CHAPTER 2

Materials and Methods

2.1 Background

Cells have frequently been used to determine the integrity of metabolic pathways for selected patients with suspected mitochondrial fatty acid ß-oxidation disorders (FAOD) when *in vivo* metabolite findings are inconclusive. However, evaluation of the rate of release of end products such as labelled carbon dioxide or water lacks diagnostic specificity. An improved investigative procedure appears to be the *in vitro* analysis of acylcarnitines, derived from accumulated acyl-CoA ß-oxidation intermediates. These are produced as a result of incubating cells in a medium enriched with precursors to the pathway, long-chain fatty acids and free L-carnitine.

Cells + Fatty Acids → Acyl-CoA esters

Acyl-CoA esters + L-Carnitine → Acylcarnitines

An enzyme deficiency is indicated by the excessive accumulation of acylcarnitine species upstream of the site of metabolic block.

Acylcarnitines have been traditionally difficult to analyse because of their polarity, zwitterionic nature, and the lack of a suitable chromophore. Sophisticated instruments such as radio-HPLC, gas chromatography chemical ionisation mass spectrometry and tandem mass spectrometry (MS/MS) are required to achieve acceptable sensitivity and specificity. Among these, MS/MS is superior in that the analytical time is short, sample handling is simple and no chromatography is required. Early methods used fast atom bombardment (FAB) MS/MS, but electrospray ionisation (ESI) MS/MS has been shown to be a more robust and more sensitive alternative technique for acylcarnitine profiling from whole blood dried on filter paper samples

(Rashed et al 1995b). In this project, ESI-MS/MS was used to study the acylcarnitines generated by cultured skin fibroblasts from healthy subjects and those from patients with a number of enzyme defects in mitochondrial fatty acid ß-oxidation and respiratory chain pathways.

2.2 Outline of the *In Vitro* Acylcarnitine Profile Assay

- Intact skin fibroblasts were cultured in a medium enriched with [15,15,16,16,16-$^{2}H_5$]hexadecanoic acid and L-carnitine, and incubated at 37^{o}C for up to 96 hours.

- The acylcarnitines accumulated in the medium were analysed using an ESI-MS/MS, scanning for the precursor ion of mass 85.2 Da by multi-channel acquisition mode. The profile data were processed using Neolynx software (Micromass) measuring the ratio of analyte to its respective internal standard multiplied by the output from the external calibration curves (when available) to obtain the acylcarnitine concentrations.

- The total protein of the fibroblasts was measured using the bicinchoninic acid (BCA) assay with bovine serum albumin as standards.

- The results were expressed as nanomoles of acylcarnitine formed per milligram of total protein (nmol/mg) during the period of incubation.

2.3 Materials

2.3.1 Materials for Tissue Culture

Ham F-10 Nutrient Mixture (factory prepared liquid medium with 25 mM

HEPES buffer packaged as 500 mL per bottle; medium composition see Appendix 1), fetal bovine serum, L-glutamine (200 mM in 0.85% sodium chloride), penicillin-streptomycin (5,000 units/mL penicillin and 5, 000 µg/mL streptomycin), Fungizone (250 µg/mL amphotericin B), trypsin-EDTA solution (0.05% trypsin w/v and 0.53 mmol/L EDTA.4Na), Dulbecco's phosphate buffered saline (DPBS), were obtained from Gibco BRL, Life Technologies (Grand Island, NY, USA). Tissue culture 25-cm^2 flasks, cell culture 24-well plate with 19.2 mm diameter and 2.9-cm^2 growth surface area per well were obtained from Costar (Corning, NY, USA). Hoechst 33258 stain and dimethyl sulphoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA).

2.3.2 Materials for the **In Vitro** *Acylcarnitine Profile Assay*

[15,15,16,16,16-$^2H_5$]hexadecanoic acid ($^2H_5$-palmitic acid) was purchased from C/D/N Isotopes (Point Claire, Quebec, Canada). Internal standards (IS) containing a mixture of deuterium (^2H) labelled acylcarnitine including $^2H_3$-acetylcarnitine ($^2H_3C_2$-), $^2H_3$-propionylcarnitine ($^2H_3C_3$-), $^2H_3$-butyrylcarnitine ($^2H_3C_4$-), $^2H_9$-isovalerylcarnitine ($^2H_9C_5$-), $^2H_3$-octanoylcarnitine ($^2H_3C_8$-), $^2H_9$-tetradecanoylcarnitine ($^2H_9C_{14}$-) and $^2H_3$-hexadecanoylcarnitine ($^2H_3C_{16}$-) were from Cambridge Isotope Laboratory (CIL) (Kit B free carnitine and acylcarnitine internal standards manufactured by CIL and distributed by NeoGen, Andover, MA, USA). Bovine serum albumin (essentially fatty acid free), Krebs-Ringer bicarbonate buffer pH 6.8 and L-carnitine were obtained from Sigma (St. Louis, MO, USA). All other chemicals including acetyl chloride (Aldrich), n-butanol (Analar), ethanol (99.7-100%; Analar) and acetonitrile (BDH) were of analytical grade.

2.3.3 Materials for Preparation of External Calibration Curves

DL-acetylcarnitine (C_2-) hydrochloride, L-propionylcarnitine (C_3-) hydrochloride**,** L-isovalerylcarnitine (C_5-) hydrochloride, DL-hexanoylcarnitine (C_6-)

hydrochloride, DL-octanoylcarnitine (C_8-) hydrochloride, DL-decanoylcarnitine (C_{10}-) hydrochloride and L-hexadecanoylcarnitine (C_{16}-) hydrochloride were obtained from Sigma (St. Louis, MO, USA).

2.3.4 Materials for Total Protein Measurement

Bicinchoninic acid protein assay reagents, bovine serum albumin standard (2.0 mg/mL in aqueous sodium chloride solution 0.9%, w/v) were obtained from Pierce (Rockford, Illinois, USA).

2.4 Cell Lines

Cultured skin fibroblasts from healthy subjects and those from patients with documented enzyme deficiencies were obtained from the archives of NSW Biochemical Genetics Department, Metabolic Research Laboratory of the Western Sydney Genetics Program, Metabolic Laboratory of Prince Margaret Hospital, Chemical Pathology of Women and Children's Hospital and Neonatal Screening Laboratory of Sheffield Children's Hospital. Patient samples referred to our laboratory for investigation of suspected fatty acid ß-oxidation disorders and from asymptomatic neonates detected by the NSW Newborn Screening Program included cell lines established by the Cytogenetics Department of the Children's Hospital at Westmead and other institutions around Australia.

2.5 Tissue Culture

2.5.1 Culture Medium

The culture medium was composed of Ham F-10 Nutrient Mixture supplemented with fetal bovine serum 10%, L-glutamine 2 mmol/L, penicillin, streptomycin and Fungizone 1% each.

2.5.2 Cell Culture Technique

All tissue culture work was carried out in a laminar flow biological safety cabinet using sterilised equipment and aseptic techniques. Archived cells were taken up from frozen stock stored at –196^{o}C suspended in 15% dimethyl sulphoxide (DMSO) and 15% fetal bovine serum in culture medium in a Nunc cryotube at a concentration of 10^{7} cells/1.0 mL. The ampoules were thawed rapidly at 37^{o}C, and the cell suspension was transferred to a sterile 11 mL Nunc centrifuge tube containing 5.0 mL warm culture medium. Cells were centrifuged at 350x*g* (2 minutes, 37^{o}C) to remove the DMSO. The supernatant was discarded, the cell pellet resuspended in 4.0 mL fresh culture medium and transferred to a 25-cm^{2} cell culture flask, capped and incubated in a water-jacketed incubator at 37^{o}C at atmospheric air. The medium was changed the next day to remove cells which had not adhered to the flask, and subsequently every 3 to 4 days until the cell monolayer became 100% confluent. These cell lines were then used for the *in vitro* acylcarnitine profiling assay (Section 2.8).

The physical appearance and general health of the cell lines were examined with a Wilovert II phase-contrast inverted light microscope. All cell lines were checked for microbial contamination using the Hoechst 33258 stain with a fluorescence microscope whenever they were trypsinised. Hoechst 33258 is a fluorescent dye, which binds specifically with DNA causing nuclei to fluoresce. All prokaryotic organisms, mycoplasma, bacteria, yeast and fungi will emit fluorescence. Contaminants were identified by noting the size, morphology and relationships to the cells. Cell lines found to be contaminated were not accepted for assay.

2.6 Reaction Mixture for the *In Vitro* Acylcarnitine Profile Assay

2.6.1 Preparation of [15,15,16,16,16-$^2H_5$]hexadecanoic acid 2.2 mmol/L

[15,15,16,16,16-$^2H_5$]hexadecanoic acid ($^2H_5$-palmitic acid) is a straight chain saturated fatty acid labelled with five deuteriums at the methyl end. To avoid problems with degradation of the deuterium label or autohydrolysis of the acylcarnitines when in solution, the stock $^2H_5$-palmitic acid (2.2 mmol/L) was stored dry until use. This was prepared by dissolving 14.4 mg of the compound in 25 mL ethanol (99.7-100%), aliquoting 1.0 mL into a 25 mL glass vial, drying under a nitrogen stream, capping and storing in a desiccator at -20°C.

2.6.2 Preparation of Reaction Mixture

The reaction mixture is comprised of $^2H_5$-palmitic acid (110 µmol/L) complexed to bovine serum albumin (0.5 mg/mL) with L-carnitine (400 µmol/L) in the culture medium. It was prepared fresh just before use as follows: 1.0 mL of 10.0 mg/mL bovine serum albumin (essentially fatty acid free) in Krebs-Ringer bicarbonate buffer pH 6.8 was added to a vial of stock $^2H_5$-palmitic acid (2.2 mmol/L; refer to section 2.6.1). The mixture was sonicated (Bransonic 220 sonicator) for 12 seconds followed by cooling under tap water for one minute and this process repeated twice to ensure a homogenous solution without denaturing the protein. It was then incubated for 30 minutes in a 37^{o}C water bath to enhance the conjugation of free fatty acid to albumin. After incubation, 800 µL of L-carnitine (100 mmol/L) and 19.8 mL of the culture medium (refer to section 2.5.1) pre-warmed to 37^{o}C were added and mixed thoroughly before use. The remaining reaction mixture was discarded.

2.7 Electrospray Ionisation Tandem Mass Spectrometry

The tandem mass spectrometer used was a Quattro LC instrument equipped with a Z-spray electrospray ionisation source (Micromass, Manchester, UK), attached to a

model 215 liquid handler and series 306 HPLC pump (Gilson Instruments) for automated loading of samples in a continuously flowing solvent system.

2.7.1 Electrospray Ionisation

This technique involves the sample solution passing along a narrow capillary tube, the end of which is maintained at high potential producing a protonated or deprotonated molecular ion. The emerging liquid forms fine droplets carrying a charge and, under the influence of heated nitrogen gas, the solvent evaporates. Eventually ions start to desorb from the surface of the droplets and move into the high vacuum area (ion source, see below) of the mass spectrometer.

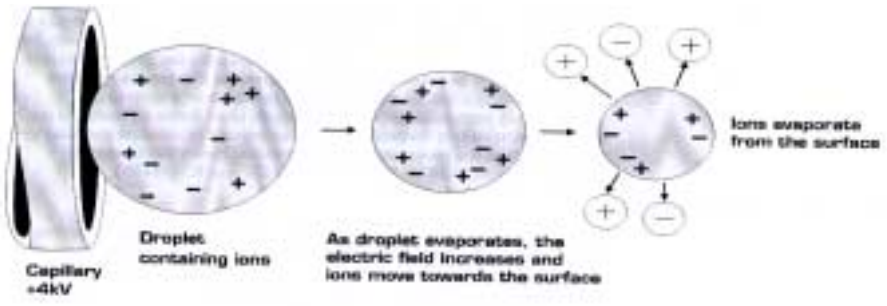

Figure 2.1 Schematic representation of electrospray ionisation. (Adapted from Micromass literature)

2.7.2 Tandem Mass Spectrometry

The tandem mass spectrometer is comprised of three quadrupole mass spectrometers connected in series, designated as MS1, collision chamber, and MS2.

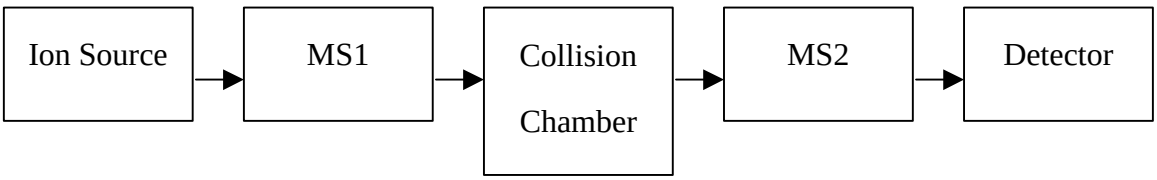

Figure 2.2 Schematic representation of tandem mass spectrometer

Ions selected by MS1 (called precursor ions) are passed to the collision chamber, where the ions are accelerated, collide with argon gas, and fragment (collision induced dissociation). Fragments formed in the collision chamber (called product ions) are separated by MS2 and detected.

2.7.3 Application of ESI-MS/MS to Acylcarnitine Profiling

Butyl esters of acylcarnitines fragment to yield a common charged fragment of 85.2 mass to charge ratio (*m/z*) corresponding to $^{+}CH_2$-CH=CH-COOH. This fragment results from the loss of elements $N(CH_3)_3$, C_4H_8 and the side chain acyl group RCOOH (Figure 2.3). By scanning MS1 between 200 and 500 *m/z* and fixing MS2 at 85.2 *m/z*, all precursor ions of 85.2 *m/z* are detected.

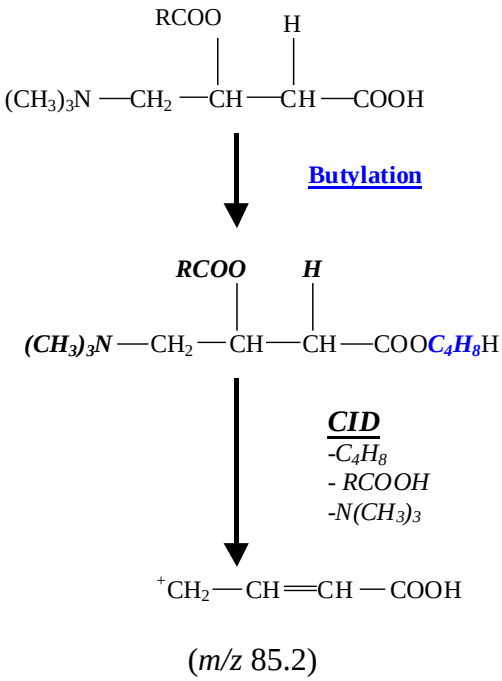

Figure 2.3 Butylation and fragmentation of acylcarnitines to yield a common fragment at *m/z* 85.2

R: acyl group e.g. $–(CH_2)n\text{-}CH_3$, *m/z*: mass to charge ratio

CID: Collision induced dissociation

2.8 The *In Vitro* Acylcarnitine Profile Assay

2.8.1 Preparation of the Cell Monolayer

The culture medium of the confluent cell line in a 25-cm^2 flask was discarded and the cell monolayer rinsed twice with 4.0 mL Dulbecco phosphate buffered saline (DPBS) to remove the excess culture medium. The cells were then incubated with 0.2 mL pre-warmed trypsin-EDTA solution for one to two minutes to detach cells from the flask, followed by the addition of 2.0 mL culture medium to inactivate the trypsin. A homogenous cell suspension was obtained by repeated uptake and expulsion of the mixture with a pipette. The cells were then counted using a phase contrast inverted light microscope at objective 10x in a Weber haemocytometer (depth 0.1 mm, 1/400 mm^2; with coverslip). Aliquots of cells (equivalent to 37-60 µg total protein) were subcultured into wells of a 24-well cell culture plate, and allowed to settle for 24 hours to form a cell monolayer.

2.8.2 Incubation of the Cell Monolayer with the Reaction Mixture

The culture medium of the cell monolayer was then replaced with 0.5 mL reaction mixture (section 2.6.2). The culture plate was manually swirled every two to three hours (during working hours) to obtain a homogenous reaction mixture. After incubation in a water-jacketed incubator at 37^{o}C, atmospheric air for up to 96 hours, the resultant reaction mixture in each well was transferred to a 2.0 mL polypropylene tube for acylcarnitine analysis, and the cell monolayer retained for protein quantitation. The cell monolayers were checked with the inverted light microscope, and only the cells adhering as a continuous sheet were judged to be viable cultures suitable for the following sample preparation and acylcarnitine profiling.

2.8.3 Sample Preparation for Acylcarnitine Analysis

Internal standards (IS) containing a mixture of deuterium (^2H) labelled acylcarnitine including $^2H_3C_2$-, $^2H_3C_3$-, $^2H_3C_4$-, $^2H_9C_5$-, $^2H_3C_8$-, $^2H_9C_{14}$- and

$^{2}H_3C_{16}$-acylcarnitine prepared in ethanol were added to each tube of resultant reaction mixture, vortex mixed and centrifuged at 29,000x*g* (five minutes, 4^{o}C) to remove the precipitate. The supernatant was evaporated to dryness under a stream of air with heating at 40^{o}C. The dried sample was derivatised with 300 µL butanolic-hydrochloric acid (10% v/v) in a heating block set at 60^{o}C for 15 minutes to form butyl esters of the -COOH group. Butanolic-hydrochloric acid (10% v/v) was prepared in a fume cupboard by slowly adding 50 mL of acetyl chloride to 450 mL of n-butanol in a one Litre Schott bottle in a bed of Vermiculite beads (absorbent to mop up any spills), with frequent swirling to dissipate the heat generated. After derivatisation, the sample was spun again to remove the further precipitate formed during acid butylation, and the supernatant transferred to another tube. The excess butanolic-hydrochloric acid was evaporated under a stream of air with heating, and the dried butylated sample redissolved in 100 µL of 50% acetonitrile:water (1:1, v/v) and transferred to a 96-well polypropylene microtitre plate for automated injection into the ESI-MS/MS.

2.8.4 Data Acquisition and Processing

Twenty microlitres (µL) of the reconstituted sample was used for each injection by the autosampler. To ensure no carry over of sample, the autosampler was programmed to perform two lots of 100 µL inside needle rinses, one lot each of 220 µL and 400 µL injection port rinses between each sample injection. The HPLC pump was set to operate with a flow rate of 150 µL per minute for 0.35 minutes, then at 40 µL per minute (during data acquisition) until two minutes post injection, and finally at 250 µL per minute for a further 30 seconds to wash out any remaining sample prior to the next injection. The electrospray capillary voltage was set at 3.5 kV and the cone voltage at 30 V. The desolvation gas temperature was set at 150^{o}C and the argon collision gas energy at 30 eVolts.

Data files were acquired using Multi-Channel Acquisition (MCA) to detect all the butylated acylcarnitines in the samples. The instrument was programmed to operate the MS1 in parent ion mode with MS2 set to 85.2 mass to charge ratio (*m/z*), scanning in the range 200–500 *m/z* for 1.3 minutes following a delay of 0.6 minutes from injection of the sample into the flowing solvent stream. During this period all scans were summed to give an improved signal to noise ratio. A spectrum for each sample was printed following baseline subtraction and smoothing of the spectrum. The acylcarnitine mass spectrum was presented as the relative intensities of ions on the Y-axis and the molecular weight of the butyl esters of the acylcarnitine species *m/z* on the X-axis. The most abundant species in the sample detected within 250–500 *m/z* was scaled to 100% of relative abundance (example Figure 2.4).

Following acquisition, all data files were processed using the NeoLynx software to give concentration data for externally calibrated analytes where available (Table 2.1) or the ratio of analyte to internal standard intensities for other components. For analytes where an identical IS was not available, an identical response to the closest counterpart was used. Table 2.2 shows the parameters in Neolynx editor for calculation for each analyte.

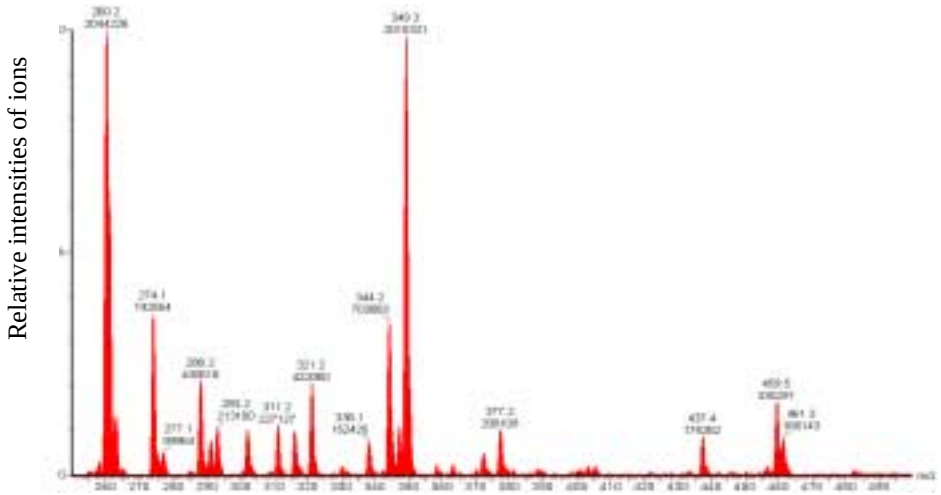

Figure 2.4: Acylcarnitine mass spectrum detected in the reaction medium of fibroblasts from a patient with medium chain acyl-CoA dehydrogenase deficiency

	Working calibration mixture (nmol/mL)				
Acylcarnitine species	**Std A**	**Std B**	**Std C**	**Std D**	**Std E**
DL-acetylcarnitine (C_2-) hydrochloride	2.0	4.0	6.0	8.0	10.0
L-propionylcarnitine (C_3-) hydrochloride	2.0	4.0	6.0	8.0	10.0
L-isovalerylcarnitine (C_5-) hydrochloride	2.0	4.0	6.0	8.0	10.0
DL-hexanoylcarnitine (C_6-) hydrochloride	0.4	0.8	1.2	1.6	2.0
DL-octanoylcarnitine (C_8-) hydrochloride	0.4	0.8	1.2	1.6	2.0
DL-decanoylcarnitine (C_{10}-) hydrochloride	0.4	0.8	1.2	1.6	2.0
L-palmitoylcarnitine (C_{16}-) hydrochloride	0.44	0.88	1.32	1.76	2.2

Table 2.1: Constituents of external calibration mixture and their concentrations for construction of standard curves.

Std: standard mixture

nmol/mL: nanomole per millilitre

Acylcarnitine from oxidation of labelled fatty acid, endogenous fatty acids or amino acids		Acylcarnitine Internal Standards used for quantitation			Calculation mode
Species	***m/z***	**Species**	***m/z***	**(nmol/mL)**	
C_2-	260.2[a plus b]	$^2H_3C_2$-	263.2	3.80	ECC
C_3-	274.2[c]	$^2H_3C_3$-	277.2	0.76	ECC
C_4-	288.2[b plus c] 293.2[a]	$^2H_3C_4$-	291.2	0.76	ISR
C_5-	302.2[c]	$^2H_9C_5$-	311.2	0.76	ECC
C_6-	316.2[b] 321.2[a]	$^2H_3C_8$-	347.2	0.76	ECC
C_8-	344.2[b] 349.2[a]	$^2H_3C_8$-	347.2	0.76	ECC
C_{10}-	372.2[b] 377.2[a]	$^2H_3C_8$-	347.2	0.76	ECC
C_{12}-	400.2[b] 405.2[a]	$^2H_3C_8$-	347.2	0.76	ISR
$C_{14:1}$-	426.2[b] 431.2[a]	$^2H_9C_{14}$-	437.2	0.76	ISR
C_{14}-	428.2[b] 433.2[a]	$^2H_9C_{14}$-	437.2	0.76	ISR
$C_{16:1}$-	454.2[b] 459.2[a]	$^2H_9C_{14}$-	437.2	0.76	NA
C_{16}-	456.2[b] 461.2[a]	$^2H_9C_{14}$-	437.2	0.76	ECC
C_{16}-OH	472.2[b] 477.2[a]	$^2H_9C_{14}$-	437.2	0.76	ISR

Table 2.2: Parameters in Neolynx editor for calculation of individual acylcarnitine concentrations.

[a]*m/z*: Acylcarnitines derived from ß-oxidation of [15,15,16,16,16-$^2H_5$]hexadecanoic acid

[b]*m/z:* Acylcarnitines derived from ß-oxidation of endogenous source of fatty acids

[c]*m/z*: Acylcarnitines derived from oxidation of branched-chain amino acids

$^{2}H_{number}$: Deuterium and the corresponding number of deuteriums labelled at the methyl end of acylcarnitine

C_{number}-: Acylcarnitine with the corresponding number of carbon atoms

C_2-: acetylcarnitine, C_3-: propionylcarnitine, C_4-: butyrylcarnitine or isobutyrylcarnitine, C_5-: isovalerylcarnitine or 2-methylbutyrylcarnitine, C_6-: hexanoylcarnitine, C_8-: octanoylcarnitine, C_{10}-: decanoylcarnitine, C_{12}-: dodecanoylcarnitine, $C_{14:1}$-: tetradecenoylcarnitine, C_{14}-: tetradecanoylcarnitine, $C_{16:1}$-: hexadecenoylcarnitine, C_{16}- hexadecanoylcarnitine, C_{16}-OH: hydroxy-hexadecanoylcarnitine

ECC: Calculated as (ratio of analyte to internal standards) x (amount of IS) x (factor obtained from external calibration curve by taking average of factors for all 5 calibrators)

ISR: Calculated as (ratio of analyte to internal standards) x (amount of IS)

NA: Not analysed

2.9 Fibroblast Total Protein Quantitation

The cell monolayer in each well was washed twice with 2.0 mL DPBS and hydrolysed with 250 µL sodium hydroxide (2.0 mol/L) at room temperature overnight, followed by 500 µL hydrochloric acid (1.0 mol/L) to neutralise the matrix. The cell hydrolysate was transferred to a 3.0 mL plastic tube (Minisorp^{TM}), centrifuged at 500*g* (three minutes, 25^{o}C) to pack the cell debris down, and the supernatant used for total protein quantitation.

Total protein was measured by the bicinchoninic acid (BCA) protein assay (Goldschmidt and Kimelberg 1989) using a Cobas BIO spectrophotometric centrifugal analyser (Roche Diagnostic, Basel, Switzerland). Cupric ions are reduced by protein to cuprous ions which then bind specifically to BCA molecules to form a purple reaction product, which exhibits a strong absorbance at 562 nm.

Fifteen µL of sample was added to 300 µL BCA reagent, mixed and the absorbance at 40^{o}C measured after 7.5 minutes, and the protein concentration determined by the autoanalyser's microprocessor against the bovine serum albumin (BSA) standard curve. The standard curve was constructed with six concentration points including zero, 30, 40, 60, 80 and 100 µg/mL, prepared by serial dilution of the stock BSA standard (2.0 mg/mL) in 0.9% aqueous sodium chloride solution (w/v) just before each batch of assays. Two samples prepared from pooled fibroblast hydrolysate with a similar matrix to that of the test samples were assayed in every batch as quality controls (QC2 and QC8). Their cumulative results are shown in Appendix 2.

2.10 Expression of Fibroblast Acylcarnitine Results

Acylcarnitine nanomole per millilitre (nmol/mL) divided by total protein milligram per millilitre (mg/mL) to give nanomole of acylcarnitine formed per milligram of total protein (nmol/mg) during the period of incubation.

$$\frac{\text{Acylcarnitine (nmol/mL)}}{\text{Total protein (mg/mL)}} = \text{Acylcarnitine nmol/mg total protein}$$

2.11 Discussion

Using ESI-MS/MS and MCA mode of data acquisition, all butyl esters of acylcarnitines and any other compounds producing product ions of 85.2 *m/z* after collision-induced dissociation (CID) may be detected. Using a labelled precursor ($^2H_5$-palmitate) enables the origin of the acylcarnitines to be traced, distinguishing between acylcarnitines derived from the oxidation of exogenous or endogenous sources of fatty acids and branched-chain amino acids. The expected metabolites include the $^2H_5$-labelled acylcarnitines derived from the ß-oxidation of $^2H_5$-palmitate, and the unlabelled species originating from endogenous sources of fatty acids and branched-chain amino acids (Table 2.2).

Acetylcarnitine (C_2-) detected in the reaction mixture consists of the products of endogenous as well as exogenous fatty acids ß-oxidation, whereas propionylcarnitine (C_3-) and the C_5-acylcarnitine, representing isovalerylcarnitine or 2-methylbutyrylcarnitine, are derived from oxidation of branched-chain amino acids present in the culture medium or within the cells. Butyl esters of acylcarnitine with 288 *m/z* ratio correspond to unlabelled C_4-acylcarnitine, which is isomeric with butyrylcarnitine and isobutyrylcarnitine originating from ß-oxidation of unlabelled fatty

acids and valine catabolism respectively. The origin of all other acylcarnitines could be identified by their mass differences.

Analytes shown in Table 2.2 are all those potentially detectable by this system. The IS $^{2}H_{3}C_{16}$- (459.2 *m/z*), which is present in Kit B free carnitine and acylcarnitine internal standards (section 2.3.2), was not used for calculation of hexadecanoylcarnitine (C_{16}-) because of potential interference from deuterium labelled hexadecenoylcarnitine ($^{2}H_{5}C_{16:1}$-) which has the same 459.2*m/z*; and IS $^{2}H_{9}C_{14}$-acylcarnitine 437.2 *m/z* was thus used instead.

Unlabelled acylcarnitines contribute no additional diagnostic information, except for C_{5}-acylcarnitine, which has been reported to be associated with multiple acyl-CoA dehydrogenase deficiency. Hence, in the following Chapters, only values of $^{2}H_{5}$-labelled butyrylcarnitine (C_{4}-), hexanoylcarnitine (C_{6}-), octanoylcarnitine (C_{8}-), decanoylcarnitine (C_{10}-), dodecanoylcarnitine (C_{12}-), tetradecanoylcarnitine (C_{14}-), hexadecanoylcarnitine (C_{16}-), hydroxy-hexadecanoylcarnitine (C_{16}-OH) and unlabelled C_{5}-acylcarnitine are reported.