ELSEVIER

Journal of Chromatography B, 702 (1997) 1–20

JOURNAL OF CHROMATOGRAPHY B

Review

L-Carnitine moiety assay: an up-to-date reappraisal covering the commonest methods for various applications

Antonio Marzo*, Salvatore Curti

I.P.A.S. S.A., Clinical Pharmacology Department, Via Mastri, Zona Stramonte, 6853 Ligornetto, Switzerland

Received 5 December 1996; received in revised form 4 July 1997; accepted 9 July 1997

Abstract

L-Carnitine and its esters are typical endogenous substances. Their homeostatic equilibria are effectively controlled by various mechanisms which include rate-limiting enteral absorption, a multicomponent endogenous pool which is regulated according to a mammillary metabolism, an asymmetric body distribution and a saturable tubular reabsorption process leading to renal thresholds. In formal pharmacokinetic and metabolic investigations, the whole L-carnitine pool should be investigated, owing to the rapid interchange process between the various components of the pool. Free L-carnitine, as well as its acyl esters, must therefore be considered from an analytical viewpoint. L-Carnitine, acetyl-L-carnitine and total L-carnitine (the latter as an expression of the whole pool) can easily be assayed by enzyme or radioenzyme methods. Propionyl-L-carnitine and other esters containing fatty acids with more than three carbon atoms can be assayed using various HPLC approaches. Tandem mass spectrometry is another excellent approach to the assay of carnitine and its short-chain, medium-chain and long-chain esters. As L-carnitine contains a chiral carbon atom, the enantioselectivity of the assays is also considered in this review. Metabolites produced by enteral bacteria, namely tri-, di- and mono-methylamine, γ-butyrobetaine, along with other systemic metabolites, namely trimethylamine *N*-oxide and *N*-nitroso dimethylamine, are very important in quantitative and toxicokinetic terms and require specific assay methods. This review covers the commonest methods of assaying carnitine and its esters, their impurities and pre-systemic and systemic metabolites and gives analytical details and information on their applications in pharmaceutics, biochemistry, pharmacokinetics and toxicokinetics. © 1997 Elsevier Science B.V.

Keywords: Reviews; L-Carnitine

Contents

*Corresponding author.

PII S0378-4347(97)00376-9

1. Introduction

The endogenous pool of L-carnitine includes L-carnitine, or free L-carnitine (LC), its short-chain esters (namely, acetyl-L-carnitine (ALC), propionyl-L-carnitine (PLC), butyryl and isobutyryl-L-carnitine, valeryl- and isovaleryl-L-carnitine), medium-chain (e.g., octanoyl-L-carnitine), and long-chain esters (e.g., palmitoyl-L-carnitine). All these esters are equilibrated with L-carnitine by carnitine acyl transferase catalysis, with different specificities for each substrate [1–3]. Among these enzymes, most investigations have been carried out on carnitine acetyl transferase (CAT), carnitine octanoyl transferase (COT) and carnitine palmitoyl transferase (CPT), which are active on short-, medium- and long-chain esters respectively [4]. Fig. 1 depicts the interconversion of L-carnitine and its ester [5].

As previously documented, L-carnitine possesses an active transport mechanism operating inter alia in enteral absorption. However, with pharmacological doses, it is rapidly saturated and operates at a very slow rate due to the ionic strength of carnitine [5,6].

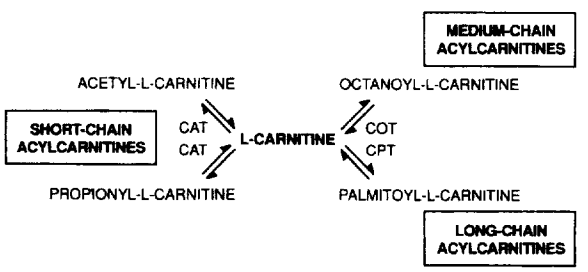

Fig. 1. Metabolism of L-carnitine.

These relatively high doses are thus absorbed through a very slow diffusion process, which is the rate-limiting step in enteral absorption kinetics [5]. Oral doses of these compounds thus scarcely affect plasma concentrations [7], as a result of their low and sluggish enteral absorption, dilution of the exogenous substance that present endogenously, and rapid urinary excretion occurring through a saturable tubular reabsorption process leading to specific renal thresholds [5,8,9], which seem to overlap with plasma concentrations of L-carnitine and each individual ester [10].

The total L-carnitine contained in human body, about 20 g, is asymmetrically distributed, 98% being present in the skeletal and cardiac muscles where it transports fatty acids and ketoacids formed by branched amino acids, 1.4% in the liver and kidneys and only 0.6% in extracellular fluids and other tissues [5].

When L-carnitine or its esters are injected i.v., their concentrations immediately increase and a rapid exchange process between the compound injected and other metabolically related substances occurs [8,9,11,12]. This behaviour accounts for the need in pharmacokinetic, metabolic and toxicokinetic studies to assay not only the compound administered, but also all compounds that can be metabolically derived from it or, alternatively, the whole pool [5,13].

The L-carnitine moiety contains a stereogenic carbon atom which generates two enantiomers, the L-form being the active one, which is marketed [4,14]. An enantioselective assay may therefore not be mandatory, bearing in mind inter alia that the stereogenic centre of L-carnitine seems to be stable and no unidirectional enzyme-mediated L to D interconversion seems to occur [15]. In any case, enzyme assays are strictly enantioselective [15]. Enan-

tioselective assays are needed to ascertain enantiomeric purity in chemistry and pharmacy procedures, and could be useful in investigating the different pharmacokinetic and metabolic behaviour of L- and D-carnitine. HPLC enantioselective assays of the L-carnitine moiety for pharmaceutical purposes have been published [16,17].

Several methods for assaying carnitine in biological fluids have been published by various authors. The most common methods are those employing enzymes and those using HPLC with precolumn derivatization and UV or fluorimetric detection. Tandem mass-spectrometry and capillary electrophoresis are other interesting approaches. Most methods have, however, been set up to solve biochemical problems, mainly those related to the deficiency of some fatty acid esters of L-carnitine. Growing interest in L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine for various therapeutic goals has led some investigators to develop comprehensive pharmacokinetic and toxicokinetic registration files which also cover the presystemic metabolites of the L-carnitine moiety. The above methods were thus carefully considered to assess whether they could satisfy the validation required for formal pharmaceutical, pharmacokinetic and toxicokinetic studies in the United States and Europe. Some of these methods, that were originally developed for biochemical purposes and have been adequately validated, complied with these requirements. They include the radioenzymatic assay, some HPLC assays with pre-column derivatization and tandem mass spectrometry.

This review covers most methods of assaying carnitine and its acylesters for pharmaceutical, pharmacokinetic, toxicokinetic and biochemical applications, including a new assay described in detail [18]. Methods of assaying the metabolites of the L-carnitine moiety, namely tri-, di-, mono-methylamine, trimethylamine-*N*-oxide, nitrosated dimethylamine, γ-butyrobetaine and possible pharmaceutical impurities (crotonoylbetaine) are also reviewed [19].

In this paper, when physiological mechanisms or enantioselective methods are described, the analytes are defined as L-carnitine, acetyl-L-carnitine, and propionyl-L-carnitine. In other cases, the above substances are described as carnitine, acetylcarnitine and propionylcarnitine.

2. Analytical approaches

The first assay procedure for L-carnitine was set up by Fraenkel [20] and was based on the growth of the worm *Tenebrio molitor* in the presence of the tested substance, compared with its growth in the presence of a known amount of L-carnitine. This assay was not capable of quantifying L-carnitine in biological fluids due to its lack of specificity, sensitivity and precision. Colorimetric procedures were developed which utilized the interaction between the quaternary ammonium group and bromophenol blue [21,22], but these approaches were still unable to assay carnitine in biological samples. The most significant step was the enzyme assay of L-carnitine developed by Marquis and Fritz in 1964 [23]. Enzyme and radioenzyme methods were continuously improved in subsequent years, enabling the problem of assaying free and total L-carnitine, and also acetyl-L-carnitine in biological fluids to be fully solved. However other short-chain, medium-chain and long-chain esters of L-carnitine needed different assays, which have been developed in the last decade. These are HPLC assays preceded by pre-column derivatization to render the carnitine esters sensitive to UV or fluorimetric detectors and tandem mass spectrometry. Methods for pharmaceutical applications are described in Section 2.5, whereas Section 2.6 is devoted to methods of assaying carnitine metabolites.

2.1. HPLC assays

HPLC assays are particularly useful for evaluating carnitine and its short-chain esters. These methods are not enantioselective and require a precolumn derivatization to quantify the analytes present in biological samples by UV or by fluorimetric detector. When applied to L-carnitine, these methods have no sensitivity problems, as L-carnitine is widely present in the body. Problems of sensitivity can, however, arise with L-carnitine esters present at lower concentrations, like acetyl- and propionyl-carnitine, which require a rigorous standardised sample clean-up procedure.

Mean plasma L-carnitine and L-carnitine ester concentrations in healthy volunteers in a baseline

Table 1
Mean plasma L-carnitine and carnitine ester concentrations expressed in nmol/ml

	Males	Females
Total L-carnitine	55	37
Free L-carnitine	48	33
Acetyl-L-carnitine	5	3
Propionyl-L-carnitine	0.8	0.6

situation, expressed in nmol/ml, are shown in Table 1 [15].

2.1.1. Pre-column derivatization for fluorimetric detection

2.1.1.1. *Derivatization reaction.* Kobayashi and Ichishima [24] have proposed the use of the water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) for fluorescent determination of uronic acids and carboxylic acids. According to this scheme of derivatization, a highly fluorescent derivative of carnitine moiety is prepared via the above carbodiimide followed by reaction with an aromatic amine to give the corresponding aromatic amide. When aminobenzene, 1-aminonaphthalene, 1-aminoanthracene and 9-aminophenanthrene were tested, only the latter two amines produced highly fluorescent derivatives. Fig. 2 depicts the derivatization scheme with EDCI and 1-aminoanthracene. An EDCI/carnitine ratio of ≥80 and a reaction time of ≥20 min gave the highest derivatization yield [18].

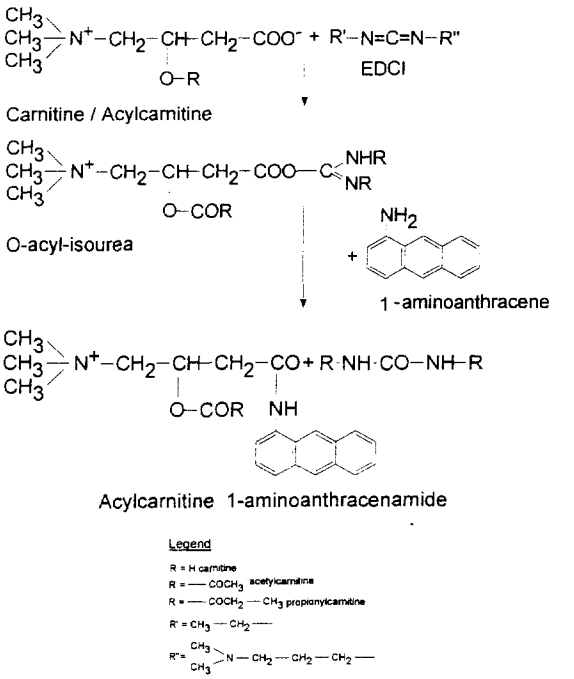

Fig. 2. Scheme of acylcarnitine reaction with EDCI and 1-aminoanthracene.

2.1.1.2. *Extraction procedure.* A 100-μl volume of plasma was transferred into a plastic test tube and 10 μl of 0.1 μmol/ml of internal standard solution and 390 μl of distilled water were added. The tube was vortex-mixed for 30 s and the sample was then applied to an Isolute SAX cartridge (100 mg), which had previously been conditioned with 0.5 ml of methanol and 1 ml of distilled water. The eluate was kept in a plastic test-tube. The cartridge was subsequently eluted with 0.5 ml of 0.01 *M* NaH_2PO_4, pH 3.5. The derivatization procedure on the combined eluates was performed in the same plastic test tube as follows: in continuous mix-vortex, 20 μl of 1 *M* HCl, 100 μl of 1-aminoanthracene (16 mg/ml) and five aliquots each of 20 μl of EDCI (160 mg/ml) were added to the sample. The tube was incubated for 20 min at 25°C. An aliquot of 300 μl of sample was transferred to a tube containing 700 μl of 0.01 *M* Na_2HPO_4 (pH 9.1) to which 5 ml of chloroform had been added. The tube was vortex-mixed for 3 min and then centrifuged at 1500 *g* for 5 min. A 20-μl aliquot of the aqueous phase was injected into the HPLC under the conditions listed in Table 2.

2.1.1.3. *Specific features.* Analytical validation was carried out on dialysed human plasma in order to eliminate all the endogenous carnitine pool from the matrix that is used as a blank. Fig. 3A depicts an HPLC recording of dialysed human plasma which proved to be completely free from carnitine and its esters. Dialysis was performed through a Spectrapor membrane (NWCO: 6-800) in Krebs-Ringer solution at 4°C for 36 h. Fig. 3B depicts HPLC recordings of dialysed human plasma spiked with carnitine (5 nmol/ml), acetylcarnitine (1 nmol/ml) and propionylcarnitine (0.25 nmol/ml). Fig. 3C shows the

Table 2
Main characteristics of the HPLC method with fluorimetric detection used to assay carnitine, acetylcarnitine and propionylcarnitine

Analytes:	Carnitine, acetylcarnitine and propionylcarnitine
Matrices:	Plasma (100 μl), urine (200 μl)
Derivatization:	1-Aminoanthracene (1-AA) or 9-aminophenanthrene (9-AP) with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
Analytical system:	HPLC with fluorescence detection; excitation, 248 nm (1-AA) or 300 nm (9-AP); emission, 418 nm (1-AA) or 366 nm (9-AP)
Column:	RP C18 250×4.6 mm I.D., 5 μm
Mobile phase:	0.1 *M* ammonium acetate, pH 3.2, and acetonitrile (70:30, v/v)
Flow-rate:	1.5 ml/min
Retention time:	Carnitine, 5 min; acetylcarnitine, 8 min; propionylcarnitine, 13 min; and I.S., 21 min
Quantification:	Either by the use of I.S. (isobutyrylcarnitine) or by external standardization
Quantification limit:	Plasma: 5, 1 and 0.25 nmol/ml, for carnitine, acetylcarnitine and propionylcarnitine, respectively; urine: 200, 20 and 1 nmol/ml, for carnitine, acetylcarnitine and propionylcarnitine, respectively
Duration:	Each analysis, also covering propionylcarnitine, takes about 18 min with external standardization and about 30 min with internal standardization
Enantioselectivity:	No
Applications:	Pharmacokinetics and toxicokinetics

HPLC recording of non-dialysed baseline human plasma assayed with this method.

When the internal standard (I.S.) was added, analyte to I.S. peak height ratios were used to obtain calibration curves through a weighted ($1/y$) linear regression analysis in six concentrations, in the ranges 5–160 nmol/ml for carnitine, 1–32 nmol/ml for acetylcarnitine and 0.25–8 nmol/ml for propionylcarnitine. Linearity, intra-day and inter-day precision and accuracy expressed in terms of mean values of precision and accuracy of five or six findings are reported in Table 3. When external standardization was used, peak heights were used to obtain a weighted ($1/y$) calibration curve. In the validation without I.S., linearity was ascertained with the above three analytes in the operating ranges by the slope, the intercept and the correlation coefficient of back calculated concentrations. Using either internal or external standardisation, overlapping validation parameters were obtained.

The limits of quantification with carnitine, acetylcarnitine and propionylcarnitine were 5.0, 1.0 and 0.25 nmol/ml, respectively. Extraction recovery averages 80% with the three analytes and was independent of their concentration.

A problem with this method is whether or not to use an internal standard (I.S.). Isobutyrylcarnitine proved to be adequate for this purpose. Its retention time, however, lengthened the assay to more than 25 min, compelling the analyst to schedule not less than 30 min for each analysis using the autosampler injector. In addition, as pointed out in a recent review devoted to the toxicokinetics of endogenous substances [5], the use of a component of the endogenous pool as an I.S. is a procedure to be discouraged, as this component is present in unknown concentrations in the samples to be assayed and it increases after exogenous administration, as ascertained in the case of carnitine family components, which behave according to a mammillary metabolism of a homologous series. The method was validated using isobutyrylcarnitine as I.S. and also without any I.S. through external standardization. Both the procedures proved to work very well. As an alternative, some trimethylalkylammonium carboxylated molecules not belonging to the endogenous pool are now under investigation as possible internal standards.

An analytical series usually contains six samples for the calibration curve, $3\times2=6$ quality control (QC) samples and about 24 unknown samples. This assay is easy to perform on a routine basis, but is relatively time-consuming. Both the reactants, 9-aminophenanthrene and 1-aminoanthracene, possess some toxicity which calls for laboratory operators to adopt adequate precautions. Table 2 summarizes the main characteristics of this assay.

A HPLC assay with fluorimetric detection of carnitine and acylcarnitine has been published by Kamimori et al. [25] who derivatized the above substrates with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone. However, the quantifi-

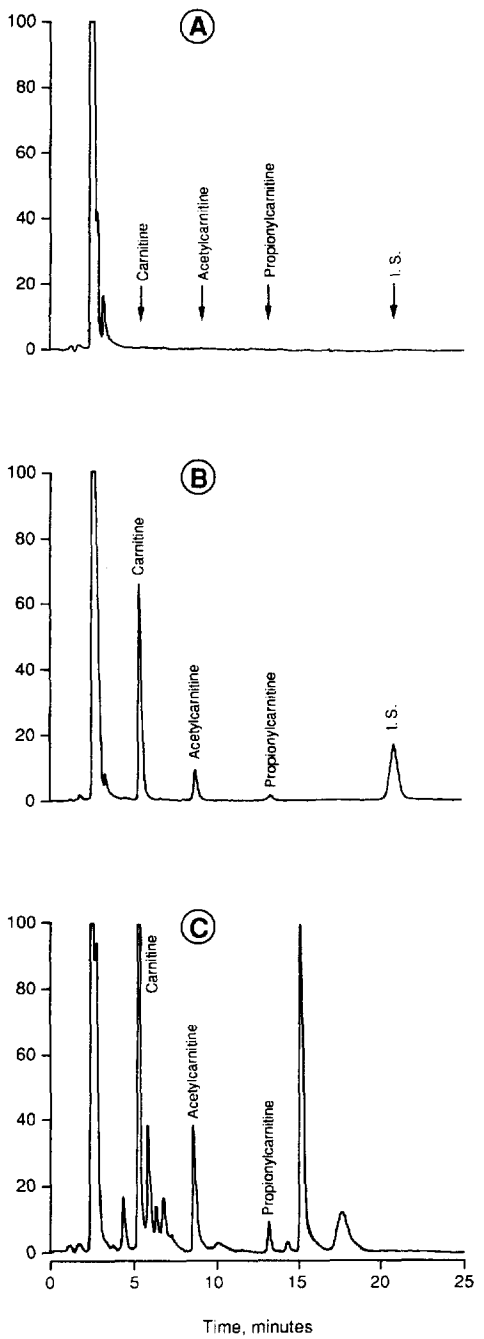

Fig. 3. HPLC recordings of L-carnitine and its short-chain esters derivatized with 1-aminoanthracene via an intermediate carbodiimide derivative. For analytical details see Table 2. (A) Dialysed blank human plasma; (B) dialysed blank human plasma spiked with authentic standards of carnitine (5 nmol/ml), acetylcarnitine (1 nmol/ml) and propionylcarnitine (0.25 nmol/ml); (C) non-dialysed blank human plasma.

cation limit confines this method to assaying the above analytes in urine, not in plasma.

2.1.2. Pre-column derivatization for UV absorbence detection

2.1.2.1. *Description of the assay.* A team headed by Dr. Hoppel in Cleveland has standardized an HPLC assay of carnitine originally applied to urine [26]. In further contributions this assay also covered plasma and tissues as matrices and carnitine esters as analytes [27–29]. According to this method, the carnitine moiety reacts with 4′-bromophenacyl trifluoromethane sulfonate to produce a derivative which is very sensitive to UV detection at 260 nm. A quaternary pump, four eluent mixtures, and disposable polypropylene chromatographic columns packed with silica gel were used. Quantification was achieved by an internal standard, namely 4-(*N*,*N*-dimethyl-*N*-ethylammonio)-3-hydroxybutanoate [27,28].

2.1.2.2. *Specific features.* According to their up-to-date validation, the authors can use this method to assay free and total carnitine and all short- and medium- chain esters of carnitine, in addition to betaine, γ-butyrobetaine and trimethyllysine, which is a metabolic precursor in L-carnitine biosynthesis. Figs. 4 and 5 show the real capability of this method when faced with biological matrices.

Clean-up of the matrices was excellent, resulting in high selectivity. However, the sensitivity of 2.5 nmol/ml seems inadequate for propionylcarnitine, which is present in plasma at concentrations of <1 nmol/ml (Table 1).

An assay of carnitine, acetylcarnitine, propionylcarnitine and internal standard using this method takes not more than 7 min, which is less than half the time required with the method described in Section 2.1.1 and allows more unknown samples to be processed in an analytical series. Table 4 summarizes the main features of this method.

The same derivatization reaction with 4-bromophenacyl triflate was used by Lever et al. [30] in a method differing from the earlier one in that the desalting process is replaced by solvent extraction into acetonitrile containing 10% methanol and with the silica-based reversed-phase columns for HPLC

Table 3
Linearity, intra-day and inter-day reproducibility in terms of mean values of precision and accuracy related to the carnitine, acetylcarnitine and propionylcarnitine assay with pre-column derivatization and HPLC-fluorimetric detection, according to the method reported in Table 2: mean values of five or six determinations

Linearity

Carnitine	Nominal concentrations (nmol/ml)						Calibration curve parameters		
	160	80	40	20	10	5	Slope	Intercept	r^2
	Back-calculated concentrations (nmol/ml)								
Mean ($n=5$)	159.72	80.47	40.99	20.35	9.97	4.88	0.1493	0.0269	0.9912
S.D.	2.12	1.61	0.88	0.61	0.29	0.23	0.0219	//	//
Precision (C.V.%)	1.33	2.00	2.15	3.00	2.91	4.71	14.67	//	//
Accuracy (%)	−0.18	+0.59	+2.48	+1.75	−0.30	−2.40	//	//	//

Acetylcarnitine	Nominal concentrations (nmol/ml)						Calibration curve parameters		
	32	16	8	4	2	1	Slope	Intercept	r^2
	Back-calculated concentrations (nmol/ml)								
Mean ($n=5$)	31.97	15.94	8.15	4.03	2.02	0.98	0.1371	0.0029	0.9921
S.D.	0.67	0.28	0.21	0.20	0.07	0.08	0.0123	//	//
Precision (C.V.%)	2.10	1.76	2.58	4.96	3.47	8.16	8.97	//	//
Accuracy (%)	−0.09	−0.38	+1.88	+0.75	+1.00	−2.00	//	//	//

Propionylcarnitine	Nominal concentrations (nmol/ml)						Calibration curve parameters		
	8	4	2	1	0.5	0.25	Slope	Intercept	r^2
	Back-calculated concentrations (nmol/ml)								
Mean ($n=5$)	7.93	4.07	2.00	1.03	0.51	0.24	0.0950	0.0080	0.9888
S.D.	0.19	0.14	0.09	0.06	0.04	0.03	0.0133	//	//
Precision (C.V.%)	2.40	3.44	4.50	5.83	7.84	12.50	13.99	//	//
Accuracy (%)	−0.88	+1.75	0.00	+3.00	+2.00	−4.00	//	//	//

Intra- and inter-day reproducibility

		Carnitine	acetylcarnitine	propionylcarnitine
Intra-day ($n=6$)	Precision	≤8.5%	≤8.5%	≤8.5%
	Accuracy	−1.32/0.85%	−5.11/0.71%	−8.89/1.13%
Inter-day ($n=5$)	Precision	≤4.7%	≤8.2%	≤12.5%
	Accuracy	−2.40/2.48%	−2.00/1.88%	−4.00/1.75%

being replaced by silica columns giving better resolution and sharper peaks.

2.2. GC approaches

Carnitine and its esters are highly polar substances which cannot volatilize. Efforts have nevertheless been made to assay these substances by GC, with a view to detecting one of their thermic decomposition products. These molecules are in fact unstable at relatively high temperatures, thus producing the β-oxybutyrolactone ring plus trimethylamine, crotonoylbetaine and the desmethyl moiety (Fig. 6). Carnitine seems to be more stable than acetylcarnitine and the latter more stable than propionylcarnitine.

Lewin et al. [31] suggested that carnitine should be decomposed at 160°C in the presence of NaOH

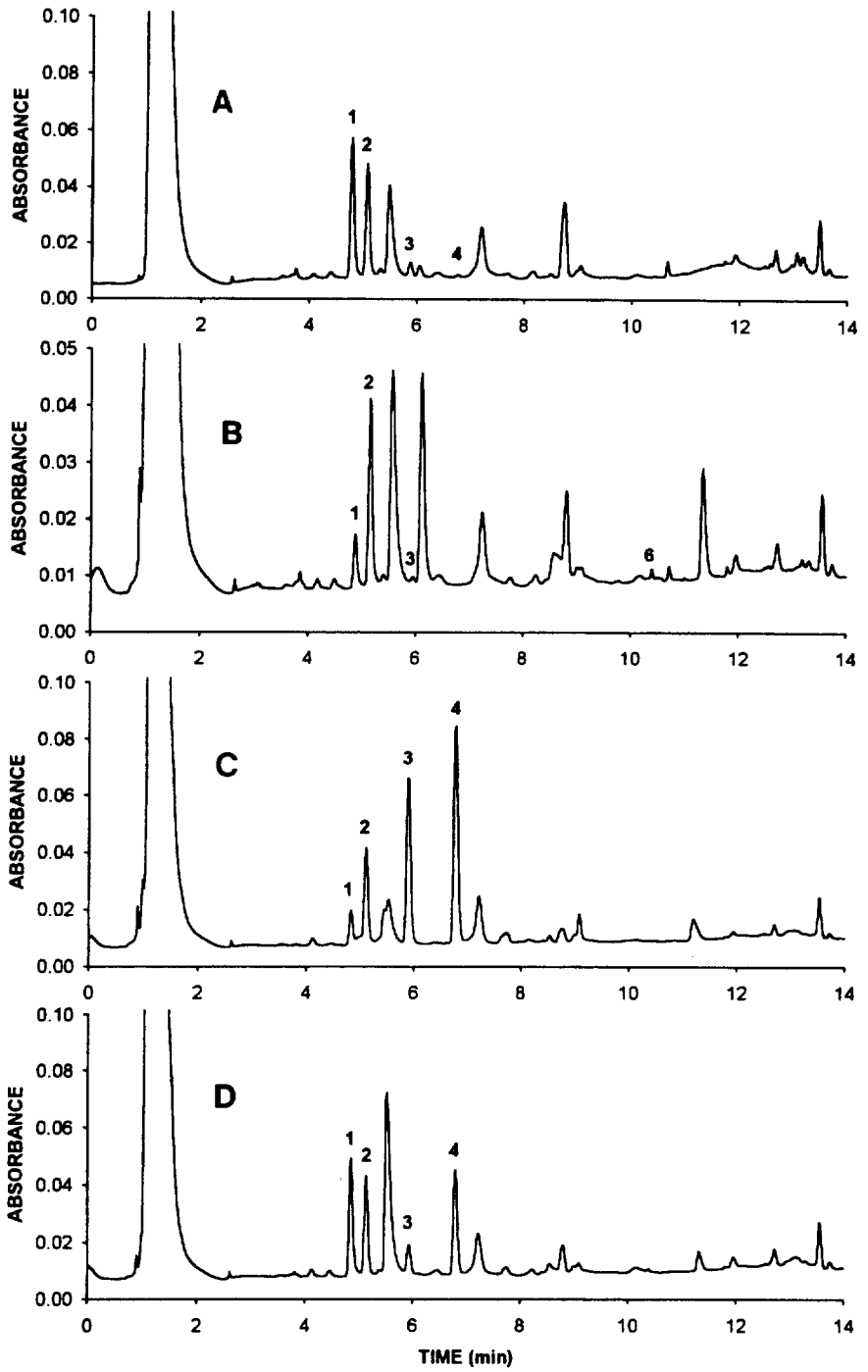

Fig. 4. HPLC assay of free L-carnitine and individual acylcarnitine esters in human plasma. (A) Normal human adult; (B) patient with medium-chain acyl carnitine deficiency; (C) patient with propionic acidemia; (D) patient with methylmalonic aciduria. Peaks are as follows: (1) carnitine, (2) e-carnitine (I.S.), (3) acetylcarnitine, (4) propionylcarnitine, (5) hexanoylcarnitine (peak missing) and (6) octanoylcarnitine. For analytical details see Table 4 (from Minkler and Hoppel, Ref. [28]).

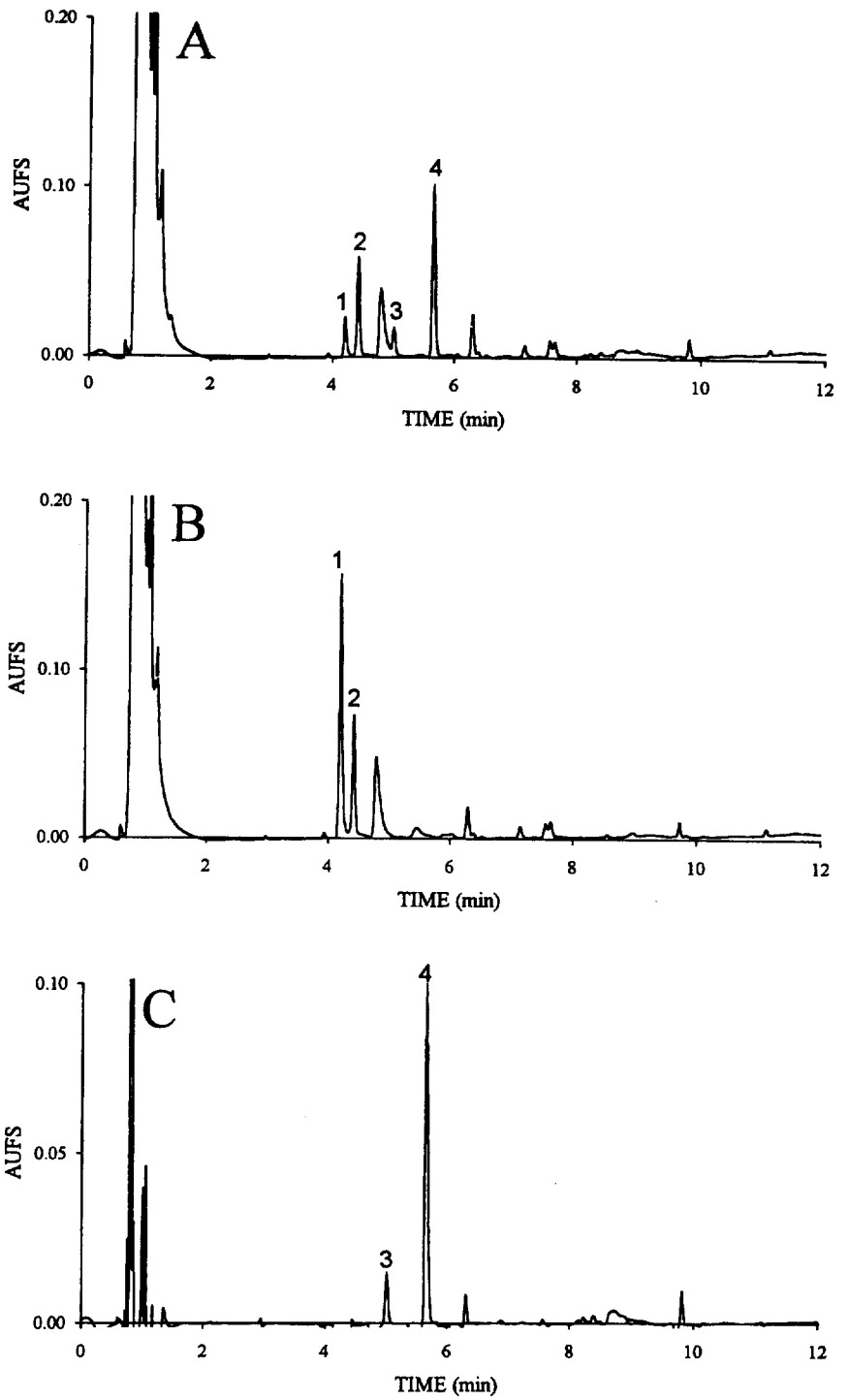

Fig. 5. HPLC assay of free carnitine and individual carnitine esters in urine obtained from a 2-year-old female patient suffering from methylmalonic aciduria. (1) Carnitine, (2) e-carnitine (I.S.), (3) acetylcarnitine, (4) priopionylcarnitine. (A) Acylcarnitines, including free carnitine, (B) total carnitine, (C) chromatogram A subtracted from chromatogram B. For analytical details see Table 4 (from Minkler and Hoppel, Ref. [27]).

Table 4
Main characteristics of the HPLC method with UV detection used to assay carnitine, its esters, the main metabolites and the metabolic precursor (from Refs. [26–28])

Analytes:	Carnitine, acetylcarnitine, propionylcarnitine, hexanoylcarnitine, octanoylcarnitine, betaine, γ-butyrobetaine, trimethyllysine, I.S.
Matrices:	Plasma (100 μl), urine (10–25 μl)
Derivatization:	4′-bromophenacyl trifluoromethanesulfonate
Analytical system:	HPLC with UV absorbance detection, 260 nm
Column:	RP C8 100×4.6 mm I.D., 3 μm
Mobile phase:	Quaternary gradient elution: (A) acetonitrile (800 ml) and water (200 ml); (B) acetonitrile (200 ml) and water (800 ml); (C) triethylamine (5 ml), phosphoric acid (4 ml), acetonitrile (200 ml) and water (800 ml); (D) triethylamine (2.5 ml), phosphoric acid (2ml), acetonitrile (900 ml) and water (100 ml)

Time program:

Time (min)	A (%)	B (%)	C (%)	D (%)
0.0	100	0.0	0.0	0.0
0.19	100	0.0	0.0	0.0
0.20	0.0	100	0.0	0.0
0.99	0.0	100	0.0	0.0
1.00	0.0	0.0	100	0.0
10.00	0.0	0.0	0.0	100
11.00	0.0	0.0	0.0	100
11.01	100	0.0	0.0	0.0

Flow-rate:	1.75 ml/min
Retention time (min):	Carnitine, 4.5; acetylcarnitine, 6; propionylcarnitine, 6.5; hexanoylcarnitine, 9; and octanoylcarnitine, 10
Quantification:	By the use of an I.S., namely 4-(*N*,*N*-dimethyl-*N*-ethylammonio)-3-hydroxybutanoate
Duration:	One assay takes about 8 min
Quantification limit:	Plasma: 5 nmol/ml for carnitine, 2.5 nmol/ml for acylcarnitines; Urine: 10 nmol/ml for all the analytes
Enantioselectivity:	No
Applications:	Biochemistry and pharmacokinetics

and $NaBH_4$ to give butyrolactone which is analysed as an expression of carnitine.

More recently Huang et al. [32] have used the *N*-desmethylated moiety in an attempt to assay carnitine and its esters. Lowes and Rose [33] have used the β-oxy(or acyloxy)-butyrolactone ring. When combined with mass spectrometry, the above approaches possess high sensitivity and selectivity, but lack reproducibility, as thermic decomposition occurs in the various directions shown in Fig. 6 [17].

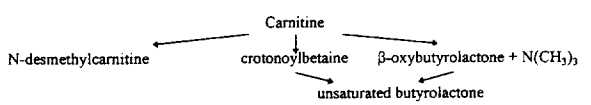

Fig. 6. Thermic decomposition of carnitine.

2.3. Tandem mass spectrometry

2.3.1. Description of assay

In the 1980s Millington and co-workers [34–37] developed mass spectrometry methods to assay carnitine and acylcarnitines, the most successful being the combination of fast atom bombardment (FAB) ionization with tandem quadrupole mass spectrometry. Esterification of the carboxylic group and the addition of an ion-pairing reagent, namely sodium octylsulfate (1%, v/v) to the FAB matrix enhances the sensitivity and selectivity of the assay. With tandem quadrupole mass spectrometry, the collision-induced dissociation of acylcarnitine methyl ester cations produces a fragment at m/z 99, derived from the loss of both the trimethylamino group and the acyl group of the analytes. The first quadrupole scans ions in the range 200–450 m/z and the third is

set at 99 m/z. This analysis gives a spectrum of the molecular ions of acylcarnitines.

2.3.2. Specific features

Using substances labelled with stable isotopes (^{3}H, ^{13}C) this method can differentiate the endogenous pool from their components administered exogenously. This is of great interest in pharmacokinetics, bioavailability and toxicokinetics, mainly when exogenous substances may affect endogenous synthesis and thus decrease the baseline.

Apart from esterification of the carboxylic group, no derivatization is needed in this assay. Sensitivity and specificity are excellent. Starting from a drop of blood, plasma or urine, the whole contents of carnitine and its acylesters can be depicted both qualitatively and quantitatively. Only a few minutes are required to assay a sample, which allows the analyst to process several hundreds of samples in a single day with an automated procedure. Fig. 7 gives typical recordings of analyses of acylcarnitines in urine with this method. Table 5 summarizes the main features of this method.

2.4. Enzyme and radioenzyme methods

2.4.1. L-Carnitine and acetyl-L-carnitine assay

Enzyme and radioenzyme methods to assay L-carnitine and acetyl-L-carnitine are extensively described in previous papers [4,15]. Although these are not chromatographic methods, they must be mentioned as they were and still are employed to assay these compounds in various biological matrices.

These methods are strictly enantioselective and highly specific and sensitive [15]. Possible interference from citrate in acetyl-L-carnitine assay is avoided as suggested by Cooper et al. [38], while interference from heparin can also be avoided, as recently stated by Marzo et al. [39]. Most authors who have published papers on the pharmacokinetic and biochemical behaviour of L-carnitine and acetyl-L-carnitine have used these methods. Alkaline hydrolysis of L-carnitine esters transforms the whole pool into free L-carnitine, which can be assayed as an expression of the whole pool. An assay of the whole endogenous pool is mandatory for endogenous substances, as pointed out by Marzo and Rescigno [13] and by Marzo [5] in two recent reviews. In any case, these assays are easy to perform and have a medium to low cost. Working in duplicate, an analytical series can contain seven samples of the calibration curve, three QCs and 100 unknown samples, covering a total of 220 individual samples. Table 6 summarizes the main features of the radioenzyme method used to assay free and total L-carnitine and acetyl-L-carnitine.

2.4.2. The radioexchange method

The above enzyme methods cannot assay L-carnitine esters with fatty acids that are longer than two carbon atoms. Kerner and Bieber [40] and Di Lisa et al. [41] have proposed a highly sensitive radioisotopic method which enables short-chain acylcarnitines to be identified and assayed. An aliquot of the sample containing a pool of free L-carnitine and its short-chain esters is incubated with labelled L-carnitine in the presence of CoASH and carnitine acetyltransferase, which catalyses an isotopic equilibrium resulting in general labelling of L-carnitine esters. L-Carnitine and its esters are then separated by HPLC or TLC and assayed. Fig. 8 shows the HPLC separation of a series of L-carnitine esters obtained with this method.

This method has recently been the subject of an interesting debate among different teams of investigators. Schmidt-Sommerfeld and Penn [42] and Schmidt-Sommerfeld et al. [43] have emphasized the advantages of the radioexchange method in measuring small amounts of L-carnitine short- and medium-chain esters in specific clinical investigations. Minkler et al. [44] and Van Kempen and Odle [45] have criticized the above two papers following a comparison with other pre-column derivatization HPLC assays. Some difficulties may in fact be encountered in quantitative assays using the radioexchange method, mainly for pharmacokinetic purposes, as the specific enzyme carnitine acetyl transferase (CAT) could give misleading results that are not fully representative of the acyl-L-carnitine pool, as acyl-L-carnitines could partly be hydrolysed as a result of an artifact during the radioexchange process [37]. The method is rather time-consuming. Due to its high sensitivity, the radioexchange method seems to be adequate for biochemical investigations, mainly when the aim of the study is to define a qualitative and semiquantitative pattern.

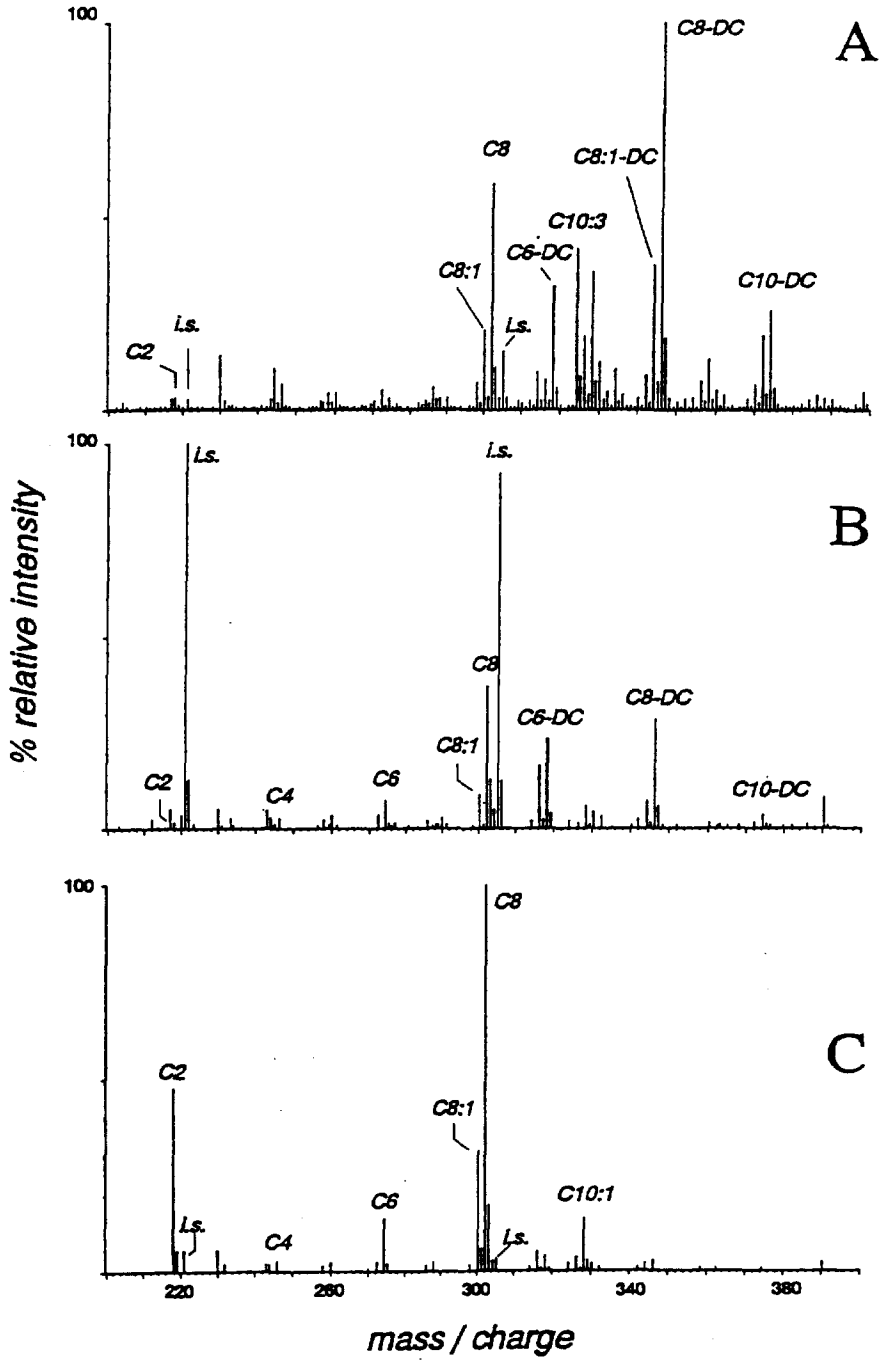

Fig. 7. Analysis of acylcarnitines in urine by tandem mass spectrometry. (A) Normal patient; (B) asymptomatic patient with medium-chain acylcarnitine deficiency; (C) the above patient after L-carnitine bolus (100 mg/kg). Peaks in the precursor ion spectra represent the intact M^+ ion of acylcarnitine methyl esters in increasing order of their molecular weights. The peaks labeled 'i.s.' are the internal standards. For analytical details see Table 5 (from Millington et al., Ref. [35]).

Table 5
Main characteristics of the tandem mass spectrometry method used to assay carnitine esters (from Ref. [35])

Analytes:	Carnitine and acylcarnitines
Matrices:	Plasma, serum, whole blood and urine, the volume of the matrix being one drop (25 μl)
Derivatization:	3 *M* HCl in anhydrous methanol to give methyl esters
Analytical system:	The combination of fast atom bombardment ionization with tandem quadruple mass spectrometry
Quantification:	By the use of deuterated I.S.
Duration:	All the analytes can be assayed in less than 3 min
Quantification limit:	0.1 nmol/ml with each analyte
Enantioselectivity:	No
Specificity:	This method cannot distinguish geometrical isomers (e.g. valerylcarnitine and isovalerylcarnitine) without previous HPLC separation
Applications:	Biochemistry and pharmacokinetics

Table 6
Main characteristics of the radioenzyme method used to assay free and total L-carnitine and acetyl-L-carnitine (from Refs. [4,15])

Analytes:	Free and total L-carnitine and acetyl-L-carnitine. Total L-carnitine is assayed after alkaline hydrolysis of the L-carnitine moiety.
Matrices:	Plasma (200 μl), urine (100 μl), tissue (5–20 mg), cerebrospinal fluid (200 μl)
Reaction:	Labelled acetyl-*S*-CoA for the assay of L-carnitine which reacts to form labelled acetyl-L-carnitine which is assayed as an expression of L-carnitine; labelled oxaloacetate for the assay of acetyl-L-carnitine in a three-reaction cascade
Analytical system:	β-Scintillation counting
Quantification:	By the channel ratio method or external standardization
Duration:	The assay of free and total L-carnitine and acetyl-L-carnitine in duplicate in 100 unknown samples takes 1 day
Quantification limit:	1 nmol/ml
Enantioselectivity:	Only L-enantiomers of the analytes are involved in the assay
Applications:	Pharmacokinetics, toxicokinetics and biochemistry

2.5. Methods for pharmaceutical applications

Carnitine and its individual *O*-acylesters can be separated by HPLC and quantified by UV detection at 205 nm without any derivatization. Using an NH_2 column, elution is inversely related to the length of the acyl group (Fig. 9), whereas this correlation is

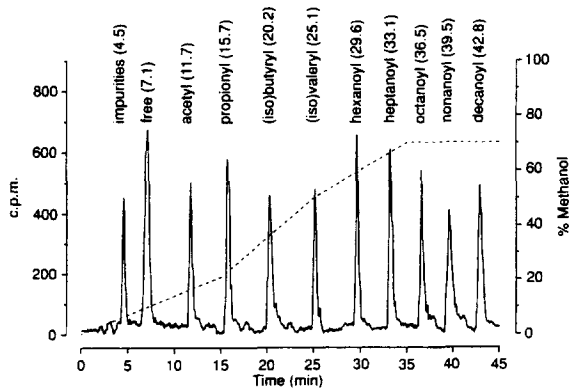

Fig. 8. Recording of short-chain and medium-chain L-carnitine esters separated and assayed by HPLC, according to the radioexchange method. Dashed line represents the percentage of methanol in the solvent system (from Di Lisa et al., Ref. [41]).

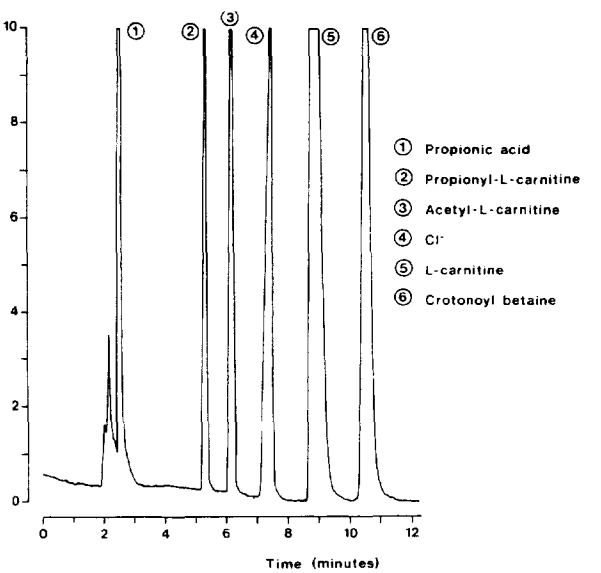

Fig. 9. Typical HPLC recording of carnitine, acetylcarnitine, and propionylcarnitine. Propionic acid, the ion Cl^- and crotonoylbetaine are also eluted and detected. A Supelcosil NH_2 (5 μm) column (250×4.6 mm I.D.) and detection at 205 nm were used. For analytical details see Table 7 (from Marzo et al., Ref. [17]).

Table 7
Main characteristics of the HPLC assay of carnitine, its esters, Cl^- and crotonoylbetaine for pharmaceutical applications (from Ref. [4])

Analytes:	Carnitine, individual short-chain acylcarnitines, chloride ion and some their impurities (e.g., acetic acid, propionic acid and crotonoylbetaine)
Matrices:	Raw materials and pharmaceutical products
Derivatization:	No
Analytical system:	HPLC with UV absorbance detection, 205 nm
Column:	NH_2 250×4.6 mm I.D., 5 μm
Mobile phase:	50 m*M* KH_2PO_4 pH 4.5 and acetonitrile (35:65, v/v)
Flow-rate:	1.5 ml/min
Retention times (min):	Propionic acid, 2.5; propionylcarnitine, 5.3; acetylcarnitine, 6; Cl^- ion, 6.4; L-carnitine, 8.8; and crotonoylbetaine, 10.8
Quantification:	By external standardization
Duration:	<10 min
Quantification limit:	200 ng
Enantioselectivity:	No
Applications:	Pharmaceutics

inverted when a reversed-phase column is used. High specificity allows this method to be the first choice as a routine assay in the pharmaceutical field [4]. Table 7 summarizes the main characteristics of the HPLC assay of carnitine and its esters.

The enantiomeric purity of L-carnitine was investigated in 1987 by nuclear magnetic resonance spectroscopy with chiral shift reagents allowing L/D-carnitine and acetylcarnitine to be enantioselectively evaluated [46]. This method has also been applied to D/L-propionylcarnitine using [Eu (hfc)$_3$] as a chiral shift reagent [4]. More recently this problem was solved with a general method which works very well in HPLC and in capillary electrophoresis (CE) [16]. This method is based on pre-column derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate [(+)FLEC] to produce a diastereomeric derivative which can be analysed both by UV absorbence and fluorescent detection (Fig. 10). Figs. 11 and 12 depict typical recordings of the separation and assay of D- and L-diastereomeric derivatives of carnitine by HPLC and capillary electrophoresis, respectively. Although this method derivatizes carnitine on the OH group, it can be applied to any *O*-acylester of carnitine which can first be hydrolysed to free carnitine [17] and then derivatized with (+)(FLEC). This procedure is fully acceptable in pharmaceutics when the enantiomeric purity of the L-enantiomer of a given component of L-carnitine family needs to be ascertained. This method has been fully validated in the range 0.2–10% of D-carnitine added to pure L-carnitine [16]. Table 8 summarizes the main fea-

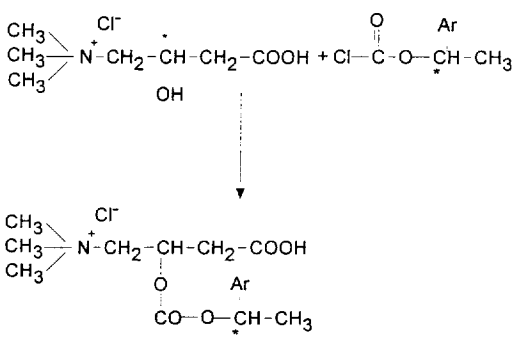

Fig. 10. Scheme of carnitine derivatization with (+)FLEC leading to diastereomeric derivatives of D- and L-carnitine. Asterisks indicate stereogenic carbon atoms.

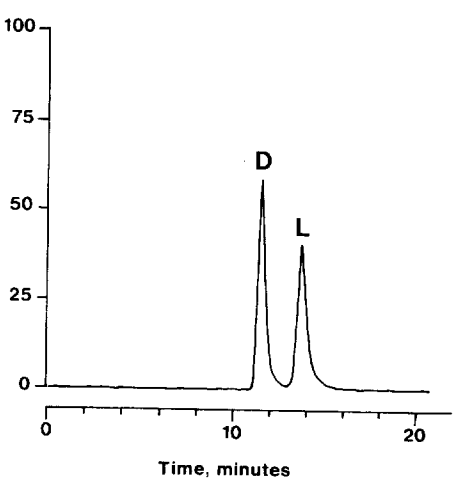

Fig. 11. HPLC separation of (+)FLEC diastereomeric derivatives of D- and L-carnitine. For analytical details see Table 8 (from De Witt et al., Ref. [16]).

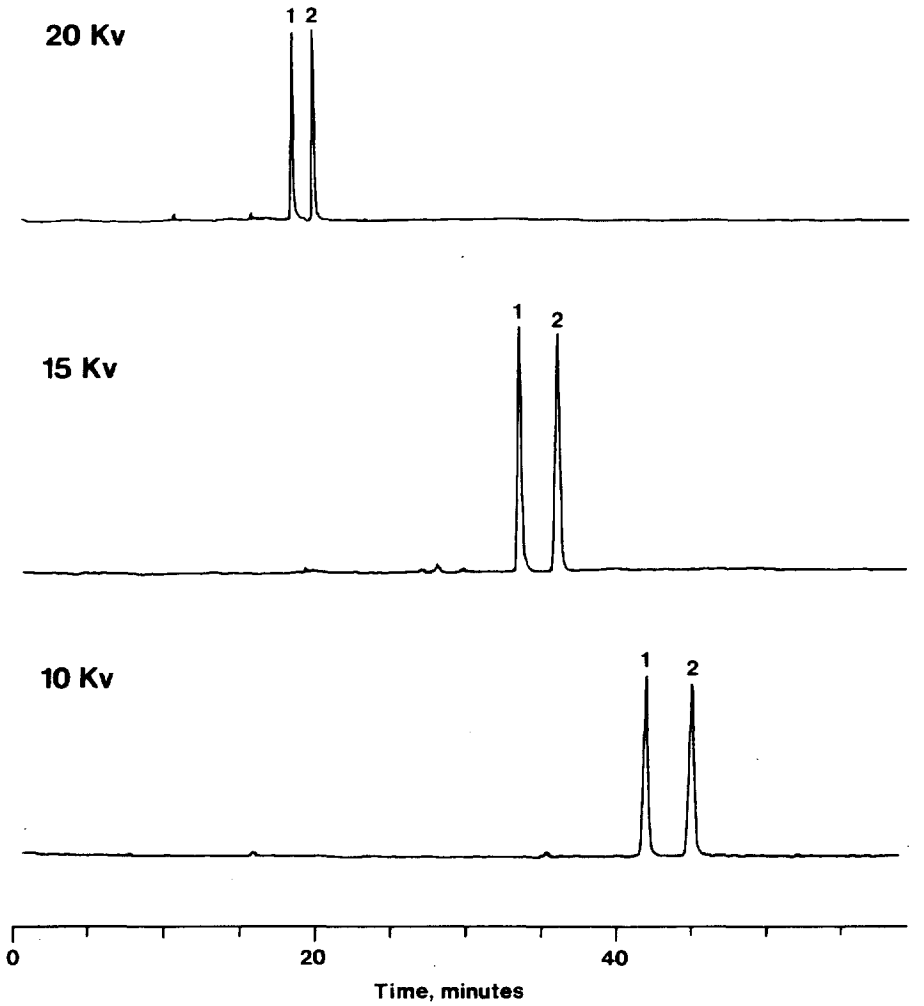

Fig. 12. Capillary electrophoresis separation of (+)(FLEC) diastereomeric derivatives of L- and D-carnitine (peaks 1 and 2, respectively) showing also the effect of increasing voltage from 10 to 20 kV. Conditions are summarized in Table 8 (from De Witt et al., Ref. [16]).

tures of the HPLC and CE enantiospecific assay of carnitine.

2.6. Methods of assaying carnitine metabolites

In the systemic circulation, L-carnitine is reversibly converted into its esters via a mammillary metabolism catalysed by carnitine acyltransferases (Fig. 1). No other systemic metabolic pathways have been described for L-carnitine moiety [7,8,11,12,15,47]. However, intestinal microorganisms extensively metabolize carnitine to γ-butyrobetaine and to (acyl)oxybutyrolactone and trimethylamine (TMA) [5,19]. TMA is rapidly absorbed and oxidized to its *N*-oxide (TMA-*N*-oxide) in the liver as a result of first-pass detoxification metabolism [48,49]. This process can however be saturated, leading to other metabolic features, like *C*-oxidation to dimethylamine (DMA) and monomethylamine (MMA); DMA can be further nitrosated (Fig. 13). TMA oxidation to TMA-*N*-oxide has proved in fact to be a process under genetic polymorphism [50–54]. In poor metabolizers the oxidation step could became saturated. This could also occur at high oral doses, e.g., in toxicokinetic studies, or in patients suffering from cirrhosis who have a reduced liver

Table 8
Main characteristics of the HPLC (a) or capillary electrophoresis (b) enentioselective assay of L- and D-enantiomers of carnitine (LC, DC) and carnitine esters for pharmaceutical applications (from Ref [16])

Analytes:	L-Carnitine and D-carnitine, L- and D-acylcarnitines
Matrices:	Raw material and pharmaceutical products
Derivatization:	(+)-1-(9-Fluorenyl) ethyl chloroformate [(+)FLEC] reacts with OH group leading to diastereomeric derivatives. Acylesters of carnitine need to have been previously hydrolyzed to free carnitine before derivatization.
Analytical system:	(a) UV absorbance (260 nm) or fluorescence detector (λ_{ex}=260 nm; λ_{em}=315 nm); (b) UV absorbance detector (214 nm)
Column:	(a) RP C18 150×3.9 mm I.D., 4 μm; (b) Fused-silica capillary (50 μm diameter, 60 cm length)
Mobile phase:	(a) Binary gradient elution:solvent A: 25% acetonitrile and 75% of an aqueous solution of 5 m*M* TBA^+ OH^- and 50 m*M* KH_2PO_4 (pH 7); solvent B: 75% acetonitrile and 25% of an aqueous solution of 5 m*M* KH_2PO_4

Time program:

Time (min)	Flow (ml/min)	Solvent A (%)	Solvent B (%)
0.00	0.75	100	0.0
20.00	0.75	100	0.0
22.00	1.00	0.0	100
35.00	1.00	0.0	100
36.00	1.00	100	0.0
40.00	1.00	100	0.0

	(b) 50 m*M* KH_2PO_4 pH 3.4; the voltage applied is 14 kV
Retention time:	(a) DC=12 min, LC=14 min; (b) LC=37 min, DC=39 min
Quantification:	Through external standardization
Duration:	(a) ≅15 min; (b) ≅40 min
Enantioselectivity:	Yes
Applications:	Pharmaceutics

function. This metabolic behaviour is common to all trimethylalkylammonium compounds, such as betaine, choline, citicoline, carnitine and its acylesters, which are poorly absorbed due to their ionic strength and thus largely undergo this metabolic behaviour [5,55–64]. This degradation can also occur from environmental sources and can affect people working with soya beans, lecithin or choline. The highly volatile TMA can easily be breathed in and absorbed into the systemic circulation without meeting liver first-pass metabolism. TMA is also present in fish as a result of the above bacterial degradation, and is then ingested and absorbed by humans feeding on the fish. The determination of TMA, DMA (as such and nitrosated) and MMA is thus of primary interest in environmental toxicology and in toxicokinetics [5], while the quantification of TMA-*N*-oxide is of significant interest in pharmacokinetics and toxicokinetics.

Assays of TMA, DMA and MMA are very simple to carry out when conducted as described by Marzo et al. in 1990 [65]. Fig. 14 shows GC separation of TMA, DMA and MMA using monoisopropylamine (MIPA) as the internal standard in GC analysis. When GC analysis is used, it should be recalled that trimethylalkylammonium derivatives in the injector are in part transformed into TMA, producing an artifact. Thus head-space or other similar techniques must be used.

The analysis of TMA-*N*-oxide is very complex, as pointed out by Marzo et al. [65]. It does not in fact volatilize, so it cannot be analysed by GC, and is completely undetectable by the most common HPLC detectors, such as UV absorbence, conductimetric, fluorimetric, refractive index and electrochemical detectors. The assay must thus be carried out after it is chemically reduced to TMA. The first approach of Lin and Hurng [66] consisted of using 0.3% $TiCl_3$ in 10% (w/v) hydrochloric acid at 80°C as a reducing agent followed by a spectrophotometric assay. In our hands, this method produced TMA, large amounts of DMA and smaller amounts of MMA, which were analysed in the GC assay. The method proposed by Maccari et al. [67] consists of chemically reducing

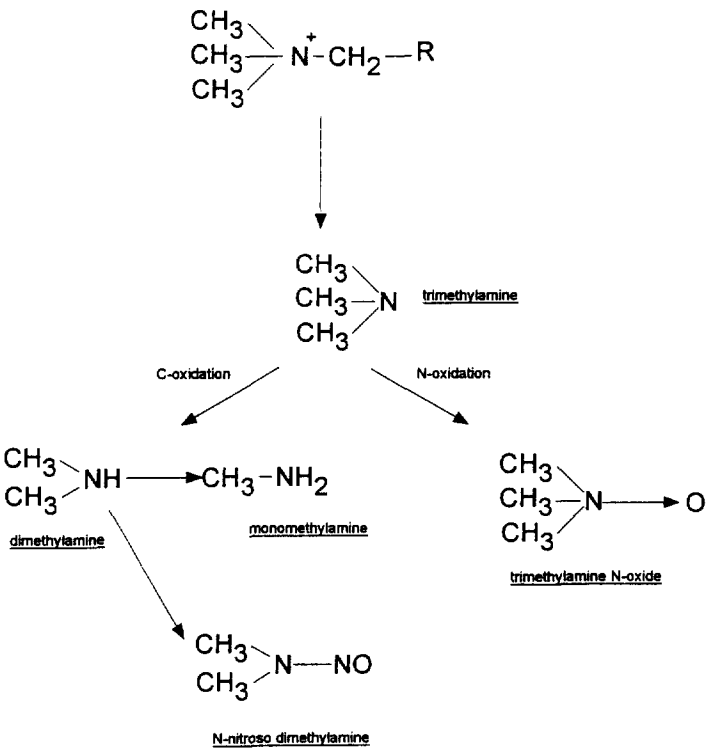

Fig. 13. Metabolic cascade of trimethylamine produced by microbic metabolism of trimethylalkylammonium compounds and further hepatic biotransformation to *N*-oxide and *N*-nitroso derivatives.

TMA-*N*-oxide to TMA by $TiCl_3$ followed by a GC analysis of the amine. However, this procedure does not overcome the simultaneous production of DMA and MMA. The method of Marzo et al. [65] consists of reducing TMA-*N*-oxide by hydrogen–palladium with a 5% Pd on charcoal catalyst, under a hydrogen flow at 100 ml/min for 30 min at 30°C. The gases are trapped in a carbotrap tube using a dynamic thermal stripper. TMA, as a reduction product of TMA-*N*-oxide, is transferred to the thermal unit to desorb the trapped compound into the GC column. TMA must be assayed before and after the reduction, the difference representing the TMA-*N*-oxide originally present. No DMA or MMA are produced with this method. Table 9 depicts the main features of the assay of TMA, DMA, MMA and TMA-*N*-oxide by GC. A similar approach was published in 1991 by Tjoa and Fennessey consisting of a chemical reduction of TMA-*N*-oxide promoted by zinc metal in powder form [68].

N-Nitroso compounds are usually assayed as pollutants, in beverages and in biological fluids with a thermal energy analyzer (TEA), which is a gas-phase technique set up in the 1970s by Fine et al. [69–71]. The *N*-nitroso derivative is placed in a catalytic flash heater where the N–NO bond is broken to release the nitrosyl radical $ON^{\bullet}$. This is oxidised with ozone to give electronically excited nitrogen dioxide, which in turn decays back to its ground state with the emission of characteristic radiation. A sensitive photomultiplier tube detects the emission intensity which is proportional to the nitrosyl radical concentration and thus to the *N*-nitroso derivative concentration. TEA has been used to assay *N*-nitroso derivatives, both volatile and non-volatile, in GC and HPLC analysis [72]. This detector has also been proposed for assaying plasma concentrations of organic nitrates following HPLC separation [73]. Compounds assayed with this method include nitroso derivatives of dimethylamine,

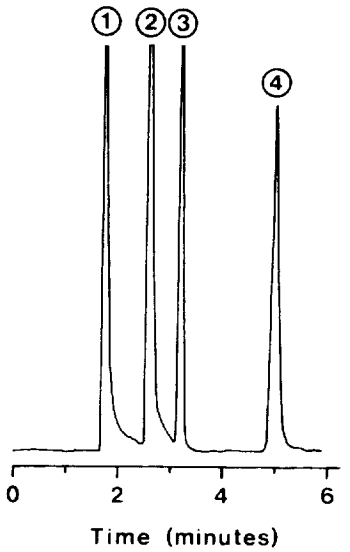

Fig. 14. Gas chromatographic separation of TMA, DMA and MMA. Peaks: (1) monomethylamine; (2) dimethylamine; (3) trimethylamine; (4) I.S. (monoisopropylamine). For analytical details see Table 9 (from Marzo et al., Ref. [65]).

diethylamine, dipropylamine, dibutylamine, piperidine, pyrrolidine, morpholine, etc. [72], which in GC can be detected at concentrations of ≤ 1 ng/ml.

3. Conclusions

This review is addressed to scientists working on the analysis of the L-carnitine family and aims to facilitate their analytical choices.

The authors have considered the most routinely employed analytical methods to investigate carnitine and its esters belonging to the endogenous pool, along with pre-systemic and systemic metabolites and likely impurities and have discussed their specific capabilities on the basis of the literature and their own experience.

Direct HPLC analysis of carnitine and its esters without any derivatization can solve pharmaceutical problems. A pre-column derivatization with (+)(FLEC) leads to diastereoisomers of L- and D-carnitine, allowing the enantiomeric excess to be evaluated up to 0.2% of the D isomer, by both HPLC or capillary electrophoresis.

In pharmacokinetics and toxicokinetics the enantioselective radioenzyme assay solves the problem of free and total L-carnitine, and acetyl-L-carnitine. Assays of these and higher esters, e.g., propionyl-L-carnitine, can be carried out by HPLC through precolumn derivatization for UV or fluorimetric detection or by tandem mass spectrometry. However, these approaches do not distinguish L- and D-enantiomers and thus are not enantioselective.

Among the metabolites of the carnitine moiety, γ-butyrobetaine can be assayed by the HPLC method of Minkler and Hoppel [26–28], and amines and TMA-*N*-oxide by the method of Marzo et al. [65] using GC and flame ionisation, or a thermoionic specific detector and *N*-nitroso dimethylamine by the method of Fine et al. [69–71] using a thermal energy analyzer as a detector in GC or HPLC apparatuses.

Several other methods described in the literature and considered in this review are also of interest, mainly for biochemical purposes. However the authors consider them less suitable for pharmacokinetic purposes.

Table 9
Main characteristics of the GC assay of tri-, di- and mono-methylamine: trimethylamine *N*-oxide is assayed with the same method after a catalytic reduction to trimethylamine (from Ref. [65])

Analytes:	Mono-, di- and trimethylamine (MMA, DMA, TMA) and trimethylamine *N*-oxide (TMA-Noxide). TMA-*N*-oxide is assayed after a catalytic reduction to TMA: the difference in TMA after the reduction, less that before, gives the TMA-*N*-oxide present
Matrices:	Raw materials, pharmaceutics and biological samples
Chemical reduction of TMA-*N*-oxide:	Hydrogen–palladium with 5% Pd on charcoal catalyst, stripped under a flow of hydrogen at 100 ml/min for 30 min at 30°C
Analytical system:	Gas chromatography with flame ionization detection (FID) or thermoionic specific detection (TSD)
Column:	Glass column (37.5 cm×2 mm I.D.) packed with 4% Carbowax 20 M+0.8% KOH on Carbopack B (60–80 mesh)
Operating conditions:	Temperature detector: 250°C; Temperature column: 100°C; Temperature injector: 160°C; Carrier gas: nitrogen, at 280 kPa
Retention time (min):	MMA, 1.76; DMA, 2.63; TMA, 3.24; and I.S., 5.07
Quantification:	By the use of I.S. (monoisopropylamine)
Quantification limit:	0.25 ng
Applications:	Pharmaceutics, pharmacokinetics and toxicokinetics

Acknowledgments

The authors are indebted to Dr. Fausta Omodeo-Salè of the Institute of General Physiology and Biological Chemistry, State University, Milan, Italy, for her invaluable suggestions on some of the biochemical aspects considered in this review.

References

[1] J. Bremer, J. Biol. Chem. 237 (1962) 3628.
[2] K.F. Lanoe, A.C. Schoolwerth, Ann. Rev. Biochem. 48 (1979) 871.
[3] I.B. Fritz, K.T.N. Yue, J. Lipid Res. 4 (1963) 279.
[4] A. Marzo, G. Cardace, N. Monti, S. Muck, E. Arrigoni Martelli, J. Chromatogr. 527 (1990) 247.
[5] A. Marzo, Arzneim. Forsch. 46 (1966) 1.
[6] P. Marciani, C. Lindi, A. Marzo, E. Arrigoni Martelli, G. Cardace, G. Esposito, Pharm. Res. 23 (1991) 157.
[7] J.G. Kelly, S. Hunt, G.D. Doyle, M.S. Laher, M. Carmody, A. Marzo, E. Arrigoni Martelli, Eur. J. Clin. Pharmacol. 38 (1990) 309.
[8] A. Marzo, G. Cardace, C. Corbelletta, E. Bassani, E. Morabito, E. Arrigoni Martelli, Eur. J. Drug Metab. Pharmacokin. Special Issue (1993) 269.
[9] A. Marzo, A. Rescigno, E. Arrigoni Martelli, Eur. J. Drug Metab. Pharmacokin. 18 (1993) 215.
[10] A. Marzo, G. Cardace, C. Corbelletta, S. Pace, S. D'Iddio, C. Verzotti, E. Cavatorta, E. Grignaffini, Gynecol. Endocrinol. 8 (1994) 115.
[11] A. Marzo, E. Arrigoni Martelli, R. Urso, M. Rocchetti, V. Rizza, J.G. Kelly, Eur. J. Clin. Pharmacol. 37 (1989) 59.
[12] R. Urso, G. Segre, G. Cardace, A. Marzo, B. Foroni, A. Rescigno, Eur. J. Drug Metab. Pharmacokin Special Issue (1993) 8.
[13] A. Marzo, A. Rescigno, Eur. J. Drug Metab. Pharmacokin. 18 (1993) 77.
[14] A. Marzo, Arzneim. Forsch. 44 (1994) 791.
[15] A. Marzo, G. Cardace, E. Arrigoni Martelli, Chirality 4 (1992) 247.
[16] P. De. Witt, R. Deias, S. Muck, B. Galletti, D. Meloni, P. Celletti, A. Marzo, J. Chromatogr. B 657 (1994) 67.
[17] A. Marzo, N. Monti, M. Ripamonti, E. Arrigoni Martelli, J. Chromatogr. 459 (1988) 313.
[18] G. Bruno, S. Curti, C. Tallarico, A. Marzo, E. Arrigoni Martelli, Sixteenth International and Symposium on Column Liquid Chromatography, Baltimora, June 14–19 (1992), Abstract 331, p. A-78.
[19] C.J. Rebouche, Am. J. Clin. Nutr. 54 (1991) 1147.
[20] G.S. Fraenkel, Arch. Biochem. Biophys. 50 (1954) 486.
[21] S. Friedman, Arch. Biochem. Biophys. 75 (1958) 24.
[22] M.A. Mehlman, G. Wolf, Arch. Biochem. Biophys. 98 (1962) 146.
[23] N.R. Marquis, I.B. Fritz, J. Lipid Res. 5 (1964) 184.
[24] M. Kobayashi, E. Ichishima, Anal. Biochem. 189 (1990) 122.
[25] H. Kamimori, Y. Hamashima, M. Konishi, Anal. Biochem. 218 (1994) 417.
[26] P.E. Minkler, C.L. Hoppel, Clin. Chim. Acta 212 (1992) 55.
[27] P.E. Minkler, C.L. Hoppel, J. Chromatogr. 613 (1993) 203.
[28] P.E. Minkler, C.L. Hoppel, Anal. Biochem. 212 (1993) 510.
[29] P.E. Minkler, E.P. Brass, W.R. Hiatt, S.T. Ingalls, C.L. Hoppel, Anal. Biochem. 231 (1995) 315.
[30] M. Lever, L. Bason, C. Leaver, C.M. Hayman, S.T. Chambers, Anal. Biochem. 205 (1992) 14.
[31] L.M. Lewin, A. Peshin, B. Sklar, Anal. Biochem. 68 (1975) 531.
[32] Z.H. Huang, D.A. Gage, L.L. Bieber, C.C. Sveely, Anal. Biochem. 199 (1991) 98.
[33] S. Lowes, M.E. Rose, Analyst 115 (1990) 511.
[34] D.S. Millington, C.R. Roe, D.A. Maltby, Biomed. Mass Spectrom. 11 (1984) 236.
[35] D.S. Millington, N. Terada, N. Kodo, D.H. Chace, A review: carnitine and acylcarnitine analysis in the diagnosis of metabolic diseases, in: Z. Matsumoto (Ed.), Advantages of Tandem Mass Spectrometry. Advances in the Chemical Diagnosis and Treatment of Metabolic Disorders, vol. 1, John Wiley and Sons, Chichester, UK, 1992, pp. 59–71.
[36] D.S. Millington, D.A. Maltby, D.S. Gole, C.R. Roe, in: T.R. Baillie, J.R. Jones (Eds.), Synthesis and Applications of Isotopically Labelled Compounds, Elsevier, Amsterdam, 1988, pp. 189–194.
[37] D.S. Millington, D.H. Chace, Carnitine and acylcarnitines in metabolic disease diagnosis and management, in: D.M. Desiderio (Ed.), Mass Spectrometry, Clinical and Biomedical Applications, vol. 1, Plenum Press, New York, 1992, pp. 299–317.
[38] M.B. Cooper, C.A. Forte, D.A. Jones, Clin. Chim. Acta 159 (1986) 291.
[39] A. Marzo, G. Cardace, C. Corbelletta, P. Verga, C. Vinciguerra, Arzneim. Forsch. 45 (1995) 1303.
[40] J. Kerner, L.L. Bieber, Anal. Biochem. 134 (1983) 459.
[41] F. Di Lisa, L.L. Bieber, J. Kerner, R. Menabò, R. Barbato, N. Siliprandi, Methods for carnitine assay, in: R. Ferrari, S. Di Mauro, G. Sherwood (Eds.), L-Carnitine and its Role in Medicine: From Function to Therapy, Academic Press, London, 1992, pp. 37–61.
[42] E. Schmidt-Sommerfeld, D.J. Penn, J. Chromatogr. B 652 (1993) 115.
[43] E. Schmidt-Sommerfeld, D.J. Penn, J. Kerner, L.L. Bieber, Clin. Chim. Acta 181 (1989) 231.
[44] P.E. Minkler, S.T. Ingalls, C.L. Hoppel, J. Chromatogr. B 652 (1994) 119.
[45] T. Van Kempen, J. Odle, J. Chromatogr. B 652 (1994) 117.
[46] R. Voeffray, J.C. Perlberger, L. Tenud, Helv. Chim. Acta 70 (1987) 2058.
[47] G. Cardace, C. Corbelletta, P. Verga, E. Bassani, E. Mapelli, S. Pace, B. Foroni, R. Urso, A. Marzo, Eur. J. Drug Metab. Pharmacokin Special Issue (1993) 133.
[48] S. Dechezlepretre, Med. Pharmacol. Exp. 16 (1967) 529.
[49] S. Dechezlepretre, Thèrapie XXIII (1968) 1357.

[50] A. Marzo, L.P. Balant, J. Chromatogr. B 678 (1966) 73.
[51] R. Ayesh, R.L. Smith, Pharmacol. Ther. 45 (1990) 387.
[52] M. Al-Waiz, R. Ayesh, S.C. Mitchell, J.R. Idle, R.L. Smith, Clin. Pharmacol. Ther. 42 (1987) 588.
[53] H.F. Hadidi, S. Cholerton, S. Atkinson, Y.M. Irshaid, N.M. Rawashdeh, J.R. Idle, Br. J. Clin. Pharmacol. 39 (1995) 179.
[54] W. Kalow (Ed.), Pharmacogenetics of Drug Metabolism, Pergamon Press, New York, 1992.
[55] R.R. Scheline, J. Pharm. Sci. 57 (1968) 2021.
[56] R.R. Scheline, Pharmacol. Rev. 25 (1973) 451.
[57] M. Al-Waitz, S.C. Mitchell, J.R. Idle, R.L. Smith, Xenobiotica 17 (1987) 551.
[58] S.H. Zeisel, J.S. Wishnoh, J.K. Blusztayn, J. Pharmacol. Exp. Ther. 225 (1983) 320.
[59] R. Marks, M.W. Greaves, C. Prottey, P.J. Hartop, Br. J. Dermatol. 96 (1977) 399.
[60] E. Strack, H. Seim, Z. Hoppa-Seyler's, Physiol. Chem. 360 (1979) 207.
[61] H. Seim, E. Strack, Hoppa-Seyler's Physiol. Chem. 361 (1980) 1095.
[62] H. Seim, H. Loester, C. Reiner, H.P. Kleber, E. Strack, FEMS Microbiol. Lett. 13 (1982) 201.
[63] A.M. Asatoor, M.L. Simenhoff, Biochim. Biophys. Acta 111 (1965) 384.
[64] T.J. Davies, J. Chromatogr. 307 (1984) 11.
[65] A. Marzo, N. Monti, M. Ripamonti, S. Muck, E. Arrigoni Martelli, J. Chromatogr. 507 (1990) 241.
[66] J.K. Lin, D.C. Hurng, Food Chem. Toxicol. 23 (1985) 579.
[67] F. Maccari, L. Ferrari, P. Pessotto, M.T. Ramacci, J. Chromatogr. 532 (1990) 124.
[68] S. Tjoa, P. Fennessey, Anal. Biochem. 197 (1991) 77.
[69] D.H. Fine, F. Rufeh, B. Gunther, Anal. Lett. 6 (1973) 731.
[70] D.H. Fine, F. Rufeh, D. Lieb, Nature 247 (1974) 309.
[71] D.H. Fine, D. Lieb, F. Rufeh, J. Chromatogr. 107 (1975) 351.
[72] C. Rühl, J. Reusch, J. Chromatogr. 328 (1985) 362.
[73] J. Maddock, P.A. Lewis, A. Woodward, P.R. Massey, S. Kennedy, J. Chromatogr. 272 (1983) 129.