>
<td></td>
<td>Isovaleryl (58)</td>
<td></td>
<td>Isovaleryl; 1.4</td>
</tr>
<tr>
<td></td>
<td>Octanoyl (57)</td>
<td></td>
<td>Octanoyl; 1.3</td>
</tr>
<tr>
<td></td>
<td>4-Phenylbutanoyl (57)</td>
<td></td>
<td>4-Phenylbutanoyl; 1</td>
</tr>
<tr>
<td>C3</td>
<td>Acetyl (47)</td>
<td>48 ± 1</td>
<td>Acetyl; 0.6</td>
</tr>
<tr>
<td></td>
<td>Isovaleryl (47)</td>
<td></td>
<td>Isovaleryl; 1.4</td>
</tr>
<tr>
<td></td>
<td>Octanoyl (49)</td>
<td></td>
<td>Octanoyl; 1.3</td>
</tr>
<tr>
<td></td>
<td>4-Phenylbutanoyl (48)</td>
<td></td>
<td>4-Phenylbutanoyl; 1</td>
</tr>
<tr>
<td>C4</td>
<td>Acetyl (84)</td>
<td>83 ± 1</td>
<td>Acetyl; 0.6</td>
</tr>
<tr>
<td></td>
<td>Isovaleryl (83)</td>
<td></td>
<td>Isovaleryl; 1.4</td>
</tr>
<tr>
<td></td>
<td>Octanoyl (83)</td>
<td></td>
<td>Octanoyl; 1.3</td>
</tr>
<tr>
<td></td>
<td>4-Phenylbutanoyl (81)</td>
<td></td>
<td>4-Phenylbutanoyl; 1</td>
</tr>
</tbody>
</table>

**TABLE 4**  Recoveries of spiked acylcarnitines from control urine samples

*Recoveries determined by comparison with a standard mixture of acylcarnitines derivatized and analysed under the same conditions (Section 6.18)
acylcarnitines was constant. For instance, the apparent average recovery for urine C2, was 57% for the four acylcarnitines. The consistency was not observed between urine samples however. Recoveries from C1 were the lowest at 31% and the highest from C4 at 83%. The results confirm the requirement for an acylcarnitine internal standard in any quantitative determinations. Ratioing the responses within a single sample to that of say 4-phenylbutanoylcarnitine (as the corresponding γ-butyrolactone derivative) allows comparisons between samples. As Table 4 shows, for each acylcarnitine the response ratio was constant from sample to sample. In the case of isovalerylcarnitine for example, the response ratio to the internal standard, 4-phenylbutanoylcarnitine, was 1.4 for all four urines examined.

Whilst the use of an acylcarnitine internal standard overcame the apparent variation in recoveries from urine and hence would allow quantitative analysis, it did not explain why the variation arose. Two possible causes were favoured. The acylcarnitines may not behave as expected during the extraction step due to some complexation process with other urinary components. This may result in loss of analyte during either the anionic- or cationic-exchange procedures. Alternatively, urine components other than acylcarnitines extracted by the sample pretreatment may be hindering the derivatization. Therefore an experiment was conducted (Section 6.3.19) whereby a urine sample spiked with isovalerylcarnitine and octanoylcarnitine was extracted and analysed as previously. The results were compared with those from the same urine sample spiked after extraction. Within experimental error, the FID detector responses for the corresponding derivatives were the same for the pre- and post-extraction spiking tests. If the acylcarnitines were being extracted to a different extent in the presence of urine, then the derivative concentrations in the final sample would be higher for the post-extraction spiking experiment than for the pre-extracted. Therefore, the results indicated that the variations observed between urine samples were not due to differing recoveries,
but may be assigned to a varying derivatization yield. This is probably because of one or more undesirable components coming through the work-up and interfering with the cyclization.

That urine components other than acylcarnitines did get through the extraction procedure was supported by observing differing amounts of solid materials present after the freeze-drying step, immediately before derivatization. Solid material was also encountered after the cyclization reaction and evaporation of the acetonitrile. The residue was insoluble in ethyl acetate and was filtered off by passing through a Millipore filter unit prior to GC analysis.

Urea was suspected as a major contaminant and this was confirmed by GC/MS. The presence of urea was not obvious from the GC results because of the poor sensitivity of the FID to compounds which do not contain C-H bonds. Using GC/MS with EI however, the urea produced a very large broad peak in the total ion current chromatogram (see TIC traces later, e.g. Figure 7).

Under EI conditions the urea was easily detected and characterized by ions with \( m/z \) 60 (M+) and 44 (H₂NC=O⁺). In all urine samples analysed by GC/MS a large overloaded peak was attributed to urea and this caused problems when co-eluting with the more volatile acylcarnitine derivatives of interest (Section 3.2.2). Attempts were made to remove this unwanted compound by enzymatic digestion with urease. Urine samples were treated with urease tablets before and after the extraction process. In both instances however, the urea was not completely removed and continued to cause problems when analysing for acetyl and propionylcarnitine, by co-eluting from the GC column with the derivatives of interest. The removal of urea warrants further investigation, possibly with pure urease rather than the tablet form.
2.5.4 Use of Bond Elut CERTIFY Columns

These "ready to use" cartridge-type units are gaining popularity in the area of biological fluid analysis. They present a convenient and rapid method of extracting many compounds of interest from urine. Their flexibility arises from a mixed-bed ion-exchange resin (cationic- and anionic-exchange resins), which on sample application, binds most charged species (i.e. cations and anions). Extraction selectivity is then obtained by careful choice of eluting solvents and control of pH. Established methods exist for extracting drugs of abuse from urine for GC and GC/MS analysis. Modifications of these methods were applied to acylcarnitine extractions (Section 6.3.20). Unfortunately the elution systems investigated were not successful and since an acceptable ion-exchange procedure (Figure 5, and Section 2.5.3) had been identified, further attempts were not made using Bond Elut CERTIFY columns. Further work with these units is recommended since they are already common in clinical screening laboratories and potentially represent a simpler extraction procedure than the double ion-exchange procedure reported here and subsequently applied to metabolic investigations (Chapter 3).

2.6 GC AND GC/MS: CONDITIONS AND TECHNIQUES
APPLIED TO ANALYSIS OF ACYLCARNITINE DERIVATIVES

The main objective of the project was to be able to analyse acylcarnitines by methods involving GC. Therefore, during method development, frequent attempts were made to apply techniques such as packed-column and capillary GC, GC/Fourier transform infrared (GC/FTIR) and capillary GC/MS to reaction products. GC/FTIR was of very limited success because high sample loading was required on wide-bore capillary columns to gain sufficient sensitivity for additional IR information over the chromatography retention time. This is unsuitable for
clinical urine samples when detection of acylcarnitines at physiological levels demands high sensitivity on limited sample volume. Sensitivity problems were also encountered with packed-column GC. Both on-column and vaporizing injection techniques were investigated but only decomposition products from the derivatives could be detected. This was probably due to active sites within the column and/or the injector port region. Whilst packed-columns are capable of handling high analyte amounts before overloaded peaks result, the disadvantages of active sites, stationary phase bleeding, low temperature limits, poor resolution and a lack of consistency between columns in their ability to separate a mixture, has severely limited their application. Modern vitreous silica capillary GC columns have largely superseded packed columns. Capillary columns are superior in separating efficiency, durability, consistency between columns and they are much simpler to interface to a mass spectrometer. The GC and GC/MS analyses reported here were all performed with wall-coated open tubular (WCOT), vitreous silica, capillary columns.

The columns used for GC and GC/MS applications were those with a non-polar stationary phase, mainly the BP5 type (5% diphenyl dimethyl siloxane) from SGE. These columns have an immobilized stationary phase, chemically bonded to the silica walls of the column and heavy cross-linking within the phase. This allows for greater film thickness, temperature limits and gas flows. A range of separations is possible using different film thicknesses rather than the conventional approach of using different polarity columns.

For GC analysis with FID detection, a 0.32 mm i.d., 25 m, BP5 column with a 0.5 μm film thickness was generally employed. This allowed for suitable flexibility in sample loading and provided the required efficiency when helium was used as the carrier gas. A linear carrier gas flow around 30-40 cm/s was used in accordance with Van Deemter curves, to maintain good efficiency. Helium was employed rather than hydrogen in order to be consistent with GC/MS analyses.
For use in GC/MS apparatus, a narrower bore capillary was required. The interface accepted a 0.25 mm, BP5 column and phase thicknesses of 0.25 and 0.5 µm were used. The thinner film thickness enabled higher efficiency separations but required lower sample loadings to avoid overloaded peaks.

Correct choice of GC injection technique was found to be very important for the analysis of derivatized acylcarnitines. The sample introduction techniques used are worth further description so that the results obtained with the respective injection methods can be fully interpreted.

2.6.1 Vaporizing Injection GC

Vaporizing a sample in an injection chamber or port before chromatography was the first method of sample introduction to be developed for capillary GC. Split and splitless injection are established as the classical means of introducing analytes into the GC column. Despite several recognized disadvantages and limitations they are still by far the most common injection techniques. The vaporizing injection methods were employed during this project as well as the alternative on-column injection techniques.

In doing so, different operating conditions were required to optimize selectivity and sensitivity depending on the sample introduction technique. An account will thus follow on how and why the conditions employed were identified as suitable for GC analysis of volatile acylcarnitine derivatives.

2.6.1.1 Split Injection

During split injection the vaporized samples is divided into two unequal parts. A small proportion is carried into the column by the flow of carrier gas, whereas the major part is vented through a split exit. Between 0.3 and 20% (depending on the split ratio) of the injected sample enters the column and is chromatographed. The
splitting process ensures that sample vapour is carried onto the column for only a short period of time. This transfer time determines the width of the solute bands and should be sufficiently short to result in sharp eluting peaks.

The major problem with split injection is the poor reproducibility encountered between injections. This was initially thought to arise from the composition of that part of the sample which enters the column being different from the original sample (discrimination). Whilst this effect does exist and has been demonstrated with some elegant experiments with high boiling point alkanes, many early difficulties arose from selective ejection of different solutes out of the syringe needle. Appropriate syringe design and handling techniques can improve reproducibility but discrimination cannot be eliminated. For meaningful quantitative results with split injection, internal standards are essential.

The design of the syringe is important in ensuring a narrow bandwidth of sample on the head of the column. Plunger-in-barrel syringes ensure rapid expulsion of sample when the plunger is depressed. This is important to avoid evaporation of sample/solvent in the needle leading to discrimination. Wire-in-needle type syringes tend to expel the sample more slowly and discrimination effects are more noticeable.

Syringe handling techniques can also have remarkable effects on resulting peak shapes and resolution. For all split injections performed during this project, the hot needle technique was employed. The required volume of sample is drawn up in the syringe, then the outside of the needle wiped clean. Next, a plug of air is drawn up until all of the sample is visible in the barrel of the syringe. On inserting the needle into the injector, a pause of a few seconds allows the needle to heat to the temperature of the injection port. Depressing the plunger ensures sample is rapidly expelled from the needle by the combined action of the plunger with the effect of boiling solvent in the needle. This is in contrast to filled- and cold-needle
techniques where the sample evaporates as the needle is heated and before the plunger is depressed, leading to discrimination against high boiling point components and band-broadening.

Split injection is an inherently insensitive technique because the majority of the sample is vented to waste prior to chromatography. Although high resolution and sharp peaks are relatively easy to achieve, if sample concentration is low (i.e. less than 100 ppm) alternative techniques are required in which more of the analyte enters the column. During development of the derivatization conditions, split injection was suitable when reactions with relatively large quantities of standard acylcarnitines were being investigated (i.e. greater than 0.1 mg). However, once the best derivation conditions had been identified and interest turned to applying the reaction to physiological levels of acylcarnitines and clinical urine samples, sensitivity became a problem with split injection. Splitless and on-column injection techniques became necessary.

2.6.1.2 Splitless Injection

The splitless injection technique is based on using a split injector with the split valve closed. The column is the only exit for the sample vapour from the vaporizing chamber during the injection. After a preset period the split exit is opened to purge the injector and chromatographic separation begins. Since virtually all of the sample material is carried onto the column, splitless injection achieves a higher sensitivity than split injection. Suitable solute concentrations vary from 0.1 to 50 ppm when an FID is used.

Most of the chromatography difficulties associated with splitless injection can be attributed to band effects, due to the large amount of sample entering the column compared to split injection. The sample transfer (at optimum carrier gas flow rate for chromatography) from the vaporizing chamber to the column takes several tens of seconds to complete. This creates excessively broad initial bands and if steps are
not taken to correct this effect, unacceptably broad peaks will result. The chromatographer must employ one of two known techniques to reconcentrate the initial bands before or at the start of separation. Both "cold trapping" and "solvent effect" act to block the first section of the sample vapour on the head of the column, until the last of the material has been transferred from the vaporizing chamber. The techniques have been described fully elsewhere\textsuperscript{121} but essentially temporary retardation may be achieved by lowering the GC oven temperature below the elution temperature of the solutes (cold trapping effect) or by an increase in film thickness of the stationary phase using the sample solvent as liquid phase (solvent effect). For reproducible results and quantitative evaluations using splitless injection, such reconcentration techniques must be employed. This is a distinct deficiency of splitless injection as it may require a skilled chromatographer with substantial experience to achieve optimum separation and sensitivity.

Like split injection, splitless injection suffers from selective expulsion of sample out of the syringe needle. When splitless injection was used in this project the hot needle method was used to minimize discrimination.

Whilst the injection technique allows sensitive analyses, as compared to split injection, care is still required not to inject too large an amount of sample in the hope of gaining extra sensitivity. The maximum sample quantity which may be injected is dependent on a number of factors including the vaporizing chamber volume. A situation must not be allowed to arise whereby the sample vapours expand backwards out of the injector port and down the gas lines. There is no method for accurately calculating the maximum tolerable sample volume and hence this must be determined experimentally, if the chromatographer wishes to work at the limit of sensitivity. Typically, 1 \textmu l volumes were used whenever splitless injection was applied to derivatized acylcarnitine investigations.
An advantage of splitless injection is the ability to handle "dirty" samples and it has thus found many applications in trace analysis. Involatile material (providing it is expelled from the syringe needle) remains on the walls of the injector liner which may be periodically removed and cleaned. This may extend the life of the column especially when compared to analysing similar samples by on-column injection techniques.

Splitless injection was often used in GC/MS applications during this project. It became apparent that an injector temperature limit had to be imposed to avoid ester pyrolysis (Scheme 16) of γ-butyrolactone compounds in the vaporizing chamber. Table 5 summarizes the results from an investigation (Section 6.3.21) into the effect of injector temperature on the analysis of derivatized octanoylcarnitine. At low injector temperatures (e.g. 150 °C) sample transfer out of the vaporizer chamber was poor. So much so, that the peak for the derivative was only just detected as a broad signal of low peak height. An injector temperature increase to 230 °C improved the size of the signal and its peak shape. This temperature was regarded as optimum for the analysis of the octanoyl-containing acyloxy lactone and became the standard setting for all acylcarnitine determinations which used vaporizing injection GC techniques. Raising the temperature above 230 °C caused substantial pyrolysis of the derivatives with the associated detection of products of this decomposition (Scheme 16). This process was unfavourable in mixture analysis as the liberated fatty acids may have retention times very similar or identical to those of other peaks of interest. Therefore, even though injector temperatures of 250 and 280 °C produced excellent peak shapes for the derivative, such conditions were best avoided for unambiguous acylcarnitine analysis. High injector temperatures may be required if the primary interest of the analyst is in the long-chain acylcarnitines, however. For instance, an injector temperature of 230 °C was not suitable for the splitless injection analysis of palmitoylcarnitine. The high boiling point of the acyloxy lactone derivative of this compound results in poor transfer out of the
### TABLE 5: Effect of injector temp. on GC behaviour of cyclized octanoylcarnitine

*Peak heights and shapes refer to compounds in the following decomposition reaction:

\[
\frac{\text{C-O}}{\text{aH}}_3\text{co} + \text{RCOOH} \rightarrow \text{5} + \text{RCOOH}
\]
vaporizing chamber and therefore a poor peak shape. Such cases warrant high injection temperatures or, as preferred for GC analysis of all acylcarnitine derivatives, on-column injection.

2.6.2 Cold On-Column Injection GC

On-column injection techniques are generally regarded as unrivalled for precise quantification and good sensitivity. As the name suggests, the sample is injected directly into the head of the column prior to vaporization. A cooling circuit around the head of the column ensures sample initially enters the column as a liquid. Carrier gas then transports the sample plug into the heated portion of the column where vaporization and separation occurs. Careful control of the oven temperature is used to reconcentrate the solute bands and enable high efficiency separations.

Cold on-column injection was particularly useful in this project for the analysis of lactones derived from acylcarnitines. As observed with vaporizing injection methods, the lactone compounds were susceptible to decomposition on hot surfaces. Cold on-column injection was found to cope with this thermal lability and it became the method of choice for analysing acylcarnitine derivatives by GC and GC/MS.

Using standard solutions of synthesised acyloxylactone compounds, a linear relationship between concentration and peak area was observed. For example, over the range 1 - 90 nmol, the correlation coefficient was 0.996 (nine points). The detection limit of the lactone compounds was concluded to be in the sub-nmol range but was not determined accurately. Accurate determinations await full evaluation of selectivity and recovery efficiency of extraction procedures for urine which will ultimately dictate the detection limits in biomedical application. At this stage however, detection limits for standards below the nmol level were regarded as suitable for attempting the determination of physiological levels of acylcarnitines in clinical samples.
Efforts were taken to minimize non-volatile components which are not "trapped-out" by on-column injection and eventually lead to deterioration of column performance by accumulating on the first few centimetres of the column. When analysing urine samples, only those which had been taken through the full extraction procedure (Figure 5) were subject to on-column injection. Despite this, the first 30 cm of column were periodically removed when the chromatography became noticeable affected.

Temperature programming parameters were deduced empirically to obtain complete resolution of eleven, standard, derivatized acylcarnitines as shown in Figure 4. These conditions were then applied to extracted and derivatized clinical urine samples. This enabled preliminary investigation of many urine samples from infants with defective fatty acid and amino acid metabolism (Chapter 3). Further confirmation and identification of components was achieved using capillary GC/MS with on-column injection or splitless injection with a vaporizer chamber maximum temperature of 230 °C.

2.6.3 GC/MS Investigations

The benefits of structural information on peaks eluting from the GC was appreciated and was often deemed necessary when examining complex mixtures. This often occurred with clinical urine samples despite the multistep sample pretreatment (Figure 6). To date, only the qualitative abilities of GC/MS have been exploited. Sufficient sensitivity was available in the full-scan mode for initial investigations of clinical urine samples. Further sensitivity and quantification is envisaged with the use of selected ion monitoring (SIM), (Section 3.2.4).

Interfacing the GC with the mass spectrometer was achieved by direct insertion of the capillary column into the ion source. Ionization and separation efficiency were not too adversely affected with this arrangement. The temperature of the interface section between the GC oven and the mass spectrometer ion source was maintained.
Figure 6. GC analysis of urine from a child with MCADD
at 10 °C above the final programmed GC temperature. This maintained transfer of analytes through the column and did not induce any observable on-column decomposition.

Two ionization methods were used: electron ionization (EI) and chemical ionization (CI). EI was the most utilized method and produced characteristic fragmentation of the acyloxy lactone compounds. However, EI rarely produced molecular ions with the compounds of interest but CI produced complementary data.

The EI mass spectra were very consistent throughout the acyl carnitine range investigated. Fragmentations of the acyl chain were used to distinguish the different derivatives. Major ions observed and proposed fragmentation pathways are summarized in Scheme 17. The EI mass spectra of acetyl-, octanoyl-, palmitoyl- and 4-phenylbutanoyl-containing derivatives were identical to the corresponding synthesised lactone compounds (Sections 2.3 and 6.5) and examples are included in the Appendix.

Characteristic ions immediately identified as originating from the acyloxy lactone compounds include the signal at $m/z = 85$. This ion was often present as the base peak in the mass spectrum of the derivatives. The peak may be attributed to cleavage of the whole acyl chain, leaving the charge on the lactone ring residue. It is interesting to note that in the case of short-chain acyl carnitines, notably acetylcarnitine and propionylcarnitine, this process is not so significant and so does not yield a base peak at $m/z = 85$. In these cases formation of the unsaturated lactone ring residue accounts for the most abundant ion at $m/z = 84$. This process does occur with acyl carnitines of longer chain length but to a decreasing extent with increasing chain length.

Another common ion in the EI spectra was that occurring at $m/z = 144$. This product of a McLafferty rearrangement was formed when the acyl chain contained four carbons or more. In the case of 4-phenylbutanoyl-containing lactone (3, $R =$
Loss of \( \text{CH}_3, \text{CH}_2\text{CH}_3, \text{CH}_2\text{CH}_2\text{CH}_3 \) etc. (depending on chain length)

\[
\begin{align*}
\text{Scheme 17}
\end{align*}
\]
the ion at \( m/z \) 144 accounted for the base peak in the mass spectrum. The aromaticity of this compound, in common with the derivative of 3-phenylpropionylcarnitine, stabilized their molecular ions, producing larger \( M^+ \) peaks.

The identification of lactones from acylcarnitines by GC/MS analysis of extracted and derivatized urine samples benefited by a knowledge of the known fragmentation patterns of the standard acyloxy lactones. In particular, the use of mass chromatograms proved very effective for locating peaks of interest. By using the data system to plot the variation of ion abundance at a specific \( m/z \) value with time, detection of several acylcarnitines was made from otherwise complex total ion current (TIC) chromatograms. In Figure 7, mass chromatograms at \( m/z \) values of 84, 85 and 144 are shown alongside the TIC trace, typical of a clinical urine analysis. Acylcarnitine presence was supported by coincident peaks in both mass chromatograms. For example, the peak eluting at scan number 544 is due to hexanoylcarnitine and may have been missed on the basis of the TIC trace alone. Further evidence was given by the retention time and mass spectrum of this peak which matched the authentic standard hexanoylcarnitine (although the mass spectrum showed additional ions due to co-eluting material). Similarly mass chromatograms at \( m/z \) 84 were invaluable for identifying acetylcarnitine and propionylcarnitine because of the co-elution of these components with an overloaded urea peak (Section 3.2.2).

Most of the lactones from physiological acylcarnitines in urine were readily recognized from their EI mass spectra by comparison with reference mass spectra from derivatized standards. However, in the case of isomers, the EI spectra were often similar. Figure 8 shows the EI positive-ion spectra of derivatized octanoyl- and valproylcarnitine. The similarities between the spectra mean that more information is required to distinguish isomers unambiguously. The task is made easy by comparing the GC retention times with standard octanoyl and
Figure 7. The use of mass chromatograms to detect acylcarnitines in a clinical urine sample
Figure 8. EI mass spectra of isomeric acylcarnitine derivatives
(a): octanoyl carnitine derivative (b): valproyl carnitine derivative
valproylcarnitine, thus demonstrating a major advantage over MS/MS techniques. Other than differentiating isomers in mixtures, the comparison with reference spectra was usually sufficient for positive identification of acylcarnitine derivatives. If reference spectra had not been available, the EI spectra could have been interpreted but may have been difficult to assign because of the very small, or often absent, molecular ion peak. Determining the molecular mass was best achieved using CI conditions, then the accumulated data from both EI and CI spectra could be used to identify the lactone compound.

Chemical ionization was performed using ammonia or methane as reactant gas. This milder form of ionization was effective in forming quasi-molecular ions especially with CH₄ reactant gas ions. Results obtained with ammonia indicated that protonated molecular ions and ammonium adduct ions ([M + NH₄]⁺) were formed. Using methane as reactant gas gave good CI spectra (Appendix). The protonated molecular ion was always evident and was usually the base peak. Relative molecular masses of the lactones were immediately evident from these spectra. From the investigations conducted into CIMS of acylcarnitine derivatives, it was concluded that the technique provided useful complementary data on the acyloxylactone compounds. It is recommended that CI is employed if the EIMS and retention time of a peak is not sufficient for unambiguous assignment.
CHAPTER 3

APPLICATION OF THE ACYLCARNITINE DERIVATIZATION:
RESULTS AND DISCUSSION
3.1 INTRODUCTION

Once the derivatization and analysis methods had been shown to work successfully on urine spiked with acylcarnitines, the procedure was applied to clinical samples. During the course of the project many urine samples were kindly provided by three hospitals with paediatric departments and/or interests: Sheffield Childrens Hospital, Southampton General Hospital and Höpitaux de Lyon. Samples were usually sent by post in standard 5-10 ml biological sample tubes. They were stored frozen prior to despatch and on arrival were immediately refrozen and stored at -30 °C. Creatinine concentrations were often determined before the samples left the hospital. For acylcarnitine evaluation, individual urine samples (2-5 ml) were thawed at room temperature, then an analysis aliquot (0.5 ml) removed and the remainder refrozen and stored. Repeat analyses on a given urine suggested that this procedure had no effect on urinary acylcarnitine content and its evaluation.

Since the application to clinical urine samples was a continuous process over much of the project, early attempts did not employ optimum extraction and derivatization conditions. Therefore, initial applications (e.g. cases of MCADD) gave complex chromatograms compared to later analyses (e.g. Figure 6 as compared to Figure 10). Care must be taken when comparing analyses and, because of the ongoing development, quantitative sample comparisons were only estimated. However, the technique has been used to identify trends in urinary acylcarnitine concentrations (see later) and current data suggest the method can be developed into a fully quantitative analysis (Chapter 5).

The majority of work carried out on clinical samples has concerned well-characterized diseases which are known to result in excretion of elevated amounts of acylcarnitines in the urine. The well-characterized nature of the disease state was especially important for early applications, when the technique needed to be proved as a suitable method of investigating metabolic disorders. Therefore, in cases of
MCADD, octanoylcarnitine and hexanoylcarnitine were expected to be present and initial efforts were made in detecting only these carnitine esters. Similarly, isovalerylcarnitine is known to be excreted in cases of isovaleric acidemia, as is propionoylcarnitine in propionic acidemia, and thus the experimental approach was centred around these expected metabolites. With the use of GC/MS and increasing experience of the analysis, however, it soon became possible to detect not only the major acylcarnitines, but also some minor and some unexpected ones as well. These observations have continued to prove interesting, enabling speculation of the metabolism (or defective metabolism) of some endogenous and two exogenous compounds. 4-Phenylbutanoylcarnitine (2, R = (CH$_2$)$_3$Ph), originally employed as a model acylcarnitine for development of the derivatization (Chapter 2), continued to be useful as a suitable internal standard and indicator of cyclization. This acylcarnitine is not known to occur naturally, nor has it been reported in the metabolism of any exogenous compounds to date. In most analyses of clinical urine samples, a known amount of the synthesised phenyl-containing acylcarnitine was added to the urine before extraction and derivatization. Subsequent GC and GC/MS analysis then identified the 4-phenylbutanoyl-containing lactone (3, R = (CH$_2$)$_3$Ph), if the sample pretreatment was successful. From previous investigations (Section 2.3.3), it may be assumed that other, physiological, acylcarnitines behave similarly to (2, R = (CH$_2$)$_3$Ph) and so the presence of (3, R = (CH$_2$)$_3$Ph) after derivatization should be accompanied by other γ-butyrolactones if urinary carnitine esters occur in the original sample at levels above the limit of detection. Comparison of respective signals (Section 6.3.19) can give an indication of acylcarnitine levels.
3.2 INVESTIGATIONS OF DEFECTIVE LIPID AND BRANCHED-CHAIN AMINO ACID METABOLISM.

3.2.1 Medium-Chain Acyl-CoA Dehydrogenase Deficiency (MCADD)

Several inherited defects of fatty acid oxidation have been recognized during the last decade. A deficiency of mitochondrial, medium-chain acyl-CoA dehydrogenase (MCAD) is one such disorder. MCADD has recently drawn considerable attention because of its relatively high incidence (compared to other fatty acid oxidation defects), high mortality, non-specific symptoms and the difficulty of diagnosing it.

The genetic deficiency of MCAD was first described by Kølvraa et al.\textsuperscript{124} in 1982 and since then, approximately 100 cases have been reported in the literature.\textsuperscript{20} In a survey conducted in the Sheffield area of England, Bennet et al.\textsuperscript{125} have recently reported that the incidence of pathological dicarboxylic aciduria (indicative of MCADD) was 1 in 5000 live births. Although further studies to confirm the origin of the dicarboxylic aciduria, are necessary, the evidence does suggest that MCADD is one of the most common inborn errors of metabolism. For example, it is probably more prevalent than phenylketonuria (PKU), a genetic defect in phenylalanine metabolism for which babies are routinely tested.\textsuperscript{52} Patients with MCADD are unable to utilize fatty acids for energy or ketone production and hence suffer energy depletion when glycogen stores are low or exhausted. The resulting hypoketotic hypoglycaemia is often life threatening. Typical clinical symptoms of MCADD include intolerance to fasting, episodic vomiting, lethargy and episodes of coma. These are usually accompanied by medium-chain dicarboxylic aciduria.

Dicarboxylic acids are by products of an alternative fatty acid oxidation pathway, microsomal \( \omega-1 \) oxidation, which becomes stressed as mitochondrial \( \beta \)-oxidation is curtailed. An episode of MCADD may occur abruptly in apparently healthy children, with a mortality rate, within the first two years of life, of 59% with the first episode.\textsuperscript{53} Unfortunately, the typical symptoms mentioned above are not always evident (or sufficiently characteristic) and this has led to considerable
confusion during diagnosis. Initially, patients with MCADD were reported to have Reye's syndrome\textsuperscript{126-128} and further studies have indicted that in some cases MCADD has been misdiagnosed as sudden infant death syndrome (SIDS).\textsuperscript{129-131} Strictly, the term SIDS should be reserved for sudden unexplained deaths (cot deaths). There is a growing agreement that fatal cases of defective oxidation diseases including MCADD may be categorized as sudden unexpected death syndrome (SUDS).

A deficiency of the MCAD enzyme causes a block in the metabolism of long-chain acyl-CoA species, as indicated in Scheme 18. Characteristic urinary hydroxyacids, dicarboxylic acids and glycine conjugates, arising from the alternative metabolic processes are also recognized for this disorder. Clinical diagnosis of MCADD, in the hospital environment at least, relies on detection of these alternative metabolic products to support the clinical symptoms. GC/MS, after TMS derivatization, is often employed to determine the organic acids present in the urine. Suberylglycine and hexanoylglycine, absent in the urine of healthy patients, are typical metabolites excreted by infants with MCADD. These compounds are often regarded as diagnostic of MCADD. However, the usefulness of this approach in asymptomatic patients has not been fully evaluated. The glycine conjugates are usually accompanied by elevated medium-chain dicarboxylic acid concentrations,\textsuperscript{132} and phenylpropanoylglycine may also be present (see Section 3.2.1). An experienced analyst, familiar with MCADD cases and the GC/MS data they present, should be able to identify the disorder with an acceptable degree of confidence. However, there remains a potential shortcoming of this approach, in that the analyst is not monitoring the actual defective metabolic process. Could it be that the glycine conjugates and elevated dicarboxylic acids may arise from another metabolism disorder, independent of MCADD?

In order to investigate the defective metabolic process itself (i.e. mitochondrial β-oxidation), it is necessary to perform enzymatic assays of MCAD activity in patient
Scheme 18. Degradation of long-chain (LC) fatty acids by mitochondrial β-oxidation. MCAD deficiency results in accumulation of medium-chain (MC)-acyl-CoA, which are excreted as MC-acylcarnitines when adequate L-carnitine is available.
or at least analyse the carnitine esters; products from the detoxification process of carnitine (Chapter 1). All current methods for assaying MCAD are cumbersome, taking several weeks to produce results and only a few laboratories provide the service worldwide. The difficulties associated with current acylcarnitine analyses have been discussed (Section 1.2), but the development of an alternative, rapid, simple and accurate method would be useful to some investigative studies of MCADD.

As indicated in Figure 10 certain acylcarnitines may be expected in the urine of patients with MCADD. Octanoylcarnitine and hexanoylcarnitine are usually the most prevalent but studies using FABMS and FABMS/MS have identified others. The application of the derivatization/GC and GC/MS method, developed in this project, initially aimed to detect the major acylcarnitines with the future objective of also confirming, or otherwise, the presence of octenoyl-, decanoyl- and decenoylcarnitine, in urine from patients with MCADD.

Urine samples from eleven patients with MCADD were analysed using the developed GC and GC/MS methods. Several samples were available from some patients, allowing interpretation of urinary acylcarnitine status at different stages of the disease. Urine from an MCADD patient undergoing L-carnitine supplementation was obtained, as were regular samples over the first four-week postpartum period of a baby with MCADD.

Early attempts at using the developed method to analyse for endogenous acylcarnitines in clinical samples involved application to urine from two babies with MCADD. Figure 6 shows the gas chromatogram obtained after subjecting 0.5 ml of urine from one of the infants to the double ion-exchange purification procedure and derivatization conditions. The peak eluting at 12.68 min had the same retention time as the lactone derivative of octanoylcarnitine. Confirmation of this result was obtained by GC/MS analysis of the same sample. Figure 9 shows the
Figure 9. GC/MS analysis of urine from a child with MCADD
total ion current trace and the mass spectrum of the peak at scan number 798 (which also corresponded, in retention time, under GC/MS conditions, to the octanoylcarnitine derivative). A comparison with the EI mass spectrum of the authentic octanoyl-containing acyloxylactone (Appendix, MS 6) clearly confirms the identification of octanoylcarnitine in the clinical sample. Using mass chromatograms at \( m/z \) 85 and 144 and examining the relevant mass spectra, a further three lactones from acylcarnitines were identified. This first clinical urine sample had been spiked with synthesised 4-phenylbutanoyl-containing \( \gamma \)-butyrolactone (3, \( R = (\text{CH}_2)_3\text{Ph} \), after derivatization, as the first step towards assessing the suitability of 4-phenylbutanoylcarnitine (2, \( R = (\text{CH}_2)_3\text{Ph} \) as an internal standard. The lactone was detected as a peak eluting at scan 1019 in the TIC trace (Figure 9) and having a corresponding mass spectrum. Another expected lactone compound was the derivative of hexanoylcarnitine found at scan 648, using mass chromatograms. This technique also identified mass spectra consistent with the lactone from 3-phenylpropanoylcarnitine (2, \( R = (\text{CH}_2)_2\text{Ph} \), originally an unexpected metabolite but subsequently explained (Section 3.3.1).

The results from this first MCADD sample validated the application of the developed method to acylcarnitine determinations in biochemical studies. The appearance of a peak for the aromatic lactone (2, \( R = (\text{CH}_3)_3\text{Ph} \) in an appropriate, uncluttered part of the chromatogram, with a convenient retention time (i.e. not excessively long), supported the use of 4-phenylbutanoylcarnitine as an internal standard for future quantitative analyses. As expected from the results of analysing standards, the technique was sensitive enough to detect the characteristic major urinary acylcarnitines of MCADD, thus prompting further application.

The general conclusion, drawn from the analysis of the MCADD sample and several normal urine samples, was that the disease state samples contained elevated quantities of acylcarnitines. Other than a very small response for acetylcarnitine,
carnitine esters were not detected in urine from normal patients. Improvements leading to a lower limit of detection may subsequently allow analysis of the low acylcarnitine levels (i.e. sub-nmol) excreted by normal, healthy persons (see Section 3.2.2). With the exception of one MCADD sample (Table 6, Patient 2), the GC and GC/MS methods identified acylcarnitines in all urine samples from patients with the disorder. Table 6 summarizes the results of acylcarnitine detection in eleven urine samples from nine patients with MCADD, collected at varying stages of the disease. Acylcarnitine concentrations, where quoted, were estimated from the relative FID response for the corresponding derivative with that for the lactone from the internal standard. As expected, octanoylcarnitine and hexanoylcarnitine were prominent metabolites detected in the analysis of MCADD samples. However, the estimated concentrations for both the major carnitine esters varied significantly between samples. Some urines when analysed by the developed method gave poor detector responses for the expected acylcarnitines. This was especially noticeable for samples collected during a crisis stage of the disorder. Failure to observe high concentrations of endogenous acylcarnitines in early applications of the technique, was attributed to a non-optimized extraction and derivatization procedure. However, as the project progressed, a pattern emerged whereby urine collected during or shortly after an episode of acute clinical symptoms (crisis condition), presented lower concentrations of carnitine compounds (even below the l.o.d.) compared with samples collected during the basal stage of the disease. The same observation was made with other disorders of mitochondrial ß-oxidation, i.e. propionic acidemia and isovaleric acidemia (see later). Without precise information on the stage of the disease, relative time of sampling, therapeutic treatment, creatinine determinations and sample treatment and storage procedures, it is only possible to speculate on explanations for the trend in acylcarnitine excretion detailed above. One explanation concerns the overall
<table>
<thead>
<tr>
<th>PATIENT</th>
<th>SAMPLE</th>
<th>SAMPLE INFORMATION</th>
<th>ACYL-CARNITINES DETECTED BY GC AND GC/MS METHODS (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 only</td>
<td>Post PPA load; collected from apparently healthy child aged 6 weeks; glycine conjugate of PPA in organic acid profile; no hexanoyl or suberyl-glycine</td>
<td>Hexanoyl Octanoyl ( \Phi ) propionyl IS</td>
</tr>
<tr>
<td>2</td>
<td>1 only</td>
<td>Post PPA load; small amounts of ( \Phi ) propionyl/glycine in organic acid profile; no hexanoyl or suberyl-glycine</td>
<td>IS</td>
</tr>
<tr>
<td>3</td>
<td>1 only</td>
<td>Crisis condition urine; suberyl + hexanoyl glycine present in organic acid profile</td>
<td>Acetyl (110) Hexanoyl (10) Octanoyl (90) IS (200)</td>
</tr>
<tr>
<td>4</td>
<td>1 only</td>
<td>Patient well but suberyl + hexanoyl glycine present in organic acid profile</td>
<td>Acetyl (30) Hexanoyl (60) Octanoyl (250) IS (200)</td>
</tr>
<tr>
<td>5</td>
<td>1 only</td>
<td>Crisis condition urine; suberyl + hexanoyl/glycine present in organic acid profile</td>
<td>Acetyl (40) Hexanoyl (&lt;10) Octanoyl (30) IS (200)</td>
</tr>
<tr>
<td>6</td>
<td>1 only</td>
<td>Hypoketotic fast; suberyl, hexanoyl and ( \Phi ) propionyl/glycine present in organic acid profile</td>
<td>Hexanoyl (70) Octanoyl (510) IS (200)</td>
</tr>
<tr>
<td>7</td>
<td>A</td>
<td>Post PPA load; basal condition</td>
<td>Acetyl (10) Octanoyl (100) ( \Phi ) propionyl (40) IS (200)</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>Just after acute episode of MCADD symptoms</td>
<td>Octanoyl (90) ( \Phi ) propionyl (30) IS (200)</td>
</tr>
<tr>
<td>8</td>
<td>1 only</td>
<td>Post PPA load; basal condition</td>
<td>Acetyl (1650) Isovaleryl (270) Octanoyl (80) IS (200)</td>
</tr>
<tr>
<td>9</td>
<td>A</td>
<td>Crisis condition urine</td>
<td>Octanoyl (&lt;10) IS (200)</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>Basal condition urine</td>
<td>Acetyl (380) Hexanoyl (170) Octanoyl (2710) IS 200</td>
</tr>
</tbody>
</table>

**TABLE 6** Acylcarnitines Detected in Urine Samples from Patients with MCADD

IS: Internal Standard
reduction in accumulating acyl-CoA groups may occur, thus leading to decreased acylcarnitine excretion. Further work, including time course studies over the crisis phases of MCADD, seems advisable to determine the reason(s) for the fluctuating levels of carnitine esters in the urine of patients with the disorder.

The urine from one patient, diagnosed MCADD (Table 6; patient 8) gave interesting GC and GC/MS results in that isovalerylcarnitine was detected along with octanoylcarnitine. Isovalerylcarnitine was not observed in any other MCADD urine and was originally thought to be specific to another metabolic disorder, isovaleric acidemia (Section 3.2.3.). Whilst octanoylcarnitine (characteristic of MCADD) was present, hexanoylcarnitine was not detected. Also worthy of attention was the large FID response for acetylcarnitine which was confirmed by GC/MS. The patient was diagnosed as MCADD on the basis of the urinary organic acid profile and decreased octanoate oxidation in fibroblasts. However, a phenylpropionic acid load did not result in detectable urinary phenylpropionylglycine (see Section 3.3) and the electron transfer flavoprotein (ETF) assay of the MCAD was normal, both results atypical of MCADD. The detection by GC/MS of excreted isovalerylcarnitine as well as octanoylcarnitine, coupled with the inconclusive results from the established clinical tests, suggests that the patient may be suffering from a multiple acyl-CoA dehydrogenase (MAD) deficiency as diagnosed for a patient reported by Kidouchi et al. Such a disorder would be expected to give unexpected and original organic acid and acylcarnitine profiles due to complex permutations of multiple enzyme deficiencies. Other urinary components could not be positively identified due to limited mass spectral information obtained with this sample. Improvements to the sensitivity of the GC/MS method and analysis of further samples from patient 8 may produce valuable results to help fully elucidate the metabolic disorder in this infant and others with MAD deficiency.

The highest concentrations of the typical MCADD acylcarnitines, were found in the urine of patient 9, collected during a basal period of the disorder. In contrast to
this, the urine from the same patient, during a crisis stage of MCADD, had one of
the lowest concentrations of octanoylcarnitine and no hexanoylcarnitine was
detected.* Using GC/MS and mass chromatograms (Section 2.4.3), two additional
compounds with typical EIMS characteristics of acylcarnitines were identified
(notably responses at $m/z$ 85 and 144). The retention times of both these
components did not correspond to any of the standard acylcarnitine derivatives
available, but it was predicted that they represent oct-2-enoyl carnitine
(2, $R = \text{CH=CH(CH}_2)_4\text{CH}_3$) and decanoylcarnitine (2, $R = (\text{CH}_2)_8\text{CH}_3$)
previously identified by FABMS/MS and HPLC/MS in other MCADD urines.102
Good quality mass spectra could not be obtained on the minute levels of these
components and therefore confirmation awaits synthesis of the standard
acylcarnitines.*

The derivatization of physiological acylcarnitines to acyloxylactones was applied to
urine from two patients receiving an oral L-carnitine load of 50 mg/kg body weight.
One was a two month old neonate with a normal fatty acid metabolism. The second
was a similar aged baby with diagnosed MCADD. Pre-load samples from both
infants were collected at the same time of day (09.00 hr) and subject to the
extraction, derivatization and GC/MS analysis (see Figure 5). Post-load urine
samples were collected from the normal and the MCADD baby, 4 hr and 2.5 hr

---

* It has recently been revealed that patient 9 was receiving L-carnitine on a therapeutic basis at the
time the "basal urine" sample was collected. The crisis sample was collected when the patient was
admitted with symptoms of MCADD and not receiving L-carnitine. This may explain, in this
patient at least, the observation of greater acylcarnitine excretion in 'basal' than in crisis samples.

+ Oct-2-enoylcarnitine and decanoylcarnitine have recently been synthesised and retention times and
EIMS of the respective derivatives confirm their presence in the urine of patient 9. The synthesis
and analysis of these compounds was performed by Dr R J Morrow, The Open University.
respectively, after the L-carnitine administration. These samples were also analysed using the developed GC/MS method.

For the normal infant, the carnitine load resulted in a signal which corresponded to acetylcarnitine. Also evident by GC/MS, was a broad peak which gave a mass spectrum consistent with β-hydroxy-γ-butyrolactone. This could be assigned to carnitine, cyclized as the acylcarnitines are derivatized. Under the GC conditions imposed, the carnitine peak eluted shortly before the lactone from acetylcarnitine and had a broad shape due to unfavourable interaction of the free hydroxy group with the stationary phase of the column. The analysis of this post-load sample, from the infant with normal β-oxidation metabolism, did not detect octanoyl- or hexanoylcaritnine. These results supported those from the previous application of FABMS/MS by others, who also found that acetylcarnitine was the only urinary acylcarnitine to increase in the normal subject upon carnitine supplementation.

Analysis of the urine samples from the MCADD patient were performed as for those from the normal baby. In this case, the urine collected after the carnitine load gave significant signals for acetyl-, hexanoyl- and octanoylcaritnine. Acylcarnitine levels were not determined but those detected in the post-load sample were not evident in the pre-load sample. The broad peak for cyclized carnitine was also observed in the post-load urine of this patient. Previous FABMS/MS analysis of these urine samples also showed an increase in octanoyl- and hexanoylcaritnine after carnitine administration, validating the GC/MS results reported here.

The results of this short study on L-carnitine loading suggest that it may be a suitable method of gaining biochemical information from patients with suspected fatty acid oxidation defects. Administration of L-carnitine is tolerated by infants and has been used as a therapeutic treatment of MCADD, although its efficacy in this role is variable. L-Carnitine loading may be particularly useful in investigating the metabolism of suspected MCADD patients, who are currently not
excreting detectable amounts of urinary acylcarnitines. It represents a technique for maximizing biochemical information for confirmatory purposes. As such, it may be applied to other metabolic defects which involve accumulation of acyl groups in the mitochondrion.

The organic aciduria which accompanies acylcarnitine excretion in cases of MCADD was studied, over the first four weeks of life for a baby with the disease, by Walker et al. Urine samples from the same patient, over the same time period, were obtained and analysed by the GC and GC/MS methods developed in this project. Aliquots of the same samples were also analysed independently by Millington and co-workers using FABMS/MS.

The male baby was born normally at 37 weeks gestation, weighing 3440 g. He was the second child of unrelated parents whose first child had died unexpectedly at 15 months. MCADD was suspected in the first child, on the basis of gross fatty infiltrations of the liver and biochemical investigations of the vitreous humour. The second child appeared healthy but urinary organic acid analysis by GC/MS indicated that he too had MCADD. Urine samples were collected at intervals from 4 hours after birth. Hexanoylglycine and suberylglycine were detected in all samples. The study concluded that (i) the organic aciduria evolved with time, (ii) the levels of hexanoylglycine and suberylglycine progressed rapidly to peak on the second day of life and then subsided rapidly, (iii) other characteristic metabolites (i.e. hydroxyacids and dicarboxylic acids) showed a similar trend, and (iv) on the second day the full range of organic acids reported in MCADD, except phenylpropionylglycine, was observed.

The results from subjecting the urine samples to the extraction, derivatization and analysis procedure depicted in Figure 5 are presented graphically in Figure 11. Acylcarnitine concentrations were estimated from GC chromatograms, comparing FID signals for the respective derivatives with that for the internal
Figure 11. GC/MS determination of four organic acids excreted by a patient with MCADD during the first week of life.
standard. Figure 10 shows a typical GC trace obtained during the time course study. It represents the analysis of the urine obtained from the child at 41 hr after birth. The lactones of acetylcarnitine (4.65 min.), hexanoylcarnitine (9.84 min), octanoylcarnitine (12.38 min) and the internal standard (15.78 min) are all evident in this trace. All proposed acyloxy lactone peaks were confirmed by GC/MS, matching authentic standard samples (Appendix MS1, MS4, MS6 and MS8). The excretion of acylcarnitines is represented graphically in Figure 12. As usual, in cases of MCADD, octanoylcarnitine was the major acylcarnitine excreted. However, the concentration of this metabolite varied from undetectable to 470 nmoles ml⁻¹ over the time period investigated. Acetylcarnitine and hexanoylcarnitine were also detected with maximum concentrations estimated at 170 and 60 nmoles ml⁻¹ respectively. In common with the results for organic acid analyses (Figure 11) the maximum acylcarnitine excretions occurred between 40 and 60 hr of age. The general trend in urinary carnitine ester concentrations obtained here was also superimposable on the excretion pattern for other diagnostic MCADD metabolites. In all cases the rapid increase in metabolite concentrations is followed by a sharp fall, at around 50 hr after birth. A slight rise in hexanoylcarnitine and octanoylcarnitine at approximately 110 hr was also observed and this was also detected by D. S. Millington (Duke University) using the established FABMS/MS method¹°² (see below).

Millington and coworkers, using FABMS/MS,¹°² were able to detect the major acylcarnitines along with butanoyl-, octenoyl-, adipyl-, suberyl- and sebacylecarnitine in the same set of samples. The detection limit of the technique was 1 nmol ml⁻¹, thus allowing analysis of acylcarnitines not detected by the current full-scan GC/MS method. However, the FABMS/MS technique does not produce unambiguous full-scan spectra of the analytes. The method involves detecting the precursors of a common fragment ion (m/z 99), consisting predominantly of acylcarnitine molecular cations. A degree of ambiguity arises
Figure 12. Estimations by GC/MS of acylcarnitines excreted by a patient with MCADD during the first week of life
because urinary components, other than acylcarnitines, could fragment and give detectable \( m/z \) 99 ions under the FABMS/MS conditions imposed. This was supported by many peaks, other than those attributed to acylcarnitines, in the parent ion scan spectra (i.e. false positives). Secondly, isomers would give the same response, so inhibiting their differentiation.

Only approximate acylcarnitine levels were determined by FABMS/MS (unpublished results). Inappropriate internal standard spiking (\( ^2\)H\(_3\)-acytethylcarnitine and octanoyl-\( ^2\)H\(_3\)-carnitine) resulted in a signal which could not be accurately compared with the signals for the physiological acylcarnitines. Despite this, the estimated concentrations were in good agreement with those from the GC and GC/MS method. The trend in acylcarnitine excretion, over the first week of life was also confirmed by the FABMS/MS estimations (Figure 13). Therefore, all results substantiate the original observations of Walker et al.\(^{135}\) in that the metabolic disturbance progressed to a peak then rapidly subsided, during the second day after birth.

Similar studies on other babies with MCADD are required to determine if the metabolic events noted in this case are characteristic of the disease. The use of the GC/MS method, developed in this project, would be ideal for acylcarnitine determinations in such a study. Further sensitivity could be achieved, at the expense of full-scan mass spectral information, by employing selected ion monitoring (SIM). This may allow detection of the minor acylcarnitines as in the FABMS/MS method reported above.

If the trend in metabolite excretion is common to cases of MCADD then this is very significant to diagnosis of the disease. Walker et al.\(^{137}\) report another symptomless newborn baby with MCADD for which, by chance, the first urine collected was at 43 hr of age. Analysis of the organic acids using GC/MS identified the full range of characteristic MCADD metabolites, except for phenylpropionyl-
Figure 13. FABMS/MS estimations of metabolites excreted by a patient with MCADD during the first week of life.
glycine. Metabolite excretion in this baby may also have been approaching a maximum at 2 days of age, since subsequent analyses of urine collected at 18 days after birth, revealed only a trace of hexanoylglycine using GC/MS. The organic acid profiles for both infants with MCADD were compared with acid excretion in 59 unaffected babies ages 0 - 72 hr, and confirmed the elevated metabolite concentrations when the disease was present.

Detection of acylcarnitines by derivatization and GC/MS of the resultant acyloxy lactones was successful over the peak excretion period. However, diagnostic carnitine esters were not detected after the first week of age in this case and hence the infant may have passed as normal on the basis of urine screening after the first week of life. These observations are in contrast to many previous analyses of basal condition MCADD urines (see Table 6), which are usually characterized by readily detected amounts of octanoyl- and hexanoylcarnitine. A comprehensive understanding of the MCADD disorder demands further studies which discover why, how and when metabolite excretion varies.

3.2.2 Propionic Acidemia

Propionic acidemia, a disorder of organic acid metabolism, results from defective propionyl-CoA-carboxylase. The disease is characterized by accumulation and urinary excretion of propionylglycine, 3-hydroxypropionate and methylcitrate. Roe et al.\textsuperscript{138} and Chalmers et al.\textsuperscript{139} also observed greater amounts of acylcarnitines in the urine of patients with this disorder compared to normal subjects. An increase in propionylcarnitine was particularly striking. Most of the metabolites excreted share a common precursor, propionyl-CoA. This compound and its metabolites are known to have several detrimental consequences on normal metabolism. These include inhibition of the tricarboxylic acid (TCA) cycle,\textsuperscript{140,141} gluconeogenesis,\textsuperscript{142} pyruvate dehydrogenase complex\textsuperscript{143} and the glycine cleavage system.\textsuperscript{144} The long term use of supplementary L-carnitine together with dietary restrictions has been
recommended for control of the disease. L-Carnitine administration provides a mechanism whereby otherwise accumulating propionyl groups are removed for urinary excretion thus (i) replenishing mitochondrial, free CoA, (ii) maintaining normal ATP production, and (iii) providing sufficient L-carnitine for the patients' metabolic requirements.

Propionylcarnitine has been detected in the urine of patients with propionic acidemia by several methods, including FABMS, thermospray LC/MS, desorption CIMS and LC. 1H NMR spectroscopy has also been used, after administration of an L-carnitine load, to a propionic acidemia case thus elevating the excreted propionylcarnitine to such a degree that the metabolite could be detected by this relatively insensitive technique. In this project, derivatization to the corresponding acyloxylactone with GC and GC/MS analysis was applied to urine samples from two patients with propionic acidemia.

Two samples from each patient were available for analysis. In all four urines, propionylcarnitine was detected. Figure 14 shows a typical gas chromatogram from a sample which was extracted, derivatized and analysed by GC as depicted in Figure 5. The major peak at 5.90 min corresponded, in retention time, to the lactone from propionylcarnitine. GC/MS confirmed the component was the lactone from propionylcarnitine (see Appendix: MS2 for standard) but the spectrum was complicated by co-eluting urea. Urea interference was a recurring problem in the GC/MS analysis of propionic acidemia samples. GC analysis was not particularly affected by the presence of urea because of the insensitivity of the FID to urea. Detection problems with GC/MS were largely overcome by the use of mass chromatograms. Plotting the variation in ion abundance of the base peak of the lactone of propionylcarnitine, m/z 84, gave a sharp peak deconvoluted from the broad and otherwise interfering signal of urea in the TIC trace. However, the interference still has to be taken into account when interpreting the mass spectrum.
Figure 14. Analysis of urine from a child with propionic acidemia
of the peak. Alternatively enhanced data may be obtained by using subtraction procedures to remove the mass spectrum of urea from the spectra of interest.

Before extraction, the urines were spiked with phenylbutanoylcarnitine as in the MCADD investigations. Approximate levels of propionylcarnitine were estimated from the relative signals from the resulting lactone compounds. As with cases of MCADD, acylcarnitine excretion appeared to vary markedly in propionic acidemia. For both patients, propionylcarnitine concentration was estimated to be in the low nmol ml\(^{-1}\) level in one sample and approaching \(\mu\)mol ml\(^{-1}\) in the other. Such variations seem to have been poorly investigated, other than the comparative results from L-carnitine loading tests. Detailed studies which take into consideration aspects such as sample collection periods and disease condition, are required to determine the reasons for the fluctuating acylcarnitine levels.

The results obtained from the investigation of the limited number of propionic acidemia samples support the observations of others. Propionylcarnitine does appear to be a major urinary metabolite, characteristic of the disease. The high concentration of the acylcarnitine detected in some samples indicates that the disorder may lead to secondary carnitine deficiency. As such, L-carnitine supplements and control of dietary fat intake are essential to maintaining a healthy metabolic status. The application of the developed acylcarnitine analysis method to these samples also demonstrated that the technique may be used to investigate fatty acid metabolism in disorders other than MCADD. The procedure was identical to that used in the MCADD investigations but the combination of high resolution gas chromatography with full scan EIMS data allowed unambiguous acylcarnitine determinations.

3.2.3 Isovaleric Acidemia

As shown by Millington et al., stable isotope experiments and FABMS analysis prove that isovalerylcaritine is derived from a defective leucine metabolism in
patients with isovaleric acidemia. In the degradation of leucine (Figure 15) a mitochondrial process is employed whereby α-ketoisohexanoic acid passes through the inner mitochondrial membrane and is converted to isovaleryl-CoA. In patients with isovaleric acidemia, further metabolic breakdown is inhibited by deficiency of isovaleryl-CoA dehydrogenase. Carnitine acts in its familiar detoxification role, to remove the potentially toxic accumulation of isovaleryl groups and excrete them as isovalerylcarnitine \((2, R = CH_2CH(CH_3)2)\) in the urine. Other urinary metabolites of isovaleric acidemia include isovalerylglycine and 3-hydroxyisovalerate.

Carnitine deficiency and the subsequent effects on fatty acid metabolism have been reported in cases of the disorder.\(^{146}\) In common with other inherited defects of mitochondrial β-oxidation L-carnitine supplementation has been recommended as efficacious treatment of patients with isovaleric acidemia.\(^{147}\)

Urine samples from three patients with the disorder were subject to the extraction, lactonization and GC and GC/MS analysis depicted in Figure 5 with the results summarized in Table 7. A typical gas chromatogram resulting from the analysis of one of the samples is shown in Figure 16. The major peak in the chromatogram, eluting at 7.82 min, had an identical retention time to standard, derivatized isovalerylcarnitine. GC/MS analysis of the same peak gave the EI mass spectrum shown in Figure 17. The spectrum shows a base peak ion at \(m/z\) 85, characteristic of the medium-chain acyloxylactone compounds. A comparison with the EI mass spectrum of standard, derivatized isovalerylcarnitine (Appendix MS3) confirmed the presence of the acylcarnitine in the urine sample analysed. The metabolite was readily detected in all the samples from patients with diagnosed isovaleric acidemia. Relatively high concentrations of the endogenous acylcarnitine were detected in these samples, compared to other metabolic disorders investigated in the project. The largest estimated concentration (about 4 µmol ml\(^{-1}\)) was determined for the sample from patient 3, collected during a stable stage of the disorder. As previously encountered with MCADD cases (Section 3.1.1), acylcarnitine excretion
Figure 15. Mitochondrial steps of leucine metabolism leading to isovalerylcarnitine formation.
<table>
<thead>
<tr>
<th>PATIENT</th>
<th>SAMPLE</th>
<th>SAMPLE INFORMATION</th>
<th>ACYLCARANITINES DETECTED BY GC + GC/MS OF LACTONE DERIVATIVES (nmol ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>Isovalerylglycine conjugate observed in organic acid profile</td>
<td>Isovalerylcarntnine</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Pooled sample collected 7 months before (1A)</td>
<td>Isovalerylcarntnine</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>Acute stage of disease</td>
<td>Isovalerylcarntnine (190)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IS (200)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Basal condition of disease</td>
<td>Isovalerylcarntnine (70)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IS (200)</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>Acute stage of disease</td>
<td>Isovalerylcarntnine (600)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IS (200)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Basal condition of disease</td>
<td>Isovalerylcarntnine (4000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IS (200)</td>
</tr>
</tbody>
</table>

TABLE 7  Acylcarnitines detected in urine from patients with isovaleric acidemia

IS: Internal standard
Figure 16. Analysis of urine from a child with isovaleric acidemia
Figure 17. Mass spectrum of isovaleryl carnitine
at a crisis stage was significantly reduced in this patient. However, the observation should be contrasted with the results for patient 2, which suggest higher excretion during crisis conditions. Further studies are required to make deductions on the relevance of the varying pattern of isovalerylcamitine excretion.

Application of the developed method to urine from patients with isovaleric acidemia further supported the value of the technique for investigating metabolic disorders. However, care should be taken if quantitative evaluations are required. The high concentrations of acylcarnitines encountered in urine from patients with isovaleric acidemia (see Table 7) may approach the limitation of the derivatization method used here. As discussed in Section 2.4.3, high acylcarnitine concentrations in the ReactiVial may suppress the cyclization process, due to intermolecular interactions. Smaller volumes of urine, i.e. less than 0.5 ml, should be analysed or a larger reaction vessel employed.

3.2.4 Other Applications And General Observations

The GC/MS method was applied to some other inherited metabolic diseases with less conclusive results. For example, urine from one patient with methylmalonic acidemia was subject to the method. The disorder has previously been characterized by urinary excretion of propionyl- and acetylcarnitine.\textsuperscript{138} The GC/MS method described here (Figure 5) did not identify propionylcarnitine although a small amount of acetylcarnitine was evident. The technique has been successfully applied to physiological quantities of propionylcarnitine in cases of propionic acidemia and therefore there is no obvious reason why it should not be applicable to methylmalonic acidemia diagnosis. Further attempts with such urine samples are envisaged for future work. Likewise, application to urine from patients with β-ketothiolase deficiency is anticipated. Millington et al.\textsuperscript{103} identified tiglylcarnitine (2, R = C(CH\textsubscript{3})=CHCH\textsubscript{3}) in the urine of a patient with β-ketothiolase deficiency by thermospray mass spectrometry (LC/MS) and resolved it
from the biological isomer, 3-methylcrotonoylcamitine (2, R = CH=C(CH₃)₂). The one application of the lactonization method to urine from a patient diagnosed with this disorder of β-oxidation did not identify any acylcarnitines. The method has proven to work with other branched-chain acylcarnitines (isovalerylcarnitine and valproylcarnitine) and unsaturated acylcarnitines (octenoylcarnitine) and therefore tiglylcarnitine should be amenable to the developed technique. The fluctuating concentrations of excreted acylcarnitines encountered throughout the analysis of clinical samples may have been responsible for these negative results. If the urine samples were collected during a low acylcarnitine-excretion period of the disease, the disease-specific acylcarnitines may occur at a concentration below the detection limit of the technique. Improving the extraction procedure in terms of recovery and selectivity may be required for such applications. Alternatively or additionally, the use of a more sensitive mass spectrometric assay for extracted and derivatized acylcarnitines may enable detection of the lower physiological levels encountered in some disease state urines and may even allow determinations of normal urine acylcarnitine levels. Using the mass spectrometer in selected ion monitoring (SIM) mode is one method of gaining substantial sensitivity over full scanning. For SIM, the mass spectrometer is tuned to detect one or a small number of ions expected in the mass spectrum of the analyte of interest. Since the instrument does not spend valuable analysis time collecting data on background and ions of no relevance to the analyte, detection sensitivity may be improved by a factor of up to 1000. The technique is especially effective in improving sensitivity of capillary GC/MS analyses. The width of fully resolved GC peaks is typically 1-2 s and therefore a mass spectrometer scanning the mass range 20-800 in 1 second may only collect data on a given peak during one scan. In that one scan there may be very few ions originating from the compound that gives the chromatogram peak. The combined ion current of those few ions determines the sensitivity of the technique and is represented by the total ion current (TIC) chromatogram. In the application of SIM to the analysis of the acyloxylactone.
compounds the instrument may be tuned to focus only the common ions at $m/z$ 84, 85 and 144 expected from the analytes of interest under EI conditions. Alternatively, using CI, the expected quasi-molecular ions may be selectively monitored for suspected acylcarnitines, depending on the disease being investigated.

One urine sample from a patient with glutaric aciduria type 1 was analysed using the GC/MS technique. The disease is caused by a defect in the oxidation of glutaryl-CoA and possibly glutaconyl-CoA which are intermediates in the catabolism of tryptophan, lysine and hydroxylysine. Analysis of urinary organic acids reveals glutaric, glutaconic and 3-hydroxyglutaric acids as the major diagnostic metabolites. Recently, using HPLC$^{87}$ glutarylcaritnine has also been detected in the urine of infants with the disease. The analysis of other dicarboxylic acid conjugates of carnitine in neonatal urine is also attracting interest.

Methylmalonyl-, glutaryl-, adipyl-, suberyl- and sebacylcarnitine have all been detected using FABMS/MS and LC/MS.$^{102}$ The relevance of these metabolites to essential metabolic processes has not been fully evaluated, a situation which would benefit from a sensitive and simple assay such as the one developed in this project.

The attempt to detect glutarylcaritnine in the urine from a glutaric aciduria type 1 patient was unsuccessful. A gas chromatographic peak of cyclized glutarylcaritnine may be expected to be broad and unsuitable for quantification because of unfavourable stationary phase interactions with the remaining free carboxyl group. No such signal, nor any other which could be assigned to an acylcarnitine, was detected by GC or GC/MS. There are several other possible explanations for this result including: (i) glutarylcaritnine was not present in detectable quantities in this sample; (ii) the metabolite was not extracted using the ion-exchange method developed for the simple acylcarnitines of monocarboxylic acids; and (iii) carnitine esters with additional carboxyl functions in the side chain do not cyclise under the
derivatization conditions. It is difficult to eliminate any one of these potential explanations without further attempts with well characterized clinical samples and/or with standard glutaryl carnitine. The purification procedure, as it stands, does appear suspect in its application to such acylcarnitines. The extraction was designed and modified to be as specific as possible for isolating conventional acylcarnitines. The likely change in ion-exchange behaviour caused by an additional acid group in the analyte may mean that the extraction procedure is inappropriate for the carnitine esters of dicarboxylic acids. A loss of the analyte may occur at either of the ion-exchange steps but the first anionic-exchange stage seems most likely to be the problem. Under the anionic-exchange resin conditions, acylcarnitines containing a carboxyl function in the side chain can carry a net negative charge unlike the overall neutral nature of conventional carnitine esters. As such, glutaryl carnitine and other dicarboxylic acid carnitine esters may remain on the anionic-exchange resin column. Careful control of pH and possible reliance on only cationic resins may provide a suitable alternative purification procedure.

Derivatization of glutaryl carnitine by conversion to the respective acyloxy-γ-butyrolactone (3, R = (CH₃)₂COOH) is not expected to be as difficult as might initially be envisaged. Only the carboxyl group of the carnitine backbone should participate in the cyclization reaction since an analogous mechanism involving the side-chain carboxyl group would, theoretically, lead to a seven-membered ring. A five-membered ring, like the butyrolactones derived from conventional acylcarnitines, will form much more rapidly than a seven-membered ring. In fact, the lactonization of Br(CH₂)ₗCOO⁻ species is known to occur at least 100 times faster when n = 3 (i.e. to give a 5-membered ring) than with any other value of n.¹⁴₈ Thus any problems which occur in the application of the GC/MS method to carnitine esters of dicarboxylic acids, should only concern the free carboxyl group still present in the side chain of the derivative. Standard trimethylsilylation (TMS
derivatization) of this group may be required in order to achieve sharp GC peaks suitable for quantification.

In addition to acyloxylactones, some other compounds were regularly encountered in both the GC and GC/MS analysis of urine samples subject to the extraction and derivatization procedure depicted in Figure 5. A broad peak, eluting towards the end of a standard GC analysis (over 15 min) was almost always observed. The mass spectrum of this peak was characteristic of a phthalate plasticiser with an abundant ion at m/z 149. This artifact probably arises from the ReactiVial derivatization step. The rubber septa used to seal the reaction vessels did have a teflon side, but the high pressures generated inside the ReactiVial may allow solvent vapour to come into contact with the soft rubber before condensing back into the reaction mixture. After derivatization, samples were stored in 2 ml glass sample vials with teflon-lined septa and screw top caps. Other, conventional precautions were taken when handling solvents for example, to avoid contamination. The presence of GC plasticizer peaks was not a problem in the studies conducted in this project since they eluted substantially later than any peaks due to derivatized acylcarnitines.

Another peak, more difficult to explain, was observed in almost every neonatal urine examined, from both normal and disease states. It occurred as a broad peak, requiring a GC oven temperature above 230 °C to elute it from a BP5 capillary column. The peak never interfered with the signal for any acylcarnitine of interest. EI mass spectra for this component were reproducible and a typical spectrum is shown in Figure 18.

The spectrum is obviously very different from the now familiar spectra of the acyloxylactone compounds and could not be interpreted as originating from any acylcarnitine. The simple nature of the derivatization procedure and the lack of reagents involved, indicates that the compound is a component of urine, or is
Figure 18. Mass spectrum of suspected cyclic prolyl-isoleucine
derived from a component of urine. Such material must behave similarly to acylcarnitines in order for it to be extracted by the ion-exchange purification method and hence reach the derivatization and analysis stages. A base peak at $m/z$ 70 is highly indicative of an amino acid, prolyl group (9), and suggests a simple peptide or proline derivative. The presence of an amino-acid type substance would explain the material being extracted along with any acylcarnitines. The zwitterionic structure, containing an anionic carboxyl group and a cationic quaternary amine group, is common to amino acid and acylcarnitine molecules. Therefore, it was expected that any peptidic material present in the urine samples would be extracted using the ion-exchange purification procedure outlined in Figure 5. If the ion at $m/z$ 210 is assigned to the molecular ion of the compound, the mass spectrum (Figure 18) may be interpreted as one of the dipeptides, prolyl-isoleucine, prolyl-leucine, isoleucyl-proline or leucyl-proline, which has lost 18 daltons, i.e. loss of a molecule of water. That is, the spectrum suggests the presence of a cyclized dipeptide: a 2,5-diketopiperazine. In fact, the spectrum matched that of a previous EIMS analysis of cyclic Pro-Ile but not of cyclic Pro-Leu. The peaks at $m/z$ 86 and 124 probably represent a rearrangement reaction within the fused ring structure as shown in Scheme 19, which has also been observed in the EIMS of other 2,5-diketopiperazines. Occasionally another component with a mass spectrum which correlated with cyclic alanine-leucine or alanine-isoleucine was also observed ($m/z$ 184 (M$^+$), 5%; 112, 100%; 99, 40%; 98, 60%; 70, 45%; 43, 50%). This substance had a GC elution time of around 16.9 min and this could interfere with medium-chain acylcarnitine derivatives. However, the compound was not consistently encountered as with cyclic Pro-Ile in neonatal urine samples. The limited number of adult urine samples analysed did not contain the postulated diketopiperazine compounds found with the normal and disease state children's samples.
Scheme 19

(m/z 210) → (m/z 86) → (m/z 124)
The detection of cyclic dipeptides may originate from urinary dipeptides extracted by the ion-exchange purification and then thermally cyclized, either during the derivatization process or during chromatography. However, commercial samples of Pro-Ile and Ala-Leu, when subject to the derivatization conditions and GC analysis did not produce peaks consistent with those attributed to the cyclic dipeptides in the clinical samples. Further work is required to confirm the identity of these components unambiguously (the sequence of the parent dipeptide is, of course, "lost" once the diketopiperazine has formed) including synthesis of standard 2,5-diketopiperazines. Such investigations seem worthwhile since the identification of urinary dipeptides may have important clinical value. It would also be of interest to investigate the origin of the dipeptides detected.

3.3 INVESTIGATIONS OF THE METABOLISM OF SOME EXOGENOUS COMPOUNDS

In recent years, certain drug treatments have been associated with reduced plasma and urinary carnitine levels. Of these, secondary carnitine deficiency induced by valproic acid (3-propylvaleric acid or 3-propylpentanoic acid) administration to patients with epilepsy is most documented. Urinary excretion of valproylcarnitine has been proposed as a mechanism for reducing carnitine levels to an extent that patients show clinical symptoms of hypocarnitineaemia. A similar process is suggested for the metabolism of the antibiotic, pivampicillin (pivaloyloxymethyl-ampicillin), increasing urinary loss of carnitine as pivaloylcarnitine (2, R = C(CH₃)₃).

Oral loading with phenylpropionic acid (PPA or, properly, 3-phenylpropanoic acid) is now widely accepted as an in vivo screening/diagnostic procedure for patients with suspected MCADD. In normal subjects, phenylpropionic acid is produced naturally from phenylalanine by intestinal bacteria. This is then further metabolized to benzoate by the action of MCAD and excreted in the urine as hippurate.
Therefore, a deficiency of the dehydrogenase enzyme results in a mitochondrial accumulation of phenylpropionyl-CoA. GC/MS of urinary organic acid profiles often identifies phenylpropionylglycine, originating from endogenous phenylalanine. Administration of PPA attempts to accentuate this process for diagnostic purposes.

The acylcarnitine analysis procedure reported here was applied to urine samples from patients undergoing treatment with phenylpropionic acid and valproic acid. The results are given in this section.

3.3.1 Acylcarnitine Determination of Urine Samples from Patients Administered Phenylpropionic Acid (PPA)

Figure 19. Mass spectrum of phenylpropanoylcarnitine detected in the urine of a patient with MCADD
The very first MCADD urine analysed with the developed method identified the expected hexanoylcarnitine and octanoylcarnitine (Section 3.1). Use of mass chromatograms, m/z 85 and 144, indicated the occurrence of another component with the typical mass spectral characteristics of an acylcarnitine lactone derivative (Figure 19). Interpretation of the spectrum suggested the compound the acylloxylactone from 3-phenylpropanoylcarnitine. Subsequent synthesis of the standard acylcarnitine and mass spectrometric analysis of the corresponding lactone derivative (Appendix MS7) confirmed the presence of the aromatic carnitine ester. The patient had not received a recent bolus of PPA and therefore the metabolite probably originated from incompletely metabolized phenylalanine, due to a deficiency of MCAD.

Further studies of MCADD urines occasionally identified the derivative of phenylpropionylcarnitine, estimated at low nmol ml\(^{-1}\) levels. However, there was little consistency between samples with regard to this observation. Post-load urine samples from patients given PPA produced equally variable analytical results. Some samples gave substantial signals, by GC and GC/MS, for the lactone of 3-phenylpropanoylcarnitine, comparable with the octanoylcarnitine response, whilst in others, even after a PPA load, the aromatic acylcarnitine was not detected. The results suggest that the carnitine detoxification process may not be the only pathway for 3-phenylpropionyl group removal, and in some MCADD patients alternative pathway(s) predominate. Conventional GC/MS organic acid profiles, which accompanied some of the urine samples, often identified hippurate, suggesting that the PPA may have been fully metabolized even with a limited and otherwise insufficient MCAD activity. Also, glycine may act to remove the toxic acyl groups
to such a degree that carnitine esters are not required, this being supported by
detection of phenylpropionylglycine in some organic acid analyses.

The identification of 3-phenylpropanoylcarnitine in a clinical urine sample was first
reported using the method developed in this project.\textsuperscript{108} The almost simultaneous
identification of the same acylcarnitine, in one other patient with MCADD, using
FABMS/MS, supports the observation reported here.\textsuperscript{155} It is reasonable to
propose that this metabolite derives from the detoxification role of carnitine
previously encountered in \(\beta\)-oxidation disorders. That is, a deficiency of MCAD
may result in accumulation of 3-phenylpropanoyl-CoA species in the mitochondria
and thus carnitine acts to alleviate this potentially critical situation by forming
carnitine esters, to be excreted in the urine. The common practice of administering
a PPA load to patients with MCADD, on the basis of the results obtained in this
project, produced variable concentrations of the acylcarnitine metabolite.
Controlled studies are required to determine if an acylcarnitine excretion pattern is
reproducible over the drug treatment period. It should be noted, however, that a
PPA load to MCADD patients with critical symptoms of secondary carnitine
deficiency should be avoided, as this represents a further mechanism for lowering
the body carnitine pool.

3.3.2 Acylcarnitine Determination in Urine Samples from Patients
Administered Valproic Acid

Valproic acid is a short-chain fatty acid widely used to treat epilepsy in paediatric
practice. A daily dose, in the range 600 to 1200 mg, is frequently used to minimize
convulsions in epileptic children. The metabolism of the drug in otherwise normal
infants and in animals has been studied and is well documented.\textsuperscript{156} In a small
percentage of patients, however, hyperammonemia and a Reye's-like syndrome
have been reported.\textsuperscript{157-159} Irreversible and even fatal liver injury has been reported
with valproate therapy. It is suggested that the use of this anticonvulsant
may induce secondary carnitine deficiency in some patients thus leading to a Reye's-like syndrome, clinical condition. An inverse relationship between plasma carnitine levels and dosage of valproate has been reported. The mechanism for carnitine reduction is thought to be due to increased urinary excretion of acylcarnitines. However, a study of patients with normal mitochondria β-oxidation, on valproate therapy and given an L-carnitine load, showed that acylcarnitine excretion increased slightly but the majority of the carnitine was excreted unesterified. The study also included analysis of excreted urinary acylcarnitines from patients treated with pivampicillin and concomitant L-carnitine administration, which indicated that the drug readily esterified to carnitine and was excreted as pivaloylcarnitine. Despite poor esterification of valproic acid to carnitine, Millington et al. first identified valproylcarnitine (2, R = CH(CH₂CH₂CH₃)₂), as a drug metabolite in the urine of a patient on valproate therapy. FABMS, with linked scanning, was first used to identify the acylcarnitine but HPLC/MS, employing thermospray, was required to distinguish it from the biological isomer, octanoylcarnitine. Patients were not receiving carnitine supplementation during the study and valproylcarnitine levels were determined in the region of 10 nmol ml⁻¹. The investigation also included assays of free carnitine and short- to medium-chain acylcarnitine totals using a radioenzymatic method. The results indicated that under 10% of the excreted acylcarnitine was accounted for by valproylcarnitine. It was concluded that, "valproylcarnitine was not the predominant acylcarnitine excreted by patients receiving valproate," but no suggestion was made for the major acylcarnitine(s). Acetyl-, propionyl- and isobutyrylcarnitine were previously identified in the urine of patients receiving valproate. When carnitine supplementation was administered, the carnitine esters were detected by hydrolysis and GC analysis of the liberated fatty acids. The ambiguous origin of the fatty acids (see Section 1.2) was of concern in these analyses. Also, the acylcarnitines identified were not specific to the valproic acid metabolism and may be simply a consequence of the carnitine load.
Kuhara et al.\textsuperscript{160} reported an altered valproic acid metabolism in a patient with diagnosed Reye's syndrome and suggested the drug should not be used to treat seizures in patients with symptoms of the disorder. This has been extended to include infants with any of the defective mitochondrial β-oxidation diseases. Therefore, the application of the acylcarnitine method reported here concerned urine samples from patients with normal fatty acid oxidation metabolism and receiving valproate therapy (i.e. unlike samples in which 3-phenylpropanoyl-carnitine was detected, (Section 3.2.1)).

Urine samples from two patients on chronic valproate therapy were subject to the extraction, derivatization and GC and GC/MS analysis as previously described. The first sample analysed gave the gas chromatogram shown in Figure 20. A peak consistent in retention time to the lactone from standard valproylcarnitine was observed at 10.89 min. Using GC/MS, the mass spectrum of this component was obtained. The low concentration resulted in a poor spectrum but it was characterized by relatively abundant ions at $m/z$ 57, 85 and 157, consistent with valproyl-containing acyloxylactone (Appendix MS5). The major peak in the gas chromatogram of Figure 20 eluting at 12.10 min, was originally regarded as not being derived from an acylcarnitine, since its retention time did not correlate with any previous carnitine ester derivatives analysed. Using GC/MS, however, the component showed familiar fragment ions in its EI mass spectrum (Figure 21). The spectrum can be interpreted as consistent with the lactone from 3-oxovalproylcarnitine (2, $R = \text{CH(CH}_2\text{CH}_2\text{CH}_3)\text{COCH}_2\text{CH}_3$).

It is proposed that the patient was excreting 3-oxovalproic acid esterified to carnitine. Further confirmation awaits synthesis and analysis of 3-oxovalproylcarnitine (Chapter 5).

The discovery of this novel metabolite of valproic acid is significant for the full elucidation of the metabolism of the drug. Analysis of the second urine sample
Figure 20. GC analysis of urine from a child receiving valproic acid therapy
Figure 21. Mass spectrum of proposed valproic acid metabolite
from a child receiving valproate therapy supported the results obtained with the first sample. In addition to signals assigned to derivatized valproyl- and 3-oxovalproylcarnitine, several other components with the characteristics of lactones derived from acylcarnitines were indicated by using mass chromatograms (m/z 84, 85 and 144). Full-scan mass spectra were not helpful because of the low concentration of these analytes. Application of the extraction and derivatization procedures to larger volumes of clinical urine sample (when available) may overcome the sensitivity problems and allow further interpretation of the metabolism of this and other acidic compounds.

The presence of the proposed metabolite is supported by the observation of others that 3-oxovalproic acid (2-propyl-3-oxovaleric acid) is a major degradation product of valproate in man. The results of the investigations reported here suggest that carnitine plays a role in removing acidic drug metabolites from the body. Even in the case of subjects with a normal fatty acid metabolism, carnitine may be involved in buffering the stresses on mitochondrial β-oxidation, imposed by exogenous compound administration. This observation may have further repercussions in evaluating the metabolism of other acidic drugs. The excretion of 3-oxovalproylcarnitine represents another mechanism for reducing body carnitine levels, thus accounting for possible Reye's-like symptoms in patients receiving chronic valproate therapy.

It is interesting to speculate as to why the metabolite discussed here has not been previously identified. The FABMS technique employed by Millington et al. would be expected to identify a signal consistent with 3-oxovalproylcarnitine, if it was present in the urine samples examined. One feasible explanation concerns the limited analytical information obtained from the FABMS method. Acylcarnitines were identified by the mass of the intact molecular ion and the ability of this ion to loose 59 mass units (i.e. loss of NMe₃). Other than this fragmentation, there is little evidence to distinguish acylcarnitines from other urinary components which
also lose 59 mass units under the mild FAB ionization conditions. The mass of
the 3-oxo-valproylcarnitine ion is 302 daltons and whilst this ion most probably
fragments by loss of 59 daltons, it may have been difficult to distinguish from
nonanoylcarnitine and its isomers, which have the same nominal mass. The
presence of the next higher homologue of an administered drug is very difficult to
explain on the basis of established metabolic routes, but with the FABMS method,
there is little additional analytical information available. Therefore, the signal may
have been simply attributed to another urinary component which lost 59 daltons.
Otherwise, the signal may have been recognized as originating from an acylcarnitine
but the limited analytical information may have prevented further structure
elucidation. This would explain the authors’ comment that valproylcarnitine was not
the predominant acylcarnitine excreted in the limited number of cases examined. \(^{105}\)
The situation illustrates the benefit of chromatography and EI full-scan mass spectra
as obtained with the derivatization approach to acylcarnitine analysis.

A similar shortcoming of the analytical method may explain the results of Mills et
al., \(^{161}\) who, using FABMS/MS with parent ion scanning, report detection of
nonanoylcarnitine and heptanoylcarnitine in the urine of a child with MCADD.
Odd-carbon chain length acylcarnitines (above C-5, i.e. isovalerylcarnine) are
unexpected and difficult to explain because of the mechanism of fatty acid β-
oxidation (see Scheme 3). Therefore the proposed identification of
nonanoylcarnitine as the second most abundant acylcarnitine metabolite in a case of
MCADD requires further attention. Application of the GC/MS method may provide
valuable additional information on such samples and with standards should
unambiguously confirm or otherwise the FABMS/MS results.
CHAPTER 4

CAPILLARY ZONE ELECTROPHORESIS AND ITS
APPLICATION TO ACYLCARNITINE ANALYSIS
4.1 INTRODUCTION TO CZE TECHNIQUES

Since the development of electrophoresis as the basis of an analytical technique in the late nineteenth century the methodology has remained essentially unchanged. In electrophoresis, charged molecules in solution migrate, upon the application of an electric field, towards the electrode of opposite charge. Early applications involved biomolecules and it is in the area of biological chemistry that the technique has maintained its devotees. Based on separation through a gel or liquid medium, electrophoresis has developed as both an analytical and preparative method.

Capillary zone electrophoresis (CZE) was originally described as free solution electrophoresis in a capillary. Hjerten demonstrated separations with the use of high electric field strengths in free solution electrophoresis using 3 mm i.d. capillaries in 1967. He also drew attention to the zone broadening effects due to thermally induced density gradients and described approaches to minimizing them for improved separation efficiency. However, it was the work of Mikkers et al. and Jorgenson and Lukacs which initiated a steadily growing interest in the subject. This was followed by further publications by Jorgenson and Lukacs and an important review of electrophoresis in capillary tubes by the same authors in 1983. They established the basic theory and practical experimental approaches and demonstrated the high separation efficiency possible with this technique. Over the past decade, the technique of CZE has found many applications with over 90% of publications on the method appearing in the last two years. At this stage in its development, it is possible to speculate that CZE will become a popular laboratory technique because of its potential to be applied to a wide variety of studies. Benefits include high resolution, simplicity, speed of analysis and versatility. Applications in areas such as recombinant protein quality control, purity evaluation of peptides and DNA fragments, checking biological degradation, pharmaceutical analysis, monitoring antibodies and studying bioactive peptides and many others have already been reported. The availability of
commercial instrumentation and the increasingly widespread use of CZE confirms the acceptance of the technique, especially in the life sciences, and should ensure further developments and applications.

4.2 CZE THEORY AND METHODOLOGY

The principle of CZE is shown schematically in Figure 22. The charged analyte or mixture containing charged molecules is introduced into a capillary containing an electrolyte solution. Under the influence of a high voltage, sample molecules differentially migrate through the capillary. Cationic species move towards the cathode whilst anionic molecules migrate towards the anode. However, the rate of migration is dependent on both charge and size of the analyte and therefore separation of ions is effected. This movement of ions is referred to as electrophoretic migration.

In free-zone capillary electrophoresis, that is, when using an open capillary tube filled with electrolyte solution, separation occurs due to the combined action of electrophoretic migration and electroosmotic flow. Electroosmotic flow is one of the most distinguishing properties of capillary electrophoresis. It is caused by the formation of a static diffuse double layer on the inner walls of the silica capillary column. Fused-silica columns present internal walls which have a negative charge in solution as a result of their surface silanol groups becoming ionized. Counter-cations are attracted from bulk solution to the walls. Most of the cations are stagnant but some extend into the mobile "diffusive" layer. These cations migrate towards the cathode, "dragging" with them the solvent. This results in bulk movement of the electrolyte plus analyte molecules towards the cathode. The plug-like flow is said to have a flat velocity distribution across the capillary diameter, deviating only within a few nanometers of the capillary surface. A nearly flat flow profile allows high separation efficiencies to be realized. The effect of net mobility
Figure 22. Schematic diagram of free-zone CZE;
small arrows: electrophoretic migration of anions and cations
large open arrow: bulk electroosmotic flow towards cathode
(i.e., elution of anionic, neutral and cationic species) means that all species eventually pass through the on-line detector.

Altoria and Simpson developed an elegant method of measuring the rate of electroosmotic flow. They arranged an analytical microbalance to monitor the change in weight of one of the buffer reservoirs. This avoided problems with adsorption on the capillary walls of otherwise unretained marker compounds. Flow rate was found to be inversely proportional to ionic strength of the electrolyte and independent of column diameter. The effect of pH between 2 and 12 and applied voltage was also determined. Increases in both were found to increase electroosmotic flow rate linearly.

Much work has been directed to manipulating the electroosmotic flow in order to optimize separations. In the simplest cases, this may involve simply adjusting applied voltages and/or pH. The addition of several chemicals to the electrolyte to alter the zeta potential of the double layer at the capillary wall has been investigated. The direction of flow can be reversed by addition of cationic surfactants. Addition of simple organic solvents can also influence electroosmotic flow dramatically. Methanol decreases the bulk flow whilst acetonitrile causes an increase. Chemical derivatization of the capillary wall has also been investigated, to change the properties of the double layer. The interaction of phosphate with the silica surface has benefited some applications in that electroosmotic flow is reduced and the surface is shielded from protein adsorption, allowing separation of proteins up to 77 kDa. Electroosmotic flow may even be eliminated by silylating with (γ-methacryloxypropyl)-trimethyloxysilane followed by cross-linking the surface-bound methacryl groups with polyacrylamide.

Coated capillaries that eliminate electroosmotic flow are used in a technique termed isoelectric focusing, an extension from a gel-slab electrophoresis technique. The sample is injected into the capillary in a mixture of ampholytes. When the voltage is
applied, the ampholytes quickly form a stable, continuous and linear pH gradient across the length of the capillary. Charged sample molecules move through the gradient towards the electrode of opposite charge (i.e. electrophoretic migration). However, because of the pH gradient, the molecules reach a point where their net charge is zero and migration stops. This is known as the isoelectric point. As a substance moves out of its isoelectric point region, it encounters a different pH, acquires a charge and is forced by the electric field back to its isoelectric point. The process results in extremely sharp bands of sample molecules which are then swept past the detector by use of pressure.

Mikkers et al.\textsuperscript{165} demonstrated that high efficiency separations are best achieved by ensuring the sample concentration is only a small fraction of the buffer concentration. Otherwise, detrimental inhomogeneities in the electric field form across the capillary, because of concentration gradients induced by differential migration of different solutes.

Applying a voltage of several kV across an electrolytic solution has the potential to generate considerable thermal energy through the Joule heating effect. Much of the early theoretical discussion concerned heat dissipation in relatively large inside diameter capillaries when an electric field was applied over such a column containing an electrolyte. Jorgenson and Lukacs\textsuperscript{166,169} demonstrated that narrower capillaries than those used by Mikkers et al.\textsuperscript{165} were more effective in minimizing the effects of Joule heating. They also developed the key relationships between separation efficiency, voltage, column diameter, column length, solute concentration, and resolution.

4.2.1 Sample Injection

The combination of minimum band broadening on the column and the short analysis times possible with CZE produces an extreme constraint on sample introduction into the capillary. The samples must be injected in an on-column
fashion with minimum volume to preserve the high separation efficiency. There are several very simple approaches to sampling, which may or may not be suitable for the CZE investigation required. Most commercial instruments are capable of two or more different injection techniques, to cope with a variety of applications. The simplest methods of sample introduction are direct electrophoretic migration of the sample onto the column (electrokinetic injection) and hydrodynamic injection via generation of a pressure difference between inlet and outlet of the column.

Electrokinetic injection is applicable to any charged species and involves placing an electrode, with the inlet to the capillary, into the sample. On applying a voltage, charged molecules start to migrate towards the electrode of opposite charge. Therefore if the outlet of the column is held at a negative potential with respect to the sample, cations will preferentially enter the column. In free-zone electrophoresis, neutrals and some anions will also be sampled because of the effect of electroosmotic bulk flow. Sample volume is determined by the applied voltage during injection and the duration of the potential whilst the inlet is in the sample. The discrimination towards species of a certain charge (i.e. it is equally applicable to anions if polarities are switched) may be useful as a crude analyte concentration procedure. For example, the technique may be useful for sampling acylcarnitines directly from urine. Carnitine compounds, under neutral or slightly acidic conditions, will be cationic and therefore should be selectivity sampled onto the capillary, minimizing possible interference by organic acids and other urinary components. However, the technique does not allow a representative sample to be analysed and therefore its application is limited. Huang et al.\textsuperscript{178} showed that electrokinetic injection induces a bias proportional to the total mobility of each ion. The peaks can be corrected for differential mobilities by normalization with their retention time.

Honda et al.\textsuperscript{179} automated a syphoning sampler for CZE. A hydrostatic pressure gradient was generated by raising the inlet of the capillary, in the sample, above the
outlet reservoir. Sample volume was determined by relative heights of the inlet and outlet reservoirs and duration of the syphoning process. Automation of the technique involves mechanical complexity, although it is available on a commercial instrument. Sample viscosity is likely to affect the volume introduced onto the column and reproducibility between samples may be a problem.

Other methods of using pressure to introduce material into a capillary include applying positive pressure on the sample vial, or negative pressure at the outlet of the capillary. Both methods are available on commercial instruments and have been used to investigate CZE analysis of acylcarnitines in this project. Both methods introduce a representative sample into the capillary, however, care has to be taken to ensure good seals on application of the pressure difference to avoid irreproducible injection volumes. The maximum length for the injected zone and the detection volume are dependent on efficiency, column length and retention time. In practice, maximum efficiency for a particular separation requires an empirical approach investigating the effect of the many variables of CZE.

4.2.2 Detection

Irrespective of the injection method, once the sample is in the capillary, the inlet of the column is immersed in a reservoir of electrolyte and electrophoresis effected by application of a high voltage as in Figure 22. After separation, a detection system is required to produce peaks in an electropherogram. As with HPLC, UV-visible absorbance detection is the most popular means of obtaining data from a CZE experiment. Due to the need for small detection volumes to preserve separation efficiency, UV detection is accomplished on-column, such that the path length is defined by the diameter of the capillary. This limits the sensitivity of absorbance methods, since sensitivity is proportional to path length. The development of UV light focusing devices has been critical in the design of commercial instrumentation. By stripping a small section of the polyimide coating off the capillary, the focused
UV light passes through the column to a photomultiplier. Foret and Bocek used fibre optics to adapt a standard HPLC UV detector for use with CZE.\textsuperscript{182} A photodiode array for on-column UV detection in CZE has also been described.\textsuperscript{183}

Laser light sources are finding application in CZE detection. The light from a laser is highly compatible with focusing onto a small capillary diameter. Helium cadmium lasers are the most popular, being relatively inexpensive and they emit light in the UV range (325 nm).\textsuperscript{183-187} The more powerful argon ion lasers, have also found application.\textsuperscript{188-190} A current disadvantage of lasers is the limited wavelengths available for use. Despite this, some impressive analyses have been performed. For example, Dovichi and co-workers used a 1W Ar⁺ ion laser to detect derivatized amino acids in human urine with a separation efficiency equivalent to 200 000 plates and a detection limit of 50 atmol of analysed analyte.\textsuperscript{191} The introduction of tuneable lasers is likely to find many further applications in CZE.

Fluorescence detection is also applicable to CZE. An important advantage of fluorescence detection is the fact that it is usually much more sensitive than UV detection (depending on the quantum yield for fluorescence of a given analyte). On-column detection is simply accomplished by focusing the excitation source onto the column and collecting the emission at an angle perpendicular to the incident light. The use of sensitive fluorescence detection has allowed analysis of DNS- and fluorescamine-labelled products form tryptic digests of proteins.\textsuperscript{192} The disadvantage of derivatization is often overcome by the advantages of increased sensitivity and selectivity.

An alternative to derivatization of non-fluorescent compounds is the use of indirect detection. With this technique, the analyte displaces a chromophore uniformly present in the background electrolyte. Thus, in CZE, a charged chromophore can be used such that analyte ions of like charge will displace the chromophore, while
ions of opposite charge may ion-pair with it. This method allows detection of species which would otherwise be transparent to the detector, since the signal produced is independent of the chromophoric properties of the analyte. Foret et al.\textsuperscript{193} used the technique to determine metabolic carboxylic acids in a background electrolyte containing benzoic or sorbic acid. A detection limit of 0.5 pmol was achieved from these weakly chromophoric analytes.

Electrochemical\textsuperscript{194} and radioisotopic\textsuperscript{195} detectors have also found application, however, the technology has largely relied on minaturisation of HPLC detectors. One of the most exciting detection developments currently attracting attention is the combination of CZE with mass spectrometry. The introduction of atmospheric pressure ionization techniques has increased the compatibility of the two techniques. Such CZE/MS methods avoid vacuum-induced-flow through the capillary. Smith et al.\textsuperscript{196} have demonstrated the use of an electrospray interface for CZE/MS. The interface makes electrical contact with the electrophoretic buffer via a thin film of silver deposited on the surface of the capillary. Electrospray ionization is performed by biasing the potential between the capillary outlet and the ion optics of the mass spectrometer inlet. The solvent in the spray produced in this manner is evaporated with hot N\textsubscript{2} before the ions enter the mass analyser of a quadrupole instrument. The technique was applied to peptide, protein and quaternary ammonium salts. Total ion electropherograms had sensitivities comparable to those obtained with UV detection. Recent improvements of the electrospray interface for CZE/MS have allowed application of the technique to large biomolecules.\textsuperscript{197} The formation of multiply charged molecular ions allowed analysis of proteins with molecular weights greater than 130 000 daltons, with a determination of MW to 50-100 ppm accuracy, on a sample injection of 0.1-10 pmol of the analyte. An extension of the technique by interfacing the CZE to a triple quadrupole mass spectrometer allows CZE/MS/MS experiments.\textsuperscript{197,199}
4.2.3 Micellar Electrokinetic Capillary Chromatography

Although CZE is a highly efficient separation technique, the basic method is only applicable to charged compounds, because the separation principle is based on the difference in electrophoretic mobilities. Typically, in free-zone capillary electrophoresis, neutral molecules do not separate but travel as a single band at the speed of electroosmotic flow. Micellar electrokinetic capillary chromatography (MECC) is performed with the same equipment as CZE but employs the chromatographic principle of separation, i.e. partition between two phases. That is, the method permits the separation of uncharged or neutral analytes by the electrophoretic technique.

The principle of MECC is shown schematically in Figure 23 (the double layer formed at the capillary wall and responsible for electroosmotic flow is not depicted for simplicity). Charged surfactants are dissolved in the electrophoretic buffer to produce charged micelles. Sodium dodecyl sulphate (SDS) forms suitable micelles and is commonly used for MECC. The micelles are represented as the open circles in Figure 23, in which neutral analyte molecules (represented by x's) may be incorporated. When a high voltage is applied over the capillary, the negatively charged SDS micelles are attracted to the positive electrode. However, the electroosmotic flow, caused by the double layer at the capillary walls, effects bulk flow towards the cathode. Under neutral pH conditions, the electroosmotic flow is greater than the electrophoretic velocity of the micelles. Separation of the neutral analyte molecules is thus determined by their relative affinities for the hydrophobic micelles. The separation occurs as a result of analyte molecules partitioning between the micelles and the surrounding electrolyte. Neutral molecules which have zero affinity for the micellar matrix remain in the aqueous bulk solution and therefore migrate through the capillary solely under the influence of the
Figure 23. Schematic diagram of micellar electrokinetic capillary chromatography (MECC)

---

Figure 24. Schematic representation of a MECC chromatogram
electroosmotic flow. However, molecules which do become incorporated into the micelles for any period of time will, overall, travel slower owing to the electrophoretic mobility of the negatively charged micelles. As such, the affinity of the neutral analyte molecules for the micelles determines their rate of migration through the column. A molecule which is totally incorporated from the time of injection will have the slowest migration time of any neutral species.

A schematic representation of the migration of three typical zones and the corresponding chromatogram/electropherogram are shown in Figure 24. The electroosmotic velocity $V_{eo}$ is assumed to be greater than the electrophoretic velocity of the micelles, $V_m$, in the opposite direction. All solutes injected at the anode end of the capillary, move through the column to the cathode, as in Figure 23. The band of water, or a tracer for water such as methanol, is, in conventional chromatography terms, unretained. It does not dissolve in the micelles, at any stage and therefore migrates through capillary at the fastest speed for a neutral molecule, $V_{eo}$. (N.B. Non-incorporated cations will travel faster due to the combined effects of electroosmotic flow and electrophoretic mobility.) At the other extreme, a tracer for the micelles which is totally incorporated in the hydrophobic micelle structure and is neutral, moves with the velocity $(V_{eo} - V_m)$. A neutral solute which is partially included is transported through the capillary at a velocity between the two extremes. The capacity factor $(K')$ of the zones is given by:

$$K' = \frac{t_R - t_0}{(1 - t_R/t_c) t_0} \quad \ldots \ldots \text{equation (1)}$$

and may be calculated from the retention times of a chromatogram such as that in Figure 27. In conventional chromatography, a capacity factor of infinity means that the solute would not be eluted because it would be totally retained by the stationary phase. In MECC, however, every solute is eluted in the retention time window between $t_0$ and $t_c$ provided they are electrically neutral.
The first results of MECC were achieved using SDS micelles, however, the use of other surfactants should find application. When cationic surfactants are used, the micelles migrate to the cathode but the electroosmotic flow is towards the anode. This is because the inner wall of the capillary becomes positively charged owing to adsorbed surfactant molecules and hence all solutes migrate towards the positive electrode, assuming electroosmotic flow is greater than the electrophoretic velocity of the micelles.

4.3 APPLICATION TO ACYL CARNITINE ANALYSIS

The charged nature of acylcarnitines makes them potential analytes for free-zone capillary electrophoresis, without derivitization. If the carboxyl group of the carnitine backbone is protonated (i.e. at low pH values) the acylcarnitine will be cationic, due to the remaining positive charge on the quaternary ammonium group. The application of CZE techniques to analyse standard solutions of acylcarnitines employed free-zone capillary electrophoresis. Solutions of individual acylcarnitines and mixtures of several carnitine esters were subject to various CZE conditions. Initial investigations were made during 'demonstration sessions' for four commercially available instruments. Different sample introduction and detection systems were evaluated in their application to acylcarnitine analysis. Experiments were also conducted to determine the effect of applied voltage, temperature, pH, column diameter and length, and acylcarnitine concentration (Section 6.3.23). An acidic pH was favoured in an attempt to protonate the carboxyl group of the carnitine backbone, presenting the acylcarnitine as a cationic species. Without protonation, carnitine compounds are neutral and therefore would not separate under free-zone electrophoresis conditions. Excessive acidification, however, may result in acid-catalysed hydrolysis of acylcarnitines, resulting in detection of carnitine as the only cationic species. Separation of peptides and amino acids has previously been achieved at pH 3-5, suggesting this may be an appropriate pH range for acylcarnitines, considering the basic similarity between the structures of...
carnitine and amino acids. A sodium phosphate/sodium borate buffer system was used as the electrolyte and to maintain the acidic conditions.

The results obtained during initial investigations with free-zone capillary electrophoresis were difficult to interpret. When individual acylcarnitine solutions were analysed, a peak was usually observed in the resulting electrophoreogram if the solute concentration of acylcarnitine was in excess of 5 mg ml\(^{-1}\). The migration time for the component which gave the UV detector response varied significantly, depending on the analysis conditions. Over the experiments performed, however, it was confirmed that separation of a mixture of acylcarnitines was not achieved to any useful degree. Depending on the CZE operating parameters, the migration time for each acylcarnitine was very similar. The time taken for the carnitine esters to elute from a capillary, under acidic conditions, suggested they were acting electrophoretically as cations since they migrated faster than the electroosmotic velocity, \(V_{eo}\) (see Figure 24). Peak shape was generally very poor and could be attributed to the high sample loading required for UV detection of the weakly chromophoric analytes. Improved peak shapes were obtained with the phenyl-containing acylcarnitines, when lower solute concentrations were analysed, having greater molar absorption coefficients in the UV region. Separation of 3-phenylpropanoyl-carnitine and 4-phenylbutanoylcarnitine could be achieved under some CZE conditions although the peaks detected were not fully resolved nor of the quality expected for CZE. It was concluded from the initial experimental investigations made to date that free-zone electrophoresis did not provide the separation efficiencies required for acylcarnitine analysis. At the concentrations required for detection, the mass-to-charge ratios were not sufficiently different to result in resolved peaks. Reducing the acylcarnitine quantities introduced into the capillary may improve the separation process, however, alternative detection systems would be necessary for analysis of the weakly UV absorbing analytes.

Chemical conversion of acylcarnitines to derivatives amenable to UV and/or
fluorescent detection may be effective for subsequent CZE analysis. Chemical derivatization of acylcarnitines has previously been achieved for HPLC determinations and has been outlined in Section 1.2. The introduction of better chromophoric groups, compared to the existing carboxyl function, will allow lower concentrations of the analyte to be detected. This may avoid the overloading effect currently encountered and enable superior peak shapes to be obtained. Then, the characteristic high separation efficiency of CZE may be realized.

Since free-zone capillary electrophoresis was not successful for acylcarnitine separation, micellar electrokinetic capillary chromatography (MECC) was investigated (Section 6.3.24). The principle was to effect separation by exploiting the partitioning of acylcarnitines between the aqueous electrolyte and sodium dodecyl sulphate (SDS) micelles. A pH 7 buffer electrolyte was used with the acylcarnitines present as zwitterionic, overall neutral, species. This is appropriate to MECC where electrophoretic migration applies to the negatively charged micelles rather than the analyte molecules themselves. Figure 25 shows an electrophoregram from the MECC analysis of a mixture of eleven acylcarnitines. The peak assignments shown in Figure 25 were based on analysis of individual standard acylcarnitines under identical MECC operating conditions. These results represent initial investigations although initial variations of the MECC conditions (i.e. SDS concentration, temperature and applied voltage) did not improve the separations. The electrophoregram in Figure 25 is characterized by poor peak shapes for the non-aromatic acylcarnitines (eg. 9.12 and 11.45 min.), attributed to saturation of the available micelles. Lowering the SDS concentration resulted in worse peak shapes, but higher concentrations of the micelles did not improve on those in Figure 25. Assuming MECC is performed at or above the critical micelle concentration (c.m.c.), the concentration of SDS affects the elution time of the carnitine esters. An increase in SDS concentration causes an increase in the capacity factor ($K$) of each solute, since $K$ represents a distribution coefficient.
Figure 25. MECC electrophoregram of a mixture of standard acylcarnitines.
between the micelles and the aqueous electrolyte phase (see equation 1).

Consequently, the higher the SDS concentration, the slower the elution within the retention time widow (i.e. between the elution times of non-incorporated component and a totally incorporated micellar component). Poor peak shapes may occur when high analyte concentrations are present in the capillary, because incorporation into the micelles will also be dependent on the concentration of the micelles available. This effect will contribute to broadening of peaks such as that observed for octanoylcamitine when SDS concentration was reduced.

The elution order of the acylcarnitine mixture shown in Figure 25 may be interpreted in terms of affinity of the solutes for the non-aqueous micelles. Octanoylcamitine has the longest retention time (11.4 min) and this can be explained by the relative hydrophobicity of the alkyl chain of this compound. The electrophoregram suggests that the longer the acyl-chain of the carnitine ester, the greater is the affinity for the micelle. The remaining peaks in the electrophoregram correspond to the other acylcarnitines in the mixture and to some unknown components. For instance, the peak at 3.38 min was thought to originate from a neutral or weakly cationic species since it elutes with or just before water, identified by the dip in the baseline. This and some of the other non-assigned peaks in the electrophoregram may represent impurities in the acylcarnitine samples.

The initial experiments conducted into MECC suggest that such CZE techniques may be applicable to acylcarnitine analysis given successful development from these early investigations. Chemical derivatization of the analytes with strongly chromophoric groups (in common with free-zone CZE) is likely to improve the peak shapes of the non-aromatic acylcarnitines, since reduced levels may be detected, avoiding saturation of the micelles. The use of alternative micelle systems also needs to be investigated if optimum separations are to be achieved.

Appropriate methods should then be applied to spiked urine samples to determine what, if any, sample purification is required.
The potential of CZE techniques to acylcarnitine determinations makes it worth pursuing. In comparison to the GC/MS method, CZE offers some relative advantages if the techniques can be suitably developed. Determinations in the aqueous phase avoids the complications involved in transfer to an organic solvent required for GC applications. Sampling directly from the urine matrix, using electrokinetic injection may avoid or allow a simpler work-up procedure than that currently employed for the derivatization and GC/MS method. CZE techniques should also be applicable to acylcarnitines with any functionality in the side chain. Problems may be encountered in applying the ion-exchange purification and GC analysis procedure (Section 3.2.4) to carnitine esters of dicarboxylic acids. Such difficulties are not envisaged for CZE techniques.

The disadvantages associated with CZE largely arise from the fact that the methods are still in the development stages. Commercial instruments have only recently become available and it is likely to take some time before CZE is an established technique in the clinical laboratory. The lack of structural information on eluting components is one which is common to all stand-alone chromatography techniques. Development of CZE/MS is gaining momentum and should prove to be a very useful analytical technique for compounds such as acylcarnitines, although it is doubtful if this technique will become widely available to hospital laboratories in the imminent future.

In the area of acylcarnitine analysis, CZE techniques and their development are seen as a viable complement to the proven GC/MS method. The potential of CZE remains promising but considerable effort is required to build on the results reported here, to produce a method applicable to all physiological acylcarnitines in body fluid samples.
CHAPTER 5

CONCLUSIONS AND FUTURE WORK
Throughout the period of this project, interest in the biological role of carnitine has maintained a flux of publications from research groups worldwide. The relevance to inherited metabolic diseases has continued to be the driving force behind such research. Acylcarnitine determinations in biological fluids are still regarded by many as important in elucidating the metabolic status and influencing therapeutic care. The derivatization method reported here has been shown to be appropriate for many such applications. As a result, several established groups involved in acylcarnitine determinations have shown an interest in the GC/MS procedure. This has resulted in fruitful collaborations from which the initial results are included in this thesis. It is hoped and expected that such contacts will continue in this developing area of clinical chemistry.

As a method for identifying common physiological acylcarnitines, the derivatization and GC/MS method can now be included as an option alongside the other existing procedures and techniques (see Section 1.2). For the simplest analysis, the major analytical instrument need only be a conventional capillary gas chromatograph with flame ionization detection. Such apparatus along with the required reagents, ion-exchange resins and standard laboratory equipment allows detection of acylcarnitine derivatives from a sample of urine. This basic setup allows separation of a mixture of acylcarnitines, including structural isomers; however, identification relies on matching peak retention times with those of authentic standards. For unambiguous analysis, structural information on the components eluting from the GC column may be obtained by interfacing to a mass spectrometer. Several clinical research establishments and hospital laboratories have GC/MS equipment and the introduction of affordable bench-top instruments makes the derivatization procedure an attractive proposition for simple and rapid determinations of urinary acylcarnitines. During this project, a bench-top GC/MS system has been used to identify acylcarnitine derivatives, detecting octanoyl- and hexanoyl- and phenylpropionylcarnitine in the urine of a patient with MCADD. The availability
of an affordable acylcarnitine analysis procedure contrasts with the high expenditure and running costs of LC/MS and MS/MS techniques previously required for comparable determinations. It is believed that this factor, together with the success of the method demonstrated during the project, will lead to continued development of the procedure.

Derivatization of acylcarnitine standards in the absence of a biological matrix has indicated that the optimized conditions give an excellent yield of the butyrolactone compounds. Yields above 70% were regarded as acceptable during the development stages. Ultimately however, the required sensitivity is dictated by the analysis being attempted and this includes extraction efficiency, derivatization yield, handling losses and detector sensitivity. Therefore, when deciding if the method is sensitive enough for acylcarnitine analysis of biological samples the complete analytical procedure has to be considered. For the future it is important to address those steps in the procedure having the worst effect on recovery (i.e. mitigate against low detection limits) and improve them for the good of the whole method.

Defining the sensitivity required depends on the application intended. Initially it was envisaged that the derivatization method would be able to determine the characteristic acylcarnitines in the urine of patients with metabolic disorders such as MCADD and isovaleric acidemia and this has now been demonstrated to be so. However, minor acylcarnitines are now known to be excreted along with the major carnitine esters in some disorders (e.g. unsaturated octenoylcarnitine in MCADD). Such metabolites are beginning to be detected with the derivatization method and it is hoped that improvements will enable routine detection of such compounds, important for full elucidation of the metabolic defect. Significant improvements to sensitivity may even enable detection of acylcarnitines in the urine of normal infants and adults (thought to be excreted at the pmol level).

The results from the studies conducted so far suggest that improvements to the extraction procedure, which may only recover about 30% of each acylcarnitine,
would lead to substantially better sensitivity and selectivity. It is likely that improved sensitivity will be dependent on greater selectivity towards acylcarnitines in biological samples. The results from the experiment described in Section 6.3.18 (see Section 2.5.3) indicate that the constituents of the biological matrix, which are co-extracted with the acylcarnitines, reduce the yield of the derivatization step. Therefore an acylcarnitine-specific extraction procedure should also lead to an improved derivatization step for physiological carnitine compounds and hence greater sensitivity of the method. In practice, this may not be straightforward since the extraction procedure needs to be applicable to a range of acylcarnitines with different chemical properties. This problem may be further compounded when attempts are made to analyse acylcarnitines with additional functional groups such as acid functionalities in the side-chain. By nature, the extraction procedure then has to cover metabolites with a wide range of polarities and ion-exchange resin behaviour. For instance, glutaryl carnitine and even long-chain acylcarnitines (i.e. above C12) are known* to have relatively high affinities for anionic-exchange resins, making the existing extraction procedure inappropriate. Further investigations of solvent extraction techniques may be required for such applications.

The large amounts of urea known to be extracted with the acylcarnitines using the double ion-exchange method may be one of the urinary components influencing the derivatization process. This has not been fully evaluated to date although several unsuccessful attempts were made to degrade this compound enzymatically prior to derivatization (see Section 2.5.3). It is believed that this area is worthy of further attention especially since urea interferes with the chromatographic resolution of short-chain acylcarnitine derivatives.

* R.J. Morrow and M.E. Rose, unpublished results.
Improvements to the speed of analysis and hence sample turnover rate may also be envisaged. This aspect is particularly important to the interests of clinical screening laboratories where automated or partially automated sample pretreatment and analysis are commonplace. The current procedure requires a degree of manual skill to achieve optimum results. Sample turnover to an extent, is dependent upon freeze-drying facilities, analyst experience, and the performing of several ion-exchange procedures simultaneously. Analysing more than five samples per day will require alternative sample pretreatment procedures. Initial investigations with a one-step, mixed-bed, ion-exchange column have been tried (see Section 2.5.4) and further studies with such commercially available units may result in a quicker analysis. Ion-exchange membrane units may also be useful and are probably worth some initial trials.

The gas chromatographic equipment and operating conditions have met all the requirements to date. Peak shapes for the acylcarnitine derivatives are excellent and the resolution of responses for all carnitine esters encountered have aided unambiguous assignments. The limit of detection of the GC step has proved adequate for trace analysis. Therefore, improvements to the chromatographic step are currently not necessary. However, with the technique being applied to an ever widening range of carnitine compounds, this may have to be reviewed in the future. Identification of the compounds eluting from the GC column will remain to be most effectively achieved by mass spectrometry, although rapidly advancing MS techniques may also lead to sensitivity improvements. For instance, ion trap instruments are gaining popularity due to their associated high sensitivity, compatibility with gas chromatography, bench-top dimensions and low cost. Such an instrument may prove to be well suited to the analysis of derivatized acylcarnitines.

The identification of urinary acylcarnitines has been an encouraging success of this project and is currently attracting attention from other research groups. However,
an emerging requirement is the availability of quantitative data on the identified metabolites. Such information is important to the deduction of clinical status and also to investigations of the acylcarnitine excretion process. Studies conducted in this project have helped to uncover some interesting variations in acylcarnitine excretion over a time period. Whilst these experiments were done on an estimation basis only, the findings warrant further exploration with truly quantitative analysis.

The use of phenylbutanoylcarnitine as a spiked internal standard has remained consistent throughout the analysis of clinical samples in this project. The aromatic acylcarnitine fulfilled the basic requirements of an internal standard in that (i) it was extracted from the spiked raw urine and derivatized along with the analyte carnitine compounds thus allowing comparison of signals and estimation of quantities, (ii) it is not a naturally occurring metabolite, (iii) it is available as an easily synthesised compound (see Section 2.2), and (iv) during chromatography its derivative eluted in a relatively uncluttered area of the chromatogram, resolved from other peaks but without an excessively long retention time. Other internal standards were not investigated although alternative non-physiological acylcarnitines such as heptanoylcarnitine may be equally acceptable. 85

When developing a quantitative analysis pure standard compounds are best used in the calibration process. Calibration mixtures of varying analyte concentrations and fixed internal standard concentration are made up and a calibration curve of the ratio of peak area versus analyte concentration is then constructed. Obtaining high purity standards may require further investigation of the acylcarnitine synthesis procedure. 111 Alternative methods of preparation and purification are available and should be considered in attempts to produce the purest standard compounds. The other published methods of acylcarnitine preparation are also based on the condensation of the relevant acid chloride with carnitine but formation of the acid chloride, reaction solvents and work-up procedures vary from the methods of Ziegler et al. 111 used in this project to date. Purities in excess of 95% should be the
objective of future syntheses for quantitative purposes. Calibration curves should cover the concentration range of physiological acylcarnitines expected in the clinical samples to be analysed. Also, when using mass spectrometry as the detection system, it is generally recognized that selected ion monitoring (SIM) is the most effective means of obtaining good quantitative data. As previously described, this can also decrease detection limits by a factor of 100-1000 and so this change at the mass spectrometric stage is most likely to be the single most efficacious means of improving detectability. When combined with an optimized extraction procedure, SIM may be applicable to acylcarnitine excretion in patients with normal β-oxidation metabolism.

For optimum quantification, the ideal internal standard and the substance to be quantified must behave identically during sample pretreatment, derivatization and work-up. This would ensure that incomplete extraction and/or derivatization would not influence the ratio of peak areas (analyte to internal standard) used in the construction of a calibration graph. It has been shown in this project that incomplete extraction and derivatization does occur with physiological acylcarnitines and that the degree of incompleteness varies with different carnitine esters. Therefore, there lies a flaw in the use of a single acylcarnitine (i.e. phenylbutanoylcarnitine) as an internal standard for the quantification of other acylcarnitines. Isotopically labelled analogues of the substance(s) to be quantified are useful internal standards for mass spectral analyses. Such compounds are practically identical in chemical properties whilst being readily distinguished by their mass differences. The use of isotopically labelled acylcarnitines as internal standards for the quantification of physiological acylcarnitines by FABMS and FABMS/MS has been reported. The same or similar approaches may be usefully employed with the derivatization and GC/MS technique. However, consideration must be given to the difference in ionization modes. For instance, under EI conditions it has been shown that the lactone derivatives readily fragment.
Selected ion monitoring of a major fragment ion such as that assigned to the lactone ring residue \((m/z = 85)\) should provide a sensitive assay of most acylcarnitines. To exploit this sensitivity in a quantitative analysis, the internal standard fragment ion must contain the label atom(s) since there will be little or no chromatographic separation between analogues. Therefore when using electron ionization the internal standard must be isotopically labelled on one of the carnitine backbone atoms, with the exception of the nitrogen atom or any carbon atom of the quaternary ammonium group since this is eliminated during the derivatization.

If CI conditions are to be employed then the same internal standards as with EI may be used or alternatively, \(^{13}\)C, \(^{18}\)O and/or \(^2\)H labelling may be incorporated in the side-chain. The wider range of labelling sites with CI arises because this mild form of ionization results in minimum fragmentation and therefore SIM may be performed on the intact ion of each derivative (i.e. \([M + H]^+\) ion). The potential disadvantage of using CI with SIM is that there is no fragment common to the majority of the acylcarnitine derivatives, unlike the situation with GC/EIMS.

Therefore, the analyst may need to perform a full-scan investigation of the sample prior to SIM quantification in order to identify the acylcarnitines present and thus select the ion or ions to be monitored. Unlike the single ion monitoring possible by GC/EIMS, the sensitivity would be reduced by the need to focus on all \([M + H]^+\) ions of derivatives known, or expected, to be present.

The objective of an accurate quantitative assay in future developments of the derivatization method should not detract from the validity and importance of the quantitative results achieved so far. The trend in acylcarnitine excretion levels over the postpartum period of an MCADD patient demonstrated that the GC/MS method and an FABMS/MS investigation of the same samples yielded comparable results. Both techniques confirmed the original hypothesis based on urinary organic acid profiling. Such a discovery, if it is shown to be common to other infants with
MCADD will have important implications for future screening programmes and therapeutic treatment. Whilst there are difficulties in arranging time-course studies of body fluids for new-born babies in a hospital environment, the potential treatment and preventative care advances which may be derived should encourage such investigations. This may even be extended to other metabolic diseases since the results obtained in this project indicate that urinary concentrations of acylcarnitines are variable for patients with any of the recognized disorders. The general observation of higher acylcarnitine levels being detected in basal condition urines as compared to those collected during or shortly after crisis needs to be addressed. Controlled studies (or as near as possible to controlled studies) should be performed whereby accurate and detailed sample information is available, so vital for correct interpretation of the analytical results. This will demand close and clear liaison between the analyst, clinician and any other persons involved with sample collection, storage, work-up and analysis.

The derivatization and GC/MS technique has been shown to be effective in detecting the diagnostic acylcarnitine metabolites of MCADD, isovaleric acidemia and propionic acidemia. It is hoped that the method will also be successfully applied to some other β-oxidation disorders which result in acylcarnitine excretion. Included in these diseases are those which gave negative results during the initial investigations conducted in this project. The analysis of two urine samples from a patient with methylmalonic aciduria may have been expected to detect propionylcarnitine as observed by Roe et al. The predicted metabolite was not observed and without detailed information on the sample it is only possible to speculate on the reason(s) for its absence or reason for going undetected (see Section 3.2.4). Further studies on urine from patients with methylmalonic aciduria are required before conclusions can be drawn on the suitability of the method to such samples. Another disease which is likely to yield urinary acylcarnitines, directly amenable to the existing derivatization method is β-ketothiolase deficiency.
As the name suggests this disorder results in an insufficiency of a mitochondrial enzyme required for the β-oxidation process (see Scheme 3). The disorder may result in the urinary excretion of tiglylcarnitine \((2, R = \text{C(CH}_3\text{)=CHCH}_3)\) as observed by Millington et al.\textsuperscript{102,103} Despite the unsaturated function in the side chain of this acylcarnitine metabolite, the existing extraction and derivatization procedures should be amenable to such a compound. As with the cases of methylmalonic aciduria, further analysis of samples from patients with diagnosed β-ketothiolase deficiency are recommended.

Recently, an interest has developed in the metabolic role of some acylcarnitines which may not be directly amenable to the existing GC/MS method. Long-chain acyl-CoA dehydrogenase deficiency (LCADD) is a disease analogous to MCADD with a metabolic block to β-oxidation resulting in formation and excretion of long-chain acylcarnitines \((C_{12-20})\) rather than the medium-chain length acylcarnitines associated with MCADD. The cyclization of long-chain carnitine esters is not expected to cause difficulties, especially since palmitoylcarnitine (i.e. \(C_{16}\) acyl chain length has been shown to be amenable to the technique during the development stages of this project (see Chapter 2).\textsuperscript{108} However the extraction of such long acyl-chain compounds from biological material may require a review of the existing ion-exchange procedure. Poor recoveries are expected from the resins\textsuperscript{202} and this may necessitate the use of alternative pretreatment techniques. Solvent extraction methods have been reported for recovering carnitine esters from biological material including urine. Simple investigations may present a suitable solvent extraction procedure for long-chain acylcarnitines.\textsuperscript{202}

In some recognized disorders, carnitine is known to conjugate with dicarboxylic acids. Conjugation by ester bond formation with one of the acid carboxyl groups leaves one free carboxyl group terminating the acyl chain of the carnitine ester. Glutaryl- \((2, R = (\text{CH}_2)_3\text{COOH})\), adipyl- \((2, R = (\text{CH}_2)_4\text{COOH})\), suberyl- \((2, R = (\text{CH}_2)_6\text{COOH})\) and sebacylcarnitine \((2, R = (\text{CH}_2)_8\text{COOH})\) are examples of
dicarboxylic acid acylcarnitines positively identified in metabolic profiles of urine from infants with perturbed fatty acid catabolism. For instance, the disorder, glutaric aciduria type 1, is known to be accompanied by the urinary excretion of glutaryl carnitine. HPLC and FABMS/MS techniques have been applied to such samples, together with their associated disadvantages (see Section 1.2). It is intended to develop the existing derivatization technique to be amenable to such metabolites. However, in order to attain this objective, several potential problems in applying the analysis procedure to carnitine esters containing a carboxyl function in the side-chain need to be evaluated and, if necessary, modifications implemented so that the advantage of the GC/MS technique are maintained. The first consideration involves the appropriateness, or otherwise, of the extraction procedure. A second free carboxyl group in the acylcarnitine molecule is likely to markedly affect the affinity for the ion-exchange resins currently employed. An alternative elution procedure may be required. Once the analyte molecules have been shown to be effectively extracted, then the consequences of the side-chain carboxyl function on the cyclization process should be determined. As already mentioned (Section 3.2.4), the favourable formation of a five-membered ring by the action of the carnitine carboxyl group is likely to result in the required butyrolactone derivative rather than a product of an intramolecular reaction involving the side-chain carboxyl. Any such reaction leads to formation of a larger ring which is kinetically less favoured. Assuming that extraction and derivatization can be suitably achieved, there may remain a problem concerning the gas chromatographic properties of an analyte containing an organic acid function. A further, simple derivatization step such as trimethylsilylation (TMS) may be necessary to produce a volatile derivative of the original dicarboxylate ester of carnitine which is amenable to GC and GC/MS.

Once the method is shown to be compatible with physiologically occurring acylcarnitines, the analyst will have a powerful tool for investigating defective
metabolisms presented by the recognized diseases of fatty acid catabolism and also uncharacterized ones such as Reyes Syndrome. Reyes-like symptoms have been reported previously without sufficient analytical information (largely due to the lack of a convenient analysis) to characterize conclusively the exact nature of the metabolic disorder. It is predicted that the derivatization method reported here, together with some "fine tuning" of the technique will be ideally suited to such studies, studies which may be conducted in many modern clinical laboratories.

In addition to the identification of acylcarnitines produced from the metabolism of dietary fatty acids and amino acids, results from the initial investigations of some exogenous compounds have also proved interesting (see Section 3.3). The excretion of urinary phenylpropionyl-carnitine from MCADD patients has subsequently been confirmed by others, but it was the present derivatization and GC/MS technique which first positively identified the metabolite, originating from the administration of phenylpropionic acid (PPA). Considering the metabolism of fatty acids in patients with MCADD, the occurrence of phenylpropionylcarnitine is easily understood and may even have been expected. It is interesting to note, however, that this metabolite was not always present in the urine of MCADD patients even when a PPA load had been administered shortly before urine collection. Controlled time-course studies combined with analysis by the GC/MS method may give further indications as to how the excretion of phenylpropionylcarnitine varies. However, interpretation of the results must also take into account the PPA produced, naturally and independently of any exogenous administration, in the gut.

The results of analysing the urine of patients receiving valproic acid treatment were less easy to explain. It is postulated that some patients being administered this anticonvulsant drug excrete acylcarnitines previously unreported. Analogous to the metabolism of PPA, valproylcarnitine may have been predicted as an excreted substance when a defective β-oxidation condition was present. Indeed, Millington
et al.\textsuperscript{105} have observed this metabolite using FABMS and thermospray LC/MS. Investigations in this project, using the derivatization method, also detected a component extracted from an appropriate urine sample which had acylcarnitine GC/MS characteristics and a GC elution time identical to synthesised valproylcarnitine. However, the analysis also indicated a larger concentration of another acylcarnitine derivative. The EI mass spectrum could not be assigned to any of the conventional acylcarnitines previously encountered. On the basis of the mass spectrum, it was postulated that the metabolite detected was 3-oxovalproylcarnitine. This assignment is supported by an earlier study of valproic acid metabolism in man which found 3-oxovalproic acid to be the major metabolite.\textsuperscript{160} Confirmation of the GC/MS identification is required and, whilst chemical ionization studies may give further support to the assignment, unambiguous identification demands synthesis of the expected acylcarnitine.

Synthesis of 3-oxovalproylcarnitine may not be as simple as previously encountered with the preparation of other acylcarnitines. A potential problem rests with the free acid, 2-propyl-3-oxovaleric acid, which is a β-keto-acid, known for readily undergoing decarboxylation under heat. To minimise this reaction, very mild conditions will be needed for the formation of the required acid chloride (i.e. 2-propyl-3-oxovaleryl chloride) and for the condensation of this species with carnitine. Here, the alternative methods of acylcarnitine preparation which do not require a heating process may be employed to good advantage. If this is unsuccessful, then a transesterification reaction may be appropriate (possibly using the ethyl ester of 2-propyl-3-oxovaleric acid) or a synthesis involving protection and deprotection of the ketone functionality. Whilst the synthesis may require considerable experimental investigation, it is currently regarded as important for the confirmation of the valproic acid metabolite. It would also be interesting to determine if other techniques such as FABMS/MS can produce complementary results. The consequences of a positive identification of acylcarnitine metabolites in
patients with normal fatty acid catabolism may prove very important for complete elucidation of the metabolism of other exogenous compounds. For example, the breakdown of pivampicillin is thought to include acylcarnitines and it proposed to confirm this and investigate other drugs which involve acidic metabolites, such as cannabis components.

To date, the analysis of physiological acylcarnitines has only concerned those occurring in urine. Carnitine and its esters have also been found to accumulate in other body fluids and tissue including, plasma, muscle liver and vitreous humour. It is intended to apply the GC/MS method to these other biological matrices. Initially this is expected to involve the analysis of samples spiked with known acylcarnitine quantities, as was the case with the urine samples. Successful developments would be followed by analysis of previously characterized samples, to verify the method prior to the application to uncharacterized body fluids or tissue. The analysis of biological material other than urine is likely to require modifications of the method in terms of the sample pretreatment. The partial purification of the various biological matrices may employ the existing methods of others for FABMS/MS analyses, relying on solvent extraction, ion exchange and/or reverse-phase C18 cartridges.

Further studies regarding the GC/MS detection of urinary components assigned to cyclic dipeptides also demands attention. The relevance of such compounds in the urine of infants is uncertain and they may be the products of a normal metabolism. Confirmation of the presence of cyclic proline-isoleucine and alanyl-leucine or alanyl-isoleucine shall require the synthesis of standards to compare the analytical data with that from the biological sample assignments. If the presence of dipeptides is verified then efforts are recommended to identify their origin and deduce their relevance on the metabolism. Such studies may be well suited to alternative techniques including capillary zone electrophoresis (CZE).
The use of CZE and the closely associated micellar electrokinetic capillary chromatography (MECC) is still regarded as a promising approach to analysing acylcarnitines in complex mixtures. The separation of a mixture of quaternary ammonium salts and the applications to peptidic material support this view. Initial investigations conducted in this project gave a good insight into the effects of varying the operational parameters. Acceptable separation and sensitivity was not achieved, however. This was attributed to the UV detector employed and the instrumental restraints imposed by the effective small UV cell volume. That is, to maintain separation efficiency the UV cell is simply a small portion of the silica capillary stripped of its polyimide coating. Acylcarnitines, having no strong chromophoric function are inherently insensitive to such a detection system. Consequently concentrations of carnitine compounds which can be detected tended to substantially overload the separation process. It is proposed that conversion to p-bromophenacylester derivatives, already employed for HPLC determinations, will enable detection of the low acylcarnitine quantities necessary for effective CZE separations. Similarly, attempts to separate a mixture of eleven acylcarnitines by MECC using SDS micelles suffered from overloading effects. Again derivatization may facilitate improvements to such analyses.

The potential advantages of capillary electrophoresis techniques warrant the continued development of a method for acylcarnitine analysis. Minimum sample pretreatment, universal application to carnitine esters (including those with side chain carboxyl functionalities), high efficiency separations and the ability to interface to a mass spectrometer are all expected to influence further developments. There is also the possibility of detecting compounds other than acylcarnitines. An interest in urinary dipeptides has previously been highlighted. The analysis of peptides using CZE techniques is widely reported by others and their findings should be of direct relevance to the study of any urinary dipeptides excreted by infants. In addition, urinary components purposely removed during the GC/MS
sample pretreatment stages may be viable analytes for analysis along with the acylcarnitines. For example, successful developments with CZE techniques may allow the simultaneous analysis of acylcarnitines, organic acids and glycine conjugates all diagnostic metabolites of β-oxidation disorders. Application to such a wide range of urinary components is not currently envisaged for the GC/MS method since the results indicate that specificity in extraction of the acylcarnitines is important for successful evaluations. A simple technique which could handle a wide range of compounds, as is theoretically possible with CZE, would be extremely useful for the metabolism studies discussed in this project.
CHAPTER 6

EXPERIMENTAL
6.1 INSTRUMENTS

Melting points were taken on a digital melting point apparatus, Electrothermal Engineering Limited.

Infrared spectra were recorded on a Perkin Elmer 1310 IR Spectrophotometer. Samples were prepared as thin films or KBr discs.

Nuclear magnetic resonance (NMR) spectra, $^1$H and $^{13}$C, were recorded on a Jeol FX 90 Q Fourier Transform NMR Spectrometer (90 MHz) by courtesy of Mr. G. Howell, (Open University, Milton Keynes). All $^{13}$C NMR spectra reported are noise-decoupled.

Split and splitless injection, capillary gas-liquid chromatography (GC) with flame ionization detection (FID) was performed on a Perkin Elmer 8410 gas chromatograph. On-column injection, capillary GC was performed on a Carlo Erba Strumentazione, Series 4160 instrument, or a Carlo Erba Strumentazione HRGC 5300 Mega Series Capillary Column GC, equipped with a MFC 500 programmable control unit and a FID 40 detector. All GC analyses employed 12 or 25 m SGE fused-silica, capillary GC columns with internal diameter 0.32 mm and film thickness 0.5 μm. Further details of operating conditions are reported in the corresponding sections (i.e. Sections 6.3.11 and 6.3.12).

A VG 305 single-focussing mass spectrometer coupled to a VG DS 2050 data system, was used for one study (see Section 6.3.7).

Xenon FAB mass spectra were recorded on a VG 20-250 single quadrupole system, equipped with an IonTech saddle-type FAB gun operated at 7-8 KeV with a tube current of about 1 mA and controlled from a PDP-11 data operating system.

Three instruments were used for GC/MS (i) A VG 20-250 quadrupole system coupled to a Hewlett-Packard Model 5890 gas chromatograph and controlled from a PDP-11 data operating system. An SGE OCI-3 on column injector was fitted to
the gas chromatograph to complement the existing split/splitless injection facility. (ii) A Finnigan-MAT INCOS 50 bench-top quadrupole instrument coupled to a Varian Model 3400 gas chromatograph equipped with a 25 m fused-silica SE54 column and a splitless injector. (iii) A Hewlett Packard 5971A Mass Selective Detector bench-top quadrupole system employing split-splitless injection. Unless stated, all GC/MS analyses used SGE fused-silica columns as for the GC applications. With all instruments electron ionization (EI) was employed, with an electron energy of 70eV and an ion current of 100 µA. Investigations using chemical ionization (CI) were also effected with the HP 5971A MSD instrument, using methane as the CI gas and the VG20-250 using ammonia.

Studies requiring an ultrasound source (Section 6.3.8) employed an MSE Soniprep 150 Ultrasonic Disintegrator equipped with a 9.5 mm diameter probe.

Capillary zone electrophoreses (CZE) and micellar electrokinetic capillary chromatography (MECC) were performed with a Beckman P/ACE System 2000 connected to an IBM PS/2 computer with windows-based control software.

6.2 MATERIALS AND REAGENTS

DL-Carnitine hydrochloride, thionyl chloride (gold label), acetyl chloride, hexanoic acid, palmitic acid, 3-phenylpropionic acid, 4-phenylbutanoic acid, triethylamine and N,N-diisopropylethylamine were obtained from Aldrich and used without further treatment. Trichloroacetic acid (gold label) was also obtained from Aldrich but was recrystallized from ethanol-free chloroform before use. Malic acid, ethyl acetate (Distol grade) and acetonitrile (Distol grade) were obtained from Fisons; dry DMF, THF, other solvents and octanoic acid from BDH; and acetyl-, octanoyl-, and palmitoyl-DL-carnitine hydrochloride, and urease tablets from Sigma. Samples of propionyl-, 2-methylbutyryl-, isovaleryl-, valeryl-, heptanoyl- and valproyl-DL-carnitine hydrochloride were provided as a gift from the laboratory of Dr. G.A. Mills, Southampton General Hospital.
Other standard chemicals and reagents used in this project were general laboratory reagent grade samples obtained from Aldrich or BDH.

Reacti-Vials (1 ml volume) were obtained from Pierce; analytical-grade ion-exchange resins from Bio-Rad; and Gelman Acro LC13 0.45 µm filter units from Millipore.

6.3 EXPERIMENTAL PROCEDURES

6.3.1 Synthesis of Acetylcarminine (2, R = CH₃)

A mixture of acetic acid (10 ml) and acetyl chloride (0.8 g; 0.01 mole) was stirred for 2 hr at 80 °C. DL-Carnitine hydrochloride (1 g, 0.005 mol) was added and the solution stirred at the same temperature for a further 2 hr. Excess acetic acid and acetyl chloride were removed by evaporation at low pressure. The residual orange oil was dissolved in hot propan-2-ol (5 ml) and the resulting solution was filtered to remove traces of unreacted carnitine. Acetone (20 ml) was added to the filtrate and the product allowed to crystallize from the solution overnight. The fine white crystals were filtered, washed with acetone and dried in an oven at 70 °C. A yield of 0.91 g (76%) was obtained, mp 208 °C (lit., 111 210 °C).

IR: wavenumber 3300-2400 (acid O-H stretch), 1735 (ester C=O stretch), 1710 (acid C=O stretch), 1240 cm⁻¹ (O-C stretch).

¹H NMR ([2H₆]-dimethyl sulphoxide, 90 MHz): δ 2.0 (s, 3H, H₃CCOO), 2.7 (d, 2H, CH₂COOH), 3.1 [s, 9H, (CH₃)₃], 3.8 (d, 2H, NCH₂), 5.4 ppm (m, 1H, CHOCHO).

FABMS +ve ion, glycerol matrix: m/z 204 ([M + H]⁺, 100%). FABMS -ve ion, glycerol matrix: m/z 238 ([M + Cl]⁻, 18%).
6.3.2 Synthesis of Hexanoylcarnitine (2, R = (CH_2)_4CH_3)

A mixture of hexanoic acid (5 ml) and freshly distilled thionyl chloride (1 g; 8.3 mmol) was stirred for 3 hr at 80 °C. DL-Carnitine hydrochloride (1 g; 0.005 mole) was added and the solution stirred at the same temperature for a further 2 hr. After cooling, diethyl ether (50 ml) was added dropwise. Once the precipitate had fully formed, it was filtered, washed with diethyl ether and redissolved in hot propan-2-ol (10 ml). Dry acetone (40 ml) was added to the propan-2-ol solution to precipitate unreacted carnitine hydrochloride. After 3 hr, the slight precipitate which formed was filtered. Dry diethyl ether (100 ml, Na dried) was added to the filtrate and the product was allowed to precipitate overnight. The white precipitate was filtered, washed with diethyl ether and dried. A yield of 0.65 g (42%) was obtained, mp 165 °C (lit. 116°C).

IR: wavenumber 3300-2500 (acid O-H stretch), 1730-1710 broad (ester C=O stretch, acid C-O stretch), 1200 cm⁻¹ (O-C stretch).

^1^H NMR ([^2^H₆]-dimethyl sulphoxide, 90 MHz): δ 0.9 (t, 3H, CH₃CH₂), 1.1-1.7 (m, 6H, CH₃CH₂CH₂CH₂CH₂), 2.3 (t, 2H, CH₂CH₂COO), 2.7 (d, 2H, CH₂COOH), 3.1 [s, 9H, (CH₃)₃], 3.8 (d, 2H, NCH₂) ppm (m, 1H, CHOCO).

^1^3^C NMR ([^2^H₆]-dimethyl sulphoxide, 90 MHz): δ 13 (CH₃CH₂), 22 (CH₃CH₂), 24 (CH₃CH₂CH₂), 30 (CH₂CH₂COO), 33 (CH₂CH₂COO), 42 (CH₂COOH), 53 [N(CH₃)₃], 65 (C₂H₂NMe₃), 67 (CHOCO), 170 (COOCH). 172 ppm (COOH).

FABMS +ve ion, glycerol matrix: m/z 260 ([M + H]^+ , 100%). FABMS -ve ion, glycerol matrix: m/z 294 ([M + Cl]^−, 100%).

6.3.3 Synthesis of Octanoyl (2, R = (CH₂)₂CH₃), 3-Phenylproplonyl (2, R = CH₂CH₂Ph), 4-Phenylbutanoyl (2, R = CH₂CH₂CH₂Ph) and Palmitoylcarnitine (2, R = (CH₂)₁₄CH₃).
A mixture of the relevant organic acid (5 ml) and freshly distilled thionylchloride (0.6 g; 0.005 mol) was stirred for 3 hr at 80 °C. DL-Carnitine hydrochloride (0.5 g; 2.5 mmol) was dissolved in trichloroacetic acid (2.5 g) at 60 °C and added to the reaction mixture. The solution was stirred at 80 °C for 3.5 hr. After cooling, dry diethyl ether (100 ml, Na dried) was added, dropwise until precipitation began. After precipitation was complete, the white precipitate was filtered, washed with diethyl ether and redissolved in hot propan-2-ol (10 ml). This solution was filtered and dry diethyl ether (50 ml) added to the filtrate. The product was allowed to precipitate overnight, then filtered, washed with ether and dried.

Octanoylcarnitine: A yield of 0.48 g (59%) was obtained mp 158 °C (lit. 111 160 °C).

IR: wavenumber, 3300-2500 (acid O-H stretch), 1735 (ester C=O stretch), 1715 (acid C=O stretch), 1180 cm⁻¹ (O-C stretch).

³¹H NMR ([²H₆]-dimethyl sulphoxide, 90 MHz): δ 0.9 (t, 3H, CH₃CH₂), 1.1-1.7 (m, 10H, CH₃CH₂CH₂CH₂CH₂CH₂CH₂), 2.3 (t, 2H, CH₂COO), 2.7 (d, 2H, CH₂COOH), 3.1 [s, 9H, (CH₃)₃], 3.8 (d, 2H, NCH₂), 5.4 ppm (m, 1H, CHOCO).

¹³C NMR ([²H₆]-dimethyl sulphoxide, 90 MHz): δ 14 (CH₃CH₂), 23-35 (five signals assigned to side chain methylene groups), 43 (CH₂COOH), 53 [N(CH₃)₃], 65 (CH₂-NMe₃), 67 (CHOCO), 170 (COOCH), 172 ppm (COOH).

FABMS +ve ion, glycerol: m/z 288 ([M + H]+, 100%). FABMS -ve ion, glycerol matrix: m/z 322 ([M + Cl]⁻; 28%), 143 ([CH₃(CH₂)₆COO]⁻, 100%).

3-Phenylpropionylcarnitine: A yield of 0.23 g (28%) was obtained; mp 162 °C.

IR: wavenumber, 3300-2500 (acid O-H stretch), 1710-1730 (ester C=O stretch, acid C=O stretch), 1190 cm⁻¹ (O-C stretch).
\(^1\)H NMR ([\(\text{^2}H_6\)]-dimethyl sulfoxide, 90 MHz): \(\delta\) 2.6-2.9 (m, CH\(_2\)COOH, CH\(_2\)CH\(_2\)Ph), 3.1 [ss, (CH\(_3\))\(_3\) acylcarnitine, (CH\(_3\))\(_3\) isopropyl esterified acylcarnitine], 3.7 (d, NCH\(_2\)), 4.2 (m, (CH\(_3\))\(_2\)CHO, isopropyl esterified acylcarnitine), 5.5 (m, CH\(_2\)CHO) and 7.2 ppm (s, Ph).

\(^1\)C NMR ([\(\text{^2}H_6\)]-dimethyl sulfoxide, 90 MHz): \(\delta\) 30 (CH\(_2\)Ph), 35 (CH\(_2\)COO), 43 (CH\(_2\)COOH), 53 [N(CH\(_3\))\(_3\)], 65 (CH\(_2\)-NMe\(_3\)), 67 (CHO), 125 (Ph, C-4), 128 (Ph, C-2 + C-3), 140 (Ph, C-1), 170 (COOH), 172 ppm (COOH).

FABMS +ve ion, glycerol matrix: \(m/z\) 294 ([M + H]\(^+\), 100%, acylcarnitine), 336 ([M + H]\(^+\), 53%, esterified acylcarnitine). FABMS -ve ion, glycerol matrix: \(m/z\) 328 ([M + Cl]\(^-\), 40%), 149 ([PhCH\(_2\)CH\(_2\)COO]\(^+\), 100%).

4-Phenylbutanoylcarnitine: A yield of 0.49 g (57%) was obtained, mp 156 °C.

IR: wavenumber, 3700-2500 (acid O-H stretch), 1740 (ester C=O stretch), 1720 (acid C=O stretch), 1180 cm\(^{-1}\) (O-C stretch).

\(^1\)H NMR ([\(\text{^2}H_6\)]-dimethyl sulfoxide, 90 MHz): \(\delta\) 1.7-2.0 (m, 2H, CH\(_2\)CH\(_2\)CH\(_2\)), 2.2-2.8 (m, 6H, PhCH\(_2\), CH\(_2\)COO, CH\(_2\)COOH), 31.1 [s, 9H, (CH\(_3\))\(_3\)], 3.8 (d, 2H, NCH\(_2\)), 5.4 (m, 1H, CHO), 7.2 ppm (s, 5H, Ph).

\(^1\)C NMR ([\(\text{^2}H_6\)]-dimethyl sulfoxide, 90 MHz): \(\delta\) 26 (CH\(_2\)CH\(_2\)CH\(_2\)), 33 (CH\(_2\)Ph), 34 (CH\(_2\)COO), 43 (CH\(_2\)COOH), 53 [N(CH\(_3\))\(_3\)], 65 (CH\(_2\)-NMe\(_3\)), 67 (CHO), 126 (Ph, C-4), 129 (Ph, C-2 + C-3), 141 (Ph, C-1), 170 (COOR), 172 ppm (COOH).

FAB +ve ion, glycerol matrix: \(m/z\) 308 ([M + H]\(^+\), 100%). FAB -ve ion, glycerol matrix: \(m/z\) 342 ([M + Cl]\(^-\), 100%).

Palmitoylcarnitine: A yield of 0.9 g (81%) was obtained, mp 154 °C (commercial sample, 157 °C; lit., 111 161 °C).
IR: wavenumber, 3500-2500 (acid O-H stretch), 1735 (ester C=O stretch), 1710 (acid C=O stretch), 1180 cm⁻¹ (O-C stretch).

¹H NMR ([²H₆]-dimethyl sulphoxide, 60 MHz) δ: 0.8 - 1.3 [m, 29H, CH₃(CH₂)₁₃], 2.8 (d, 2H, CH₂COOH), 3.1 [s, 9H, (CH₃)₃], 3.8 (m, 2H, NCH₂) and 5.4 ppm (m, 1H, CHOCO).

FAB +ve ion, glycerol matrix: m/z 400 ([M + H]⁺, 100%). FAB -ve ion, glycerol matrix: m/z 434 ([M + Cl]⁻, 21%), 255 ([CH₃(CH₂)₁₄COO]⁻, 100%).

6.3.4 Fast Atom Bombardment Analysis of Standard and Synthesized Acylcarnitines

FAB mass spectra were recorded for all synthesised acylcarnitines and those presented as a gift from Dr. G.A. Mills.

A beam of fast Xe atoms was directed onto a FAB probe coated with a glycerol solution of the acylcarnitine to be analysed in the ion source of a single quadrupole mass spectrometer. Typically, around 0.1 mg of sample was dissolved in approximately 10 μl of glycerol on the gold plated probe target area. The FAB gun was operated with a Xe translational energy of 8 kV and 1 mA beam current. Data acquisition was activated immediately on sample introduction into the source and full scan spectra over the mass range 50-450 (1 s scan, 0.1 s interscan time) were collected over 30 s.

6.3.5 Synthesis of β-Hydroxy-γ-lactone (4)

The preparation of β-hydroxy-γ-lactone was carried out using a modification of the method of Henrot et al.¹¹³

a) Preparation of Cyclic Anhydride (7) : Scheme 20

DL-Malic acid (6.7 g, 50 mmol) was dissolved in acetyl chloride (60 ml) and the solution stirred at 40 °C overnight. The reaction mixture was filtered and the orange
filtrate concentrated under reduced pressure. A crystalline material was obtained which was washed with ethanol-free chloroform. The off-white crystals were filtered, recrystallized from chloroform, washed with dry diethyl ether and dried. A yield of 6.9 g (87%) was obtained, mp 82 °C. ¹H NMR (CDCl₃, 90 MHz): δ 2.2 (s, 3H, CH₃COO), 3.0 (dd, 1H, H-2a), 3.4 (dd, 1H, H-2b), 5.5 ppm (dd, 1H, H-3).

![Scheme 20](image)

**b) Conversion to Diester-acid Compound (6): Scheme 21**

Cyclic anhydride (7) (6.32 g, 40 mmol) was stirred with methanol (80 ml) overnight at room temperature. The solvent was evaporated under reduced pressure to leave a viscous oil. A yield of 5.52 g (80%) was obtained. ¹H NMR (CDCl₃, 90MHz): δ 2.1 (s, 3H, CH₃COO), 2.9 (d, 2H, H₃+b), 3.7 (s, 3H, CH₃COO), 5.4 (t, 1H, H₃), 9.3 ppm (s, 1H, COOH).
c) Reduction of (6) to β-Hydroxy-γ-lactone (4): Scheme 22

Sodium borohydride (4.7g, 0.12 mol) was added to freshly distilled tBuOH (70 ml) and the mixture heated to reflux. Compound (6) (5.9g, 31 mmol) was dissolved in tBuOH (25ml) and MeOH (5ml). The solution of (6) was added very slowly to the refluxing NaBH₄ solution over a period of 1.5 hr. Reflux conditions were maintained for 20 hr. The reaction was quenched by the dropwise addition of a solution of acetyl chloride (11 ml) in MeOH (75 ml). Solvents were removed by heating under reduced pressure. The product was extracted with EtOAc (100 ml) and the salts removed by filtration. The filtrate was neutralized with solid Na₂CO₃, filtered and concentrated under reduced pressure to give a yellow oil. After chromatographic purification on silica gel (EtOAc : Hexane, 4:1), 1.04 g (33%) was obtained.

IR Wavenumber: 3700-3100 (alcohol O-H stretch), 2990, 2970, 2960 (C-H stretch), 1770 (lactone C=O stretch), 1180 cm⁻¹ (O-C stretch).

¹H NMR (CDCl₃, 90 MHz): δ 2.3-3.0 (m, 2H, H-2a/b), 3.9 (d, 1H, OH), 4.2-4.5 (m, 2H, H-4a/b), 4.7 ppm (m, 1H, H-3).
6.3.6 Synthesis of Acyloxy-γ-butyrolactone Compounds (3): Scheme 12

Acetyl-, octanoyl-, 4-phenylbutanoyl- and palmitoyl-containing acyloxylactone compounds (3) were all prepared from the condensation reaction of β-hydroxy-γ-butyrolactone (4) with the respective acid chloride. The following method was used to prepare octanoyl-containing lactone [3, R = (CH₂)₆CH₃] and similarly the other standard lactone compounds were synthesised.

β-Hydroxy-γ-butyrolactone (4) (0.5 g, 4.9 mmol) was dissolved in dry tetrahydrofuran (THF) (10 ml) and added dropwise to a solution of octanoyl chloride (1.36 ml in 10 ml of THF) at 0 °C. After warming to reflux, the reaction was allowed to proceed for 24 hr. The solvent was removed and the product purified by chromatography on silica gel (EtOAc : hexane, 1:1). Traces of octanoic acid were removed by washing a solution of the product in diethyl ether with three equal volumes of a saturated solution of NaHCO₃. The diethyl ether was dried and removed to leave a clear oil (0.78 g, 70% yield).

Satisfactory analytical data were obtained for all four lactones prepared by use of IR, ¹H and ¹³C NMR and EI mass spectra.
Acetyloxy-γ-butyrolactone (3, R = CH₃), 53% yield. IR wavenumber, 3000-2860 (C-H stretch), 1790 (lactone C=O stretch), 1740 cm⁻¹ (ester C=O stretch).

¹H NMR (CDCl₃, 90 MHz): δ 2.1 (s, 3H, CH₃), 2.5-3.0 (m, 2H, CH₂CO), 4.4 (m, 2H, CH₂OCO) and 5.5 ppm (m, 1H, CHOCO).

EIMS: m/z 144 (M⁺, 1%), 84 (C₄H₄O₂⁺, 100%)

Octanoyloxy-γ-butyrolactone [3, R = (CH₂)₆CH₃], 70% yield.

IR: wavenumber, 2980-2860 (C-H stretch), 1790 (lactone C=O stretch), 1740 cm⁻¹ (ester C=O stretch).

¹H NMR (CDCl₃, 90MHz): δ 0.8-1.7 [m, 13H, (CH₃)CH₃], 2.3 (t, 2H, side-chain CH₂CO), 2.5-3.0 (m, 2H, ring CH₂CO), 4.4 (m, 2H, CH₂OCO), 5.5 ppm (m, 1H, CHOCO).

¹³C NMR (CDCl₃, 90 MHz): δ 14 (CH₃), 23 (CH₂), 25 (CH₂), 28 (CH₂), 29 (CH₂), 32 (CH₂), 34 (CH₂CO), 35 (CH₂CO), 69 (CH₂OCO), 74 (CHOCO), 173 (ester C=O), 174 ppm (lactone C=O).

EIMS: m/z 228 (M⁺, 2%), 85 (C₄H₅O₂⁺, 100%)

4-Phenybutanoyloxy-γ-butyrolactone (3, R = CH₂CH₃CH₂CH₂Ph), 44% yield.

IR: wavenumber, 3060-3020 (aromatic C=O stretch), 3000-2850 (aliphatic C-H stretch), 1795 (lactone C=O stretch), 1740 cm⁻¹ (ester C=O stretch).

¹H NMR (CDCl₃, 90 MHz): δ 1.8-3.0 [m, 8H, (CH₂)₃ and ring CH₂CO], 4.3 (m, 2H, CH₂OCO), 5.4 (m, 1H, CHOCO), 7.2 ppm (m, 5H, Ph).

¹³C NMR (CDCl₃, 90 MHz): δ 26 (CH₂CH₂CH₂), 33 (PhCH₂), 34 (CH₂CO) 35 (CH₂CO), 70 (CH₂OCO), 74 (CHOCO), 126 (Ph), 128 (Ph), 141 (Ph), 173 (ester C=O), 174 ppm (lactone C=O).

EIMS: m/z 248 (M⁺, 23%), 144 (C₆H₈O₄⁺, 100%).
Palmitoyloxy-γ-butyrolactone [3, R = (CH₂)₄CH₃], 70% yield.

IR: wavenumber, 2960-2860 (CH stretch), 1790 (lactone C=O stretch), 1740 cm⁻¹ (ester C=O stretch).

¹H NMR (CDCl₃, 90 MHz) : δ 0.8-1.7 [m, 29H, (CH₂)₁₃CH₃], 2.3 (m, 2H, side-chain CH₂CO), 2.7 (m, 2H, ring CH₂CO), 4.4 (m, 2H, CH₂O0), 5.4 ppm (m, 1H, CHOCO).

¹³C NMR (CDCl₃, 90 MHz) : δ 11 (CH₃), 23-32 (13 x CH₂), 34 (CH₂CO), 35 (CH₂CO), 69 (CH₂O0), 74 (CHOCO), 173 (ester C=O), 174 ppm (lactone C=O).

EIMS: m/z 340 (M⁺, 7%), 85 (C₄H₅O₂⁺, 100%).

6.3.7 Attempted Cyclization of Octanoylcarnitine in Hot Septum Inlet of a Mass Spectrometer.

Commercial octanoylcarnitine hydrochloride (100 mg) was dissolved in warm ethanol (0.5 ml). The solution (10 μl) was injected into the septum inlet of a VG 305 single-focussing mass spectrometer. The temperature of the septum inlet was 70 °C on injection. The inlet valve was slowly opened to allow any vaporized species into the ion source. After the evaporation of ethanol had been completed the inlet temperature was increased in 10 °C increments whilst continually monitoring any products released.

6.3.8 Use of Ultrasound in Derivatization Reaction

Octanoylcarnitine hydrochloride (6 x 10⁻⁵M) solutions in EtOAc, THF and CH₃CN were made (20 ml of each). Each solution was subject to ultrasound using an MSE Soniprep 150 Ultrasonic Disintegrator (b power, tuned to 14 microns), for 3 mins. A 1 ml aliquot was removed and analysed by split injection GC (6.3.13). The procedure was repeated five times for the remaining solution, so that the final aliquot had experienced 15 min of sonication.
6.3.9 Derivatization Reaction in DMF

4-Phenylbutanoyl-DL-carnitine hydrochloride (20 mg, 58 µmol) was placed in a 50 ml round-bottomed flask. Dimethyl formamide (20 ml, dried over BaO) was added and the acylcarnitine allowed to dissolve. (Base was added at this stage, 10-100 µmol, if required.) The reaction flask was fitted with a reflux condenser and CaCl₂ drying tube. The apparatus was supported with the flask in an oil bath at 100 °C. The reaction mixture was magnetically stirred for 3 hr at this temperature.

Reaction monitoring over the 3 hr period was achieved by TLC. The mixture was spotted onto a silica TLC plate and solvent removed by heating in an oven (~ 90 °C, 90 sec). Chromatography was achieved with EtOAc: hexane, 1:1 and the spots observed under a UV lamp.

After 3 hr the orange/brown reaction mixture was concentrated under vacuum to give an oil. Dry diethyl ether (0.5 ml, Na-dried) was used to extract the lactone product for further analysis by GC.

6.3.10 Initial Derivatization Reaction in CH₃CN

Initial investigations were performed in the same apparatus as for 6.3.9.

4-Phenylbutanoyl-DL-carnitine hydrochloride (20 mg, 58 µmol) was dissolved in acetonitrile (20 ml). Triethylamine (1 µl) was added and the mixture stirred under reflux conditions for varying periods from 3 to 18 hr. Solvent was removed under vacuum and the product extracted from the remaining colourless oil with diethyl ether (0.5 ml). Analysis of the product was achieved by TLC, IR and ¹H NMR spectroscopy.

6.3.11 Optimized Derivatization Conditions

Quantities of standard acylcarnitines (<0.1 mg) or acylcarnitines extracted from urine (see Section 6.3.15-6.3.17) were placed in a 1ml ReactiVial. When quantitative investigations were being attempted aqueous solutions of acylcarnitines...
were concentrated by lyophilization and transferred into a 1-ml ReactiVial for
complete drying, again by freeze-drying. In all cases the resulting acylcarnitine
residue was treated with acetonitrile (0.5 ml) and N,N-diisopropylethylamine (0.5
µl) and the ReactiVial Sealed with a Teflon-lined septum. The solution was
subjected to various reaction times and temperatures until the optimum conditions
were identified for cyclization of the acylcarnitines. Heating at 125 °C for 35 min
was found to be the most efficacious method of forming the lactone derivatives.
After the reaction period, solvent was removed under a stream of nitrogen until a
damp residue was obtained. [Evaporation to complete dryness caused some loss of
the more volatile lactones (3, R = Me, Et).] Ethyl acetate (0.2 ml) was added to
dissolve any acyloxylactone products. Any remaining solid material was removed
by filtering through a Gelman Acro LC13, 0.45 µm filter unit and the filtrate
analysed for acylcarnitine derivatives by GC and GC/MS.

6.3.12 Estimation of Derivatization Yields by On-Column Injection GC

Standard solutions of acetyl-, octanoyl- and palmitoyl-containing acyloxylactones
(3) were made up in EtOAc:

\[
\begin{align*}
\text{acetyl}-; & \quad 114 \, \mu\text{g} \, \text{ml}^{-1}; \quad 0.79 \, \mu\text{mol} \, \text{ml}^{-1} \\
\text{octanoyl}-; & \quad 116 \, \mu\text{g} \, \text{ml}^{-1}; \quad 0.51 \, \mu\text{mol} \, \text{ml}^{-1} \\
\text{palmitoyl}-; & \quad 100 \, \mu\text{g} \, \text{ml}^{-1}; \quad 0.29 \, \mu\text{mol} \, \text{ml}^{-1}
\end{align*}
\]

A mixture of acetylcarnitine (14 µg, 60 nmol), octanoylcarnitine (14 µg, 44 nmol)
and palmitoylcarnitine (14 µg, 32 nmol) was derivatized and reconstituted in 0.2 ml
EtOAc as in Section 6.3.11. The derivatized acylcarnitine mixture and the three
standard solutions of synthesised acylactones were analysed by cold on-column
injection capillary GC.

A Carlo Erba Model 5300 Mega series gas chromatograph was used. A BP5
fused-silica column from SGE, 25 m x 0.33 mm id. and with a film thickness of
0.5 µm was employed. Helium carrier gas flowed through the column with an average linear velocity of 35 cm s⁻¹. The hydrogen and air inlet pressures (for the flame ionization detector) were 70 and 120 kPa, respectively. The detector was maintained at 280 °C. The oven temperature was programmed from 87 to 250 °C at a rate of 10 °C min⁻¹. The temperature ramp rate started immediately after injection and on reaching 250 °C was maintained at this temperature for 15 min. Cold on-column injections (1 µl in all cases) were performed using a 5 µl gas-chromatographic syringe (Hamilton; Grob design) with a 7.5 cm needle. Retention times were measured from the time of injection and integral peak areas were determined by a Spectrophysics integrator.

6.3.13 Split-Splitless Injection Capillary GC

Experiments which required analysis of products by vaporizing injector (i.e. split and splitless injection) capillary GC employed a Perkin Elmer 8410 chromatograph with the exception of some GC/MS experiments which used splitless injection on a Hewlett-Packard 5890 GC (see Section 6.3.22).

Split ratios were only estimated because (i) accurate splitting of the carrier gas is difficult to achieve and (ii) an accurate split ratio was not required since quantitative evaluations employed an internal standard or preferably, on-column injection was performed. Estimates of the split ratio were calculated from determined values for flow rates through the split exit valve and through the capillary column. The flow rate of carrier gas through the split exit valve was measured with a bubble meter. The flow rate through the column was calculated from the retention time of an unretained component (methane). Generally, the split ratio was set around 10:1, that is, only one tenth of the sample injected entered the column, the remainder was exhausted through the split exit valve.

For increased sensitivity, splitless injection was employed.
The carrier gas split was kept closed on injection, and for a pre-selected time afterwards, to ensure most, if not all, of the sample entered the column. A splitless period between 30 s and 1 min was used before the split valve opened to allow the chromatographic process to proceed as with split injection GC.

With both injection methods, sample was injected using the hot-needle technique (see Section 2.6.1.1). Sample volumes varied between 0.1 and 1.5 µl. Injection temperatures employed were between 200 and 280 °C, however, see Section 6.3.22 for an evaluation of the effect of this parameter.

6.3.14 Attempted Derivatization of Urinary Acylcarnitines Without Extraction

Urine (2 ml) was spiked with acetylcarnitine (3 mg, 13 µmol), octanoylcarnitine (4 mg, 12 µmol) and palmitoylcarnitine (6 mg, 14 µmol). The water was removed by lyophilization and the resulting residue derivatized as in Section 6.3.11 (with the exception that 8.0 µl triethylamine was used to facilitate the cyclization process). The resulting residue, after removal of the reaction solvent, was extracted with diethyl ether (10 ml). The ether fraction was washed with an equal volume of saturated sodium bicarbonate solution and then concentrated under reduced pressure to give an oil with a white suspension. Diethyl ether (1 ml) was added and the immiscible oil removed with a pipette. The ether was concentrated by blowing off approximately 80% of the solvent with dry argon.

Analysis was performed by cold on-column injection GC using similar conditions to Section 6.3.12 on a Carlo Erba 4160 instrument.

6.3.15 Extraction of Urinary Acylcarnitines Using Anion-Exchange Resin

Bio-Rad AG1-X8, 100-200 mesh, anionic-exchange resin (1.3 cm³) in either the chloride or formate ion form was used to pack a column of 1 cm diameter. The column was equilibrated with distilled-deionized water and the urine sample...
(0.5 ml) applied to the head of the column. Acylcarnitines and other cationic, neutral and weakly bound anionic species were eluted with distilled-deionized water (2 ml). The water was removed by lyophilization and the resulting residue subject to derivatization (Section 6.3.11) and GC analysis of any lactone compounds formed (Section 6.3.12).

6.3.16 Extraction of Urinary Acylcarnitines Using Cation-Exchange Resin

(i) Bio-Rad AG1X8, 100-200 mesh, hydrogen form, cationic-exchange resin (1.3 cm³) was used to pack a column of 1 cm diameter. After equilibrating the column with distilled-deionized water, 1M pyridine solution was applied to the head of the column and allowed to flow through until a permanent pyridine smell could be detected in the column effluent. Excess pyridine was washed out with distilled-deionized water.

Urine (1 ml) spiked with acetylcamitine (0.25 mg, 1.0 µmol) and octanoylcamitine (0.25 mg, 0.8 µmol) was applied to the cationic-exchange column prepared as above. Neutral and anionic species were eluted with HCl (1 ml, 0.01 M) and distilled-deionized water (2 ml). Acylcarnitines were eluted with a 50/50 (V/V) mixture of methanol and 1.5M, pH 6 pyridinium acetate, (3.5 ml). The eluent was lyophilized, derivatized (Section 6.3.11) and analysed by capillary GC (Section 6.3.12).

(ii) An alternative extraction procedure, applied to spiked urine and aqueous solutions of acylcarnitines was the same as (i) detailed above with the exception that the acylcarnitines were eluted from the column with 0.5M NH₄OH (3 x 5 ml fractions).

6.3.17 Optimum Extraction Procedure for Urinary Acylcarnitines

The double ion-exchange procedure detailed here was based on the method of Millington et al.¹⁰²
Bio-Rad AG1-X8, 100-200 mesh, formate (or chloride) form, anion-exchange resin (2 cm$^3$) was used to pack a column of 1 cm diameter. The column was equilibrated with distilled-deionized water and the urine sample (0.5 ml) applied to the head of the column. Acylcarnitines and other cationic and neutral species were eluted with distilled-deionized water (2 ml). The eluent was acidified with 1 M HCl (0.2 ml).

Bio-Rad AG50-X8, 100-200 mesh, hydrogen form, cationic-exchange resin (2 cm$^3$) was used to pack a 1 cm diameter column. The acylcarnitine-containing eluent from above was applied to the column. Neutral and loosely bound cationic species were washed off with HCl (5 ml, 0.01 M) and distilled-deionized water (5 ml). Acylcarnitines were eluted with NH$_4$OH (2M, 20% aqueous ethanol), the first 1 ml being discarded and the following 6 ml collected and freeze-dried.

The resulting residue was dissolved in distilled water (0.5 ml), transferred into a 1 ml ReactiVial and freeze-dried in this vessel. The residue was then subject to derivatization (Section 6.3.11) and analysis by capillary GC (Section 6.3.12).

6.3.18 Estimation of Acylcarnitine Recoveries from Double Ion-Exchange Method

Urine (0.5 ml) was spiked with acetylcarnitine (0.25 mg, 1.0 µmol) and octanoylcarnitine (0.25 mg, 0.8 µmol) and extracted as detailed in Section 6.3.17. Similarly an aliquot of distilled-deionized water (0.5 ml) was spiked and extracted. A third, aqueous solution of acetylcarnitine and octanoylcarnitine, at the same concentrations as for the extracted urine and water samples, was simply freeze-dried.

All three samples were derivatized (Section 6.3.11) and analysed (Section 6.3.12) under identical conditions. Recoveries were estimated by comparing the FID responses of derivatives from the extracted and unextracted acylcarnitines.
6.3.19 Relative Extraction Efficiencies from Control Urines

A stock solution of four acylcarnitines in distilled-deionized water was made:

- Acetylcarnitine: 0.2 mg ml\(^{-1}\) (0.84 µmol ml\(^{-1}\))
- Isovalerylcarnitine: 0.2 mg ml\(^{-1}\) (0.71 µmol ml\(^{-1}\))
- Octanoylcarnitine: 0.2 mg ml\(^{-1}\) (0.62 µmol ml\(^{-1}\))
- 4-Phenylbutanoylcarnitine: 0.2 mg ml\(^{-1}\) (0.58 µmol ml\(^{-1}\))

The above stock solution (0.1 ml) was used to spike each of four control urines (0.5 ml); C1, C2, C3 and C4. Each spiked urine sample was extracted as in Section 6.3.17, derivatized as in Section 6.3.11 and analysed by capillary GC as in Section 6.3.12. An aliquot (0.1 ml) of the stock solution was also freeze-dried, derivatized and analysed, as for the spiked urine sample. In accordance with Section 6.3.18, recoveries were determined by comparing analysis results from the extracted urines with those from the matrix-free, unextracted sample.

6.3.20 FID Responses for Derivatives of Acylcarnitines Spiked into Urine Before and After Extraction

Urine (0.5 ml) was spiked with isovalerylcarnitine (20 µg, 71 nmol), octanoylcarnitine (20 µg, 70 nmol), and 4-phenylbutanoyl-carnitine (20 µg, 58 nmol). The urine was then extracted, derivatized and analysed by GC as described previously. A second aliquot of the same urine (0.5 ml) was also spiked with the same quantities of isovalerylcarnitine and octanoylcarnitine but the phenyl-containing acylcarnitine was not introduced until the sample had been extracted and was ready for derivatization. The same quantity of 4-phenylbutanoylcarnitine (20 µg, 58 nmol) was then added and the sample derivatized and analysed as previously.
6.3.21 Attempted Extraction Using Bond Elut CERTIFY™ Columns

The attempted extraction procedure was based on the established method of extracting basic drugs of abuse (e.g. amphetamines, LSD and codeine) from urine using Bond Elut CERTIFY columns.

Urine (5 ml) was spiked with octanoylcarnitine (0.9 mg, 2.8 µmol) and 4-phenylbutanoylcarnitine (1.2 mg, 3.5 µmol). Potassium orthophosphate buffer (2 ml, 0.1M, pH 6.0) was added and the sample vortex mixed and the pH checked to be between 5.0 and 7.0 with pH paper.

The column was prepared by drawing through, under vacuum, methanol (2 ml) then phosphate buffer (2 ml, 0.1M, pH 6.0) ensuring that the column did not run dry at this stage. Sample, prepared as above, was applied to the head of the column and allowed to pass through under gravity. Neutral and weakly bound species were washed from the resins with phosphate buffer (0.1M, pH 6.0): methanol, 80:20 (1 ml) then dried for 5 min by drawing through air, followed by acetic acid (1 ml, 1.0M) and a further drying period of 5 min. The column was fully dried by rinsing with hexane (1 ml) then drying for 2 min. Dichloromethane (4 ml) and methanol (6 ml) were passed through the column and these two fractions collected for analysis. The fraction expected to contain acylcarnitines was obtained by elution with NH₄OH (20% in EtOH, 2 ml) to give a yellow solution. Aliquots of this fraction were freeze-dried and analysed for acylcarnitine content by derivatization (Section 6.3.11) and on-column injection GC (Section 6.3.12).

6.3.22 Effect of Injector Temperature on GC Behaviour of Cyclized Acylcarnitines

A standard (0.1%) solution of synthesised octanoyloxy-γ-butyrolactone \([3, R = (CH₂)₆CH₃]\) was prepared. This sample was analysed using splitless-injection GC/MS at four injector temperatures, 150, 230, 250 and 280 °C.
Analysis was performed with a VG 20-250 quadrupole instrument coupled to a Hewlett-Packard Model 5890 gas chromatograph equipped with a fused-silica BP5 capillary column of dimensions 12.5 m x 0.25 mm i.d. and a stationary phase film thickness of 0.25 μm. The direct-line interface was maintained at 260 °C and a source temperature of 200 °C was used. Electron ionization (EI) with an electron energy of 70 ev and an ion current of 100 μA was used. Splitless injection (0.2 μl) were performed using a closed split valve period of 45 s. Other GC conditions were as in Section 6.3.12.

Peak heights, areas and shapes were determined from the total ion current chromatogram.

6.3.23  Free Zone Capillary Electrophoresis

Capillary zone electrophoresis (CZE) methods were performed using a Beckman P/ACE System 2000. Using this apparatus, initial attempts were made to separate various aqueous mixtures of acylcarnitines. The use of CZE to analyse acylcarnitines has not been reported to date and therefore the approach was one of method development, investigating the effect of the operational parameters on any signal responses and separations achieved.

Attempts were made to analyse standard aqueous solutions of individual acylcarnitines and mixtures of several standard acylcarnitines. Concentrations of the solutions varied from 10-1000 μg/ml for each carnitine ester. Uncoated, deactivated silica capillaries were employed with dimensions of 50 cm length (43 cm effective length) and 75 μm internal diameter. Standard electrolytic buffer systems of 0.1 M sodium phosphate/sodium borate adjusted to pH values from 2.0 to 8.0 were obtained with the P/ACE System 2000 instrument and used in these initial studies. Detection was performed at either 200 or 210 nm using the inbuilt UV detection system. Electrophoresis was achieved when 8 - 12 kV was applied across the capillary column. Sample was introduced into the capillary by either
positive pressure injection for fixed time periods of 2 - 8 s or by electrokinetic injection using the running potential for similar time periods as the pressure injections. Data were recorded and manipulated by an IBM PS/2 PC with Beckman System Gold software package.

Between injections the capillary column was conditioned with 0.1 M NaOH, flushed through the column by positive pressure for 15 s, followed by distilled-deionised water for 20 s and then re-equilibrated with the run buffer before the next injection.

6.3.24 Micellar Electrokinetic Capillary Chromatography (MECC)

The same CZE equipment as that used in the experiments of Section 6.3.23 was used for MECC investigations. Similar acylcarnitine solutions were also subject to MECC analyses. The essential difference from the free zone capillary electrophoresis studies, was the presence of sodium dodecyl sulphate (SDS) in the electrolytic run buffer. SDS concentrations of 20-400 mM (i.e. above the critical micellar concentration) were employed during the studies conducted. Other operating parameters and procedures were as for the experiments of Section 6.3.23.
REFERENCES

8. G. Fraenkel and S. Friedman, *Vitamins and Hormones*, 1957, 15, 73.


133. Personal communication with R.J. Pollit, Sheffield Childrens Hospital.


149. M.E. Rose, unpublished results, Open University, Milton Keynes.


202. K. Bartlett, personal communication. Recent studies (R.J. Morrow and M.E. Rose) have confirmed the irreversible adsorption of long-chain acylcarnitines on the ion-exchange resin and suggest that extraction with hexan-2-ol is a better isolation method.

IR1: Infrared spectrum of carnitine

IR2: Infrared spectrum of octanoylcarnitine
IR3: Infrared spectrum of 4-phenylbutanoyl-γ-butyrolactone
IR4: Infrared spectrum of octanoyl-γ-butyrolactone
$^1$H NMR: Proton nmr spectrum of carnitine.
$^1$H NMR3: Proton nmr spectrum of 4-phenylbutanoyl-$\gamma$-butyrolactone
$^1$H NMR: Proton nmr spectrum of octanoyl-$\gamma$-butyrolactone
MS1: (a) EI mass spectrum of acetyl-γ-butyrolactone
(b) CI +ve [methane] mass spectrum of acetyl-γ-butyrolactone
MS2: (a) EI mass spectrum of propionyl-γ-butyrolactone
(b) CI +ve (methane) mass spectrum of propionyl-γ-butyrolactone
MS3: (a) EI mass spectrum of isovaleryl-γ-butyrolactone
(b) CI +ve (methane) mass spectrum of isovaleryl-γ-butyrolactone
MS4: (a) EI mass spectrum of hexanoyl-γ-butyrolactone
(b) CI+ve (methane) mass spectrum of hexanoyl-γ-butyrolactone
MS5: (a) EI mass spectrum of valproyl-γ-butyrolactone
(b) CI +ve (methane) mass spectrum of valproyl-γ-butyrolactone
MS6: (a) EI mass spectrum of octanoyl-γ-butyrolactone
   (b) CI +ve (methane) mass spectrum of octanoyl-γ-butyrolactone
MS7: (a) EI mass spectrum of 3-phenylpropionyl-γ-butyrolactone
(b) CI +ve (methane) mass spectrum of 3-phenylpropionyl-γ-butyrolactone
MS8: (a) EI mass spectrum of 4-phenylbutanoyl-γ-butyrolactone
   (b) CI +ve [methane] mass spectrum of 4-phenylbutanoyl-γ-butyrolactone