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Determination of Urinary Acylcarnitines by ESI-MS coupled with Solid-phase Microextraction (SPME)

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The determination of selected short-, medium- and long-chain acylcarnitines by electrospray ionization mass spectrometry (ESI-MS) is discussed. The differences in fragmentation behaviour and ionization efficiency are described in dependence on collision induced dissociation (CID) conditions and mixture composition. A new method combination, solid-phase microextraction (SPME)-ESI-MS, is introduced to characterize acylcarnitines in body fluids. This method utilizes SPME for pre-concentration of acylcarnitines from complex biological samples and ESI-MS for a selective and sensitive detection. The method is presented by standard experiments determining of acylcarnitines in aqueous solutions and urine samples from patients with secondary carnitine deficiency syndromes or related disorders. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Carnitine (3-hydxroxy-4-N-trimethylammonium butyrate) is an essential factor in the fatty acid metabolism of organisms. It causes the penetration of long-chain fatty acids through the inner membrane of mitochondria where the β-oxidation of fatty acids occurs. The penetration of fatty acids is realized by the 'carnitine shuttle system,' which includes the enzymes carnitine palmitoyltransferase I and II and the carnitine–acylcarnitine translocase. The enzyme systems regulate the formation of acylcarnitines and their transport through the inner membrane of the mitochondria. The β-oxidation occurs after transesterification of the acylcarnitine via long-chain acyl-CoA (carnitine palmitoyltransferase II) to acetyl-CoA, which is metabolized for ATP production.[1–4]

Numerous disorders in this system have been described, especially carnitine deficiency syndromes,[5,6] which lead to disturbances in energy production and in the intermediary metabolism of the organism. Many of them are disorders in cardiac metabolism. Others are disturbances in the production of unusual acylcarnitines which are excreted and lead to the so-called 'organoacidurias.'[7,8] The individual pattern of such unusual acylcarnitines can be of diagnostic and therapeutic importance.[9,10] The diseases will be manifested in childhood and have severe consequences for these patients because of their neurological symptoms.[11,12]

The betaine structure of carnitine requires some effort for detection in biological tissues. The current methods employed for the determination of free carnitine and its esters involve spectrophotometric or radioenzymatic assays in various modifications using [acetyl-1-^{14}C]-CoA.[13–15] Among the often discussed high-performance liquid chromatographic (HPLC) procedures[16–18] or capillary electrophoresis[19] with pre-derivatization of the carnitine and acylcarnitine species are combinations with other methods, including mass spectrometry (MS). Since conventional coupled MS techniques such as gas chromatography/MS[20,21] are of limited use owing to the ionic nature and the lack of volatility of the carnitine compounds, some alternative MS approaches for carnitine determination have been examined. Fast atom bombardment (FAB) MS,[22–25] desorption chemical ionization MS,[25] atmospheric pressure ionization MS[26,27] and matrix-assisted laser desorption/ionization MS[27] require a minimum of sample preparation. Thermospray MS[28] in combination with LC using a resin-based stationary phase was shown to be suitable for the determination of quaternary ammonium drugs. The reported sensitivities were comparable to those of radioenzymatic assays. Nevertheless, the interpretation of the mass spectra of complex biological mixtures is sometimes difficult and suffers to a certain extent from matrix interference and signal suppression caused by accompanying substances. Examples of the important influence of biological matrices on the results of the ESI-MS analysis of acylcarnitines have been reported

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by Kelly et al.[26] In order to overcome problems caused by matrix influences in blood and plasma analysis, the diagnostic capabilities of electrospray tandem MS techniques were demonstrated to be a useful tool in the diagnosis of organic acidaemias.[29,30]

This paper describes the ESI-MS determination of acylcarnitines present in various matrices and the combination with solid-phase microextraction (SPME) for biological applications. SPME is used for the rapid and selective separation and pre-concentration of acylcarnitines from water, urine and blood plasma samples followed by direct ESI-MS (without any chromatographic pre-separation).

The solvent-free microextraction technique developed by Arthur and Pawliszyn[31] was mainly used for environmental analysis of aqueous systems. The first application of an SPME/HPLC interface[32] dealt with the analysis of polycyclic aromatic hydrocarbons. Also in coupling with HPLC, the advantages of SPME as a fast, solvent-free and inexpensive technique are maintained and the application range of SPME could be extended to the determination of semi-and non-volatile organic compounds in aqueous systems, a problem that is difficult to solve with gas chromatography.

EXPERIMENTAL

Materials

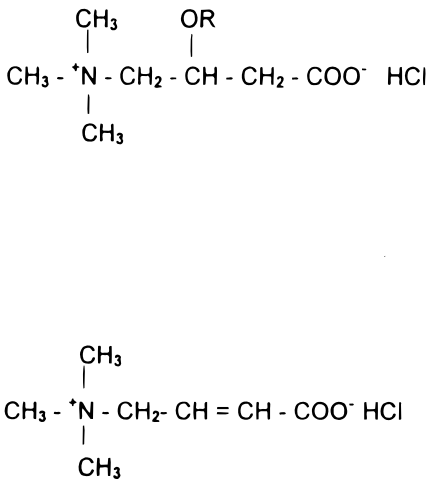
The acylcarnitine hydrochlorides and crotonobetaine shown in Fig. 1 were prepared by Löster and Müller[33] and used as reference compounds. Thin-layer chromatography was used to check the purity of the acylcarnitines synthesized. Individual stock standard solutions containing 100 μg ml^{-1} of each acylcarnitine were prepared by dissolving the pure compounds in methanol purchased from Merck (Darmstadt, Germany). The SPME fibres adjusted for the extraction of the acylcarnitines were coated with Carbowax of 65 μm thickness and also with poly(dimethylsiloxane) as a 100 μm film, both supplied by Supelco (Deisenhofen, Germany). The syringe devices were conditioned prior to use in acidic methanol (pH 3, adjusted with hydrochloric acid) at ambient temperature for 1 h. Before each analysis, noise and carryover of the fibre used were checked with blanks.

Calibration

The stock standard solutions of acylcarnitines were diluted with methanol by factors ranging from 10 to 1000. The series of dilutions were used to determine the detection limits of the individual acylcarnitines. Furthermore, defined amounts of the individual stock standard solutions were mixed to obtain a stock standard solution of the acylcarnitine mixture applied for comparative studies. Dilution with methanol led to further working standard solutions of the mixed acylcarnitines in the concentration range from μg ml^{-1} to ng ml^{-1} used for the determination of detection limits of the acylcarnitines in mixtures. The calibration for loop injection analysis was achieved by measuring five replicates at five different concentrations. The slopes and intercepts of the calibration graphs were obtained when the amounts injected were plotted against the area counts.

For calibration of SPME experiments, microlitre amounts of the stock acylcarnitine mixture mentioned above were spiked into 4 ml of distilled water to prepare working standard solutions. Concentrations of each acylcarnitine in the μg ml^{-1} range in water were determined.

A reference urine sample (4 ml) was spiked in the same way to examine the influence of the matrix on the recovery of acylcarnitines by SPME. The quantitation of acylcarnitines in urine samples was carried out using the standard addition method. The mass spectrometer was tuned and calibrated with a mixture of 5 pmol μl^{-1} apomyoglobin (Sigma) and 20 pmol μl^{-1} of a peptide (MRFA from Finnigan MAT) dissolved in methanol–water (1 : 1, containing 0.1% of acetic acid).



		quasi-molecular ion (ESI^+)
R = H	carnitine	162
R= OCR'	*acylcarnitines*	
R'=	acetyl	204
	propionyl	218
	n/iso-butyryl	232
	valeroyl	246
	heptanoyl	274
	octanoyl	288
	decanoyl	316
	lauroyl	344
	myristoyl	372
	palmitoyl	400
	crotonobetaine	144

Figure 1. Structures of acylcarnitines and crotonobetaine studied.

Instrumentation

The solutions were introduced into the electrospray ion source of an SSQ 7000 single-quadrupole mass spectrometer (Finnigan, San Jose, CA, USA). ConstaMetric 4100 coupled pumps (Thermo Separation Products, Gelnhausen, Germany) produced an eluent flow of 0.1 ml min^{-1} of methanol–water (1:1). For quantitative purposes, the pumps guaranteed a constant flow for loop injection (5 μl) of the samples into the mass spectrometer.

In order to study the fragmentation behaviour of the individual acylcarnitines, the samples were introduced into the ion source with an 'infusion only' syringe pump (Model 22, Harvard Apparatus) at a flow rate of 7 μl min^{-1}.

The ESI capillary was adjusted to +4 and −4 kV spray voltage for the positive and negative ionization mode, respectively. The temperature of the spray capillary was held at 200 °C and a spray current between 0.6 and 1.3 μA was achieved depending on the polarity of the ionization voltage. The pressure of the sheath gas was 380 kPa of nitrogen. The octapole potential was adjusted to 6 V to reduce the solvent cluster ions. Collision-induced dissociation (CID) spectra were obtained by setting the octapole to a potential of 20 V.

Procedures

Full-scan spectra were recorded in the mass range between 50 and 700 u per 1 s in the profile mode. The spectra presented here were obtained by averaging five spectra. The target ions listed in Fig. 1 were used in the single-ion monitoring (SIM) mode (maximum five ions per run) to determine the detection limits and for all experiments including SPME considering quantitative aspects. Furthermore, all SIM analyses were performed at a CID potential of +6 V, which proved to be the optimum for the observed ion abundances.

The syringe assembly consisted of a 1 cm silicon rod coated with a polymer and was fitted to a stainless-steel tube. This part was placed inside a syringe needle, protecting the fibre while the needle penetrated the septa of, e.g. the sample vial. After penetrating the septum of a sample vial, the fibre was pushed out to ensure exposure to the sample. A sample volume of 4 ml was chosen to guarantee that the coated fibre was immersed completely. The solution was stirred during the extraction time of 1 h at a rate of 1000 rpm. Before the fibre was removed from the sample, it was withdrawn into the syringe needle again. The fibre was then ready for the injection of the analyte sample.

The design and operating functions of the SPME/HPLC interface were described in detail by Chen and Pawliszyn.[32] The interface uses a Rheodyne Model 7725 regular six-port injection valve, in which the loop is replaced by a hree-way tee, the so-called desorption chamber (Fig. 2). Two outlets of the tee are connected to the former loop input and output, respectively. The third tee outlet houses the SPME port. The tee part is mounted so that the eluent flow from the injection port is forced up through the bottom of the tee and continuously immerses the SPME fibre placed inside. The

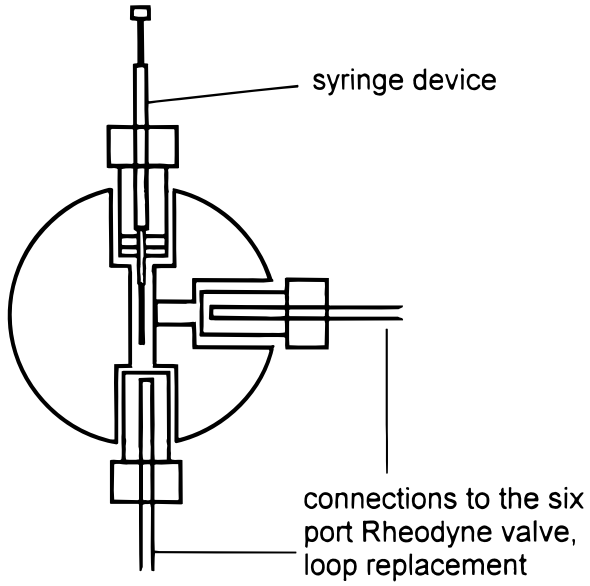


Figure 2. Scheme of the SPME desorption chamber.[36]

SPME device is carefully inserted through a ferrule and a Teflon tube into the tee until the outer needle comes to rest on the ferrule. Then the fibre is pushed out and the ferrule fixes the inner needle and provides a seal. The total volume of the interface we used was 70 μl. While the Rheodyne injection valve was in the load position, 70 μl of the desorbing solvent, a methanol–ethanol mixture (80:20, v/v), was injected via syringe inlet. The insertion and fixation of the SPME device followed immediately and after 2 min the valve was switched to injection. Now the analytes desorbed could enter the flow path to the MS instrument. The desorbing methanol was adjusted to pH 3 with 25% hydrochloric acid.

Validation

The validation of the SPME/ESI-MS experiments was based on the determination of the total carnitine and free carnitine content as described by Borum.[14] A blood plasma sample from a healthy subject was used to compare the two methods. The blood sample was centrifuged at 9000 rpm for 10 min, then 1 ml of the separated plasma was prepared for enzymatic analysis.

For SPME/ESI-MS, the plasma was treated with 0.5 M perchloric acid to sediment the proteins. After keeping it for 30 min in a refrigerator at 4 °C, the plasma was centrifuged at 4000 rpm for 7 min. A 100 μl volume of 0.5 M potassium carbonate was poured into 0.8 ml of plasma. Before analysis, the sample was adjusted to pH 7 with 25% hydrochloric acid.

RESULTS AND DISCUSSION

Mass spectrometry of acylcarnitines

The carnitine esters were analysed separately to characterize their MS fragmentation behaviour and to specify

the appropriate MS conditions for further trace detection in urine samples.

The positive ion ESI mass spectra of all the acylcarnitines studied are dominated by the quasi-molecular ions $[M - Cl]^+$, which can be used for identification.

The favoured fragmentation pathways are comparable to those observed in pyrolytic decomposition[34] and FAB mass spectra.[35] Generally, the fragment ions that appeared were of low abundance, as the example of *n*-butyrylcarnitine in Fig. 3 (top) shows.

The positive charge localized on the ammonium group determines the initial point of fragmentation. The ion at *m/z* 173 is formed by loss of the trimethylammonium group from protonated *n*-butyrylcarnitine, the quasi-molecular ion. The ion at *m/z* 162 represents the carnitine moiety caused by loss of the butyryl constituent as C_3H_6CO. The slightly increased abundance of the ion under CID conditions proves the classification as a fragment ion, although the ion at *m/z* 162 is anyway missing in published FAB fragment ion spectra.[35] The ion at *m/z* 144 arises from subsequent decomposition of the carnitine moiety. In mixture analysis, the ions at *m/z* 162 and 144 can be interfered with, e.g. by carnitine and its metabolite crotonobetaine ($M^+ = 144$, Fig. 1). The ion in the lower mass range at *m/z* 85 is of little diagnostic value because it appears in the spectra of all the acylcarnitines studied. This ion is associated with fragmentation of precursors such as the ions at *m/z* 173 and 144.

When operating under CID conditions with an octapole potential of 20 V, increasing fragmentation (Fig. 4, top) was noticed. The CID spectra differ mainly in ion abundance and not in fragmentation pattern. The quasi-molecular ion remains one of the most characteristic ions of the spectrum, accompanied by a strongly increased ion at *m/z* 85. The high abundance of this ion points to a fairly stable ion structure formed by different fragmentation pathways. In addition to a structure of an unsaturated carboxylic acid, a cyclic lactone can also be assumed (Fig. 5).

Unfortunately, no additional ions were recorded that point to cleavage of the *n*-butyryl moiety. Also, the CID spectra of the medium- and long-chain acylcarnitines contain no indications of acyl-chain fragmentation.

A comparison of the conventional ESI mass spectra of *n*-acyl- and the corresponding isoacylcarnitines hardly showed significant differences in their fragmentation pathways and ion abundances. Also, under CID conditions the spectra of the isomeric acylcarnitines show identical fragmentation pathways, as the examples of *n*- and isobutyrylcarnitine indicate (Fig. 4). In the lower mass range of both spectra, low-abundance ions appear at *m/z* 104 and 99, probably related to the fragmentation of the carnitine moiety (*m/z* 162

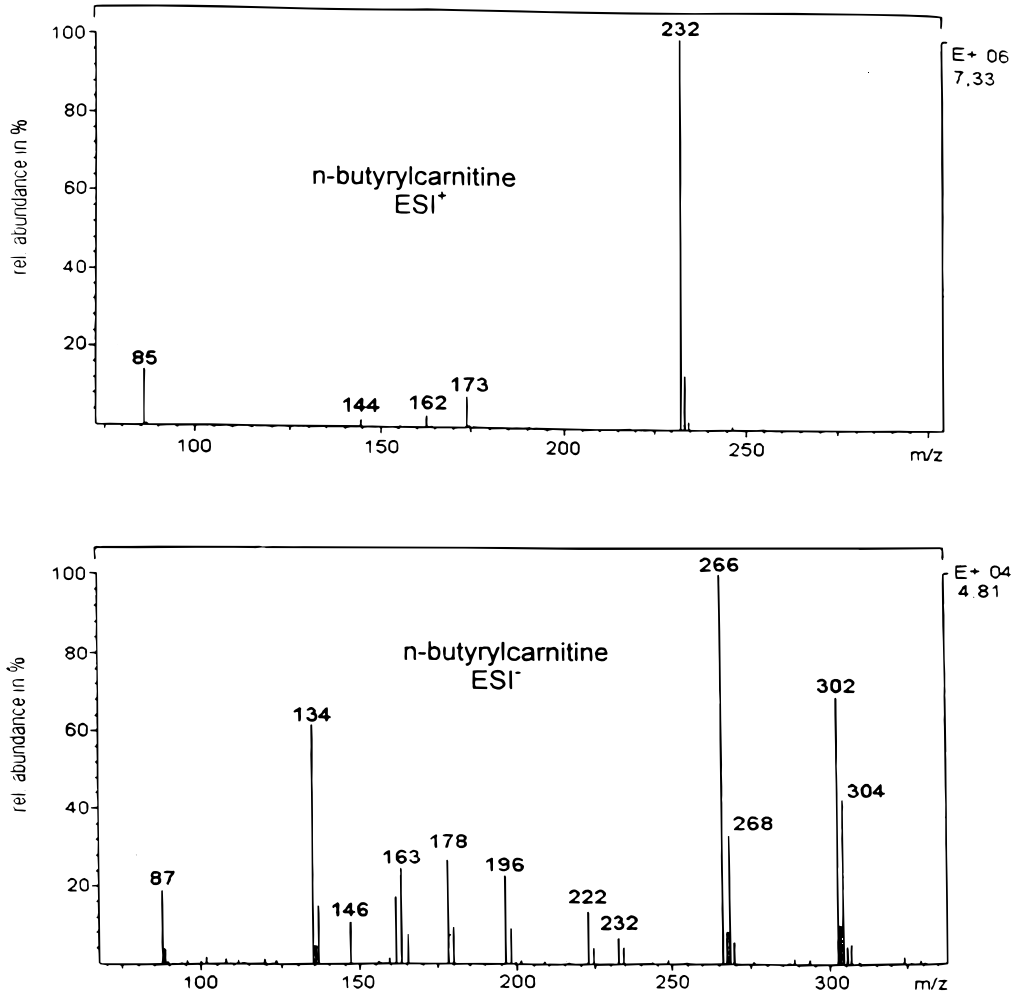


Figure 3. ESI^+ mass spectra of *n*-butyrylcarnitine hydrochloride (top) and the corresponding ESI^- mass spectrum (bottom).

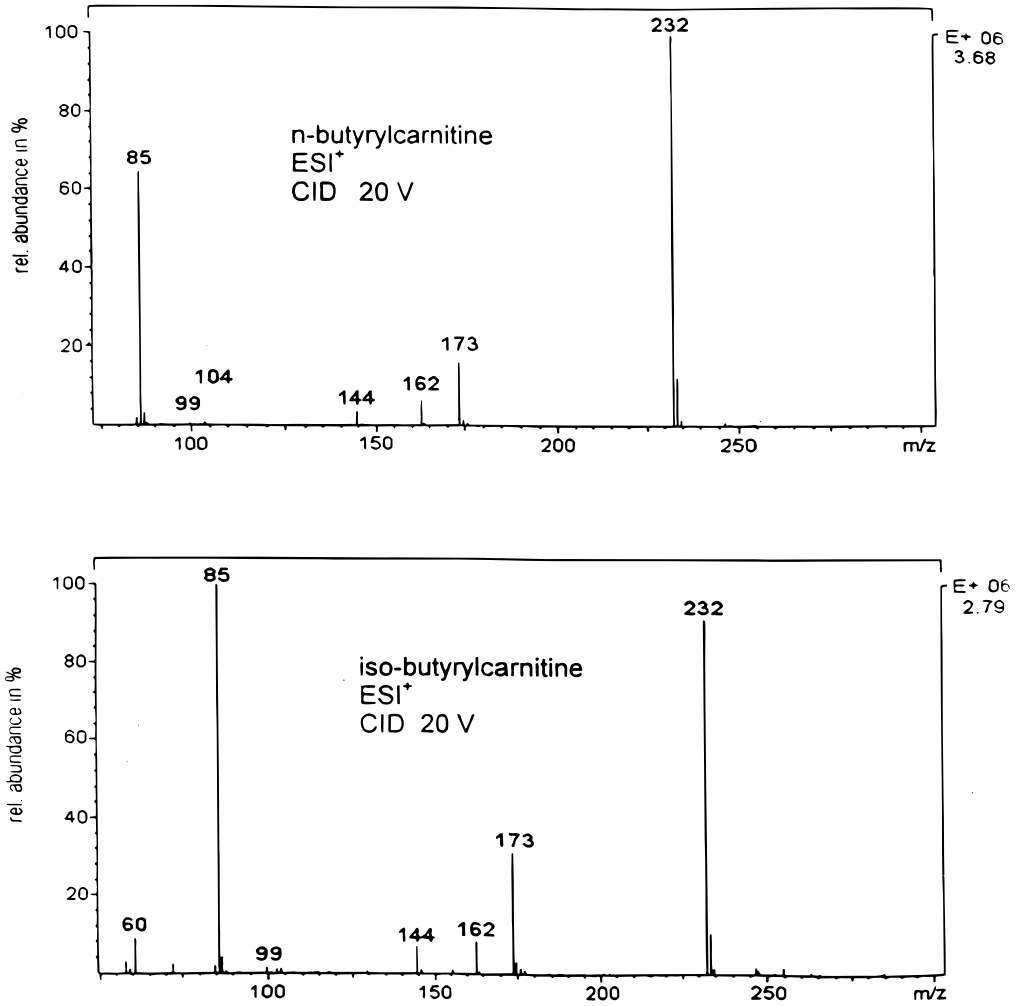


Figure 4. ESI$^+$-spectra of *n*-butyrylcarnitine and iso-butyrylcarnitine under CID conditions.

$-CH_2COO$) and of the precursor ion at *m/z* 144 (crotonobetaine structure $-COOH$).

A comparison of the abundances and ratios of diagnostic ions (Table 1) can be useful in the attempt to differentiate between the isomers. The reproducibility of the ion abundances is between 2 and 5% (RSD) at a concentration of 2 μg ml^{-1} and permits such comparisons. Assuming that the ion structures discussed differ only in the acyl substituent, the relative abundance of the quasi-molecular ion is higher in the *n*-butyryl ester. The more abundant fragmentation of the branched isomer (Fig. 4, bottom) seems to be caused by the lower stability of the iso-structure, which becomes apparent in more abundant ions at *m/z* 173 and 85. Relating the ion abundances to the quasi-molecular ions at *m/z* 232, the differences between the isomeric butyrylcarnitines are distinctive.

Table 1. Comparison of relative ion abundances and selected ion ratios of *n*- and iso-butyrylcarnitine under CID conditions

Ion ratio (*m/z*)	*n*-Butanoyl carnitine	Isobutanoyl carnitine
232/173	6.3	2.9
232/162	14.3	11.4
232/144	25.0	13.0
232/85	1.5	0.91
m/z	Relative abundance (%)	
232	100	91
173	16	31
162	7	8
144	4	7
85	64	100

Generally, only small differences in the fragmentation frequency of certain ions reflect the structural nature of the acyl group. This observation confirms the assumption that the preferred decomposition pathways of carnitine esters are influenced less strongly by the acyl substituents. The size and branching of the ester groups affect the fragmentation only with regard to the abundance of ion formation. These results differ from FAB/CID fragment ion scans[35] that indicated fragmentation also in the acyl moiety of the carnitine esters. Thus, a distinction between isomeric acylcarnitines is not possible using conventional ESI spectra. Under ESI/CID conditions the low-energy collisions occurring in the octapole region do not permit cleavages in the alkyl chains of the acyl substituents and, consequently, assignment of isomers is hindered. Indeed, the differences in ion abundances can be useful for describing the individual acylcarnitines but in mixture analysis these

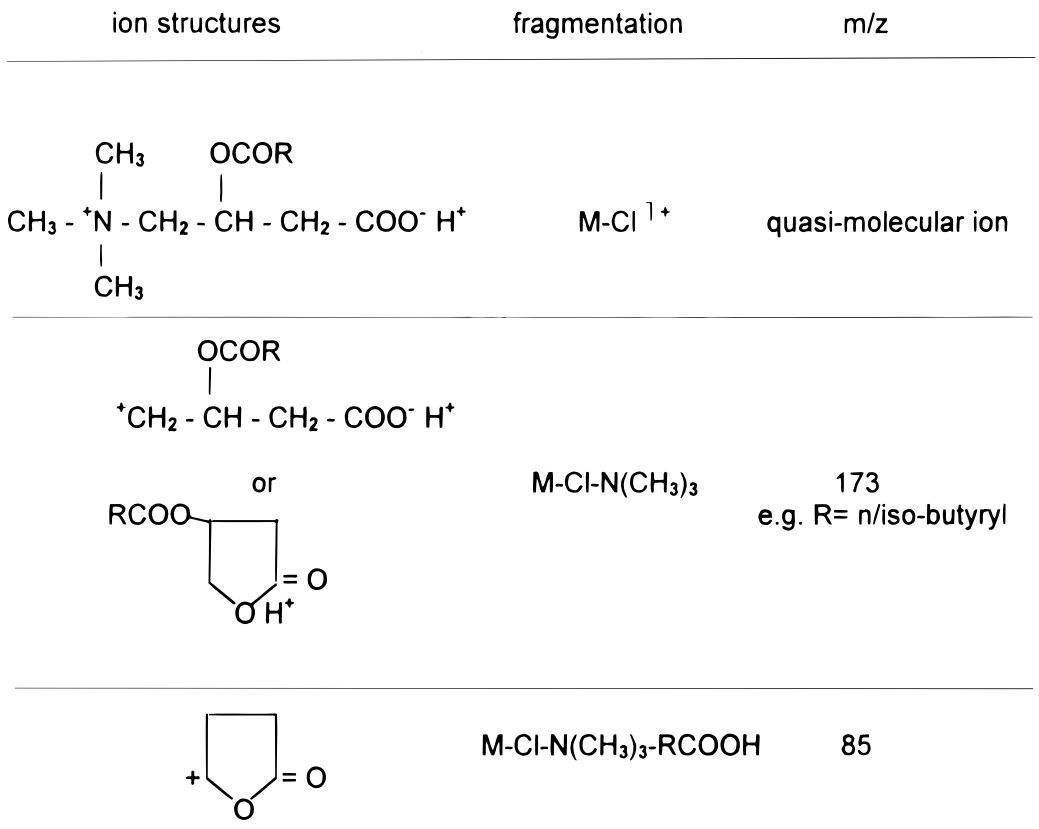


Figure 5. Characteristic ion structures proposed for *n*-butyrylcarnitine.

features are not adequate and a combination with derivatization and pre-separations is then unavoidable.

The positive ion ESI spectra of the aylcarnitines provide sufficient reproducibility over a wide concentration range from $\mu g\ ml^{-1}$ to $ng\ ml^{-1}$.Cluster ions formed during ionization could be partially or even completely destroyed by adjusting the octapole potential to 6 V. Significant changes in ion abundance were not observed under these conditions.

Owing to the betaine structure of the acylcarnitines, positive and negative electrospray (ESI^+ and ESI^-) should likewise be suitable for ionization. However, comparison of the two ionization modes shows that they are not equivalent in their capability for identification and quantitation of acylcarnitines. The example of *n*-butyrylcarnitine demonstrates the differences caused by ionization (Fig. 3).

Generally, the negative ion spectra of the individual acylcarnitines suffer from a lower total intensity than the positive ion spectra. The quasi-molecular ion at *m/z* 266 consists of the butyrylcarnitine molecule and a chloride ion as counterpart to the ammonium group. The higher mass range of the ESI^- spectrum contains ions formed by adding one hydrochloric acid (*m/z* 302). Also, the application of a low octapole potential (6 V) could not prevent the appearance of hydrochloride clusters, as the example of *n*-butyrylcarnitine shows (Fig. 3, bottom). A mass difference of 70 u from *m/z* 302 indicates the loss of the ester moiety and gives the ion at *m/z* 232. The same decomposition starting from the quasi-molecular ion forms the ion at *m/z* 196. The fragment at *m/z* 178 arises from the cleavage of *n*-butanoic acid from the quasi-molecule.

Subsequent fragmentation reactions produce ions such as *m/z* 163, 161 and 146, comparable to the pathway found in the ESI^+ mode. The ion at *m/z* 134 can be produced by loss of a CO_2 moiety from *m/z* 178. The isotopic patterns of most ions show that one chlorine is present. It is assumed that the fragmentation starts at the carboxyl terminus and in the most cases the trimethylammonium group is retained as a stable ion part.

The low intensity of the ESI^- spectra cannot guarantee sensitive detection and a distinction between isomeric acylcarnitines was also not supported. Therefore, the discussion of this work is focused on the results obtained by positive electrospray ionization.

Quantitation

The full-scan spectra of the individual acylcarnitines are very reproducible over the concentration range 10–0.1 $\mu g\ ml^{-1}$. At lower levels only the quasi-molecular ions $[M - Cl]^+$ are available as a characteristic feature for identification and target analyses.

The detection limits of the individual acylcarnitines were determined by loop injection using the target ions listed in Fig. 1. The detection limits range from 0.2 to 12.8 $ng\ ml^{-1}$ (Table 2) and the mean relative standard deviations (RSDs) obtained for the individual acylcarnitines were about 9%. Near the detection limits the RSD values fluctuated more and were found to be between 5% for palmitoylcarnitine and 18% for carnitine.

In order to characterize the influence of an accompanying matrix, first a series of acylcarnitine mixtures were prepared and analysed.

Table 2. Detection limits and corresponding relative standard deviations of the acylcarnitine salts obtained for individual and mixture analysis

R[a]	Detection limit (ng ml^{-1}) (individual)	RSD (%) (n = 5)	Detection limit (ng ml^{-1}) (mixture)	RSD (%) (n = 5)
H	0.2	12.70	2.0	10.40
Acetyl	0.6	11.50	17.6	1.70
Propionyl	0.6	8.80	10.4	7.10
n-Butyryl	10.4	7.30	3.4	7.50
Isobutyryl	0.2	8.90	—	
Isovaleroyl	8.0	9.80	12.6	4.60
Heptanoyl	12.8	9.80	4.0	0.10
Octanoyl	3.0	7.20	3.4	3.60
Decanoyl	0.8	12.30	4.4	6.2
Lauroyl	0.2	15.50	10.4	8.20
Myristoyl	0.4	2.80	16.4	6.80
Palmitoyl	2.0	2.40	15.0	9.10
Intermediate: crotonobetaine	0.8	10.50	25.0	1.30

[a] See Fig. 1.

The detection limits determined from the mixture differ from those obtained from the determination of the individual acylcarnitines. The response of most of the acylcarnitines studied deteriorated but in individual cases an improved response was observed (n-butyryl- and heptanoylcarnitine). It is proposed that competitive processes such as intermolecular interactions between the ionic structures of the acylcarnitines make the release of the analyte ions during the spray ionization more difficult and the abundances of the diagnostic ions are reduced. As a further approach to the determination of acylcarnitines in biological material, a comparison with a spiked urinary sample was carried out. Acylcarnitine mixtures of ng μl^{-1} concentration in methanol–water (1 : 1) and methanol–urine (1 : 1) were prepared for loop injection. The comparison in Fig. 6 shows the areas in counts related to 1 ng of the corresponding carnitine ester. The mean RSDs determined for the water–methanol mixture are about 5% and for the spiked urine 14%. The abundances of nearly all target ions decrease in presence of the urine matrix and it is confirmed that the more complex is the matrix the lower are the ion abundances of the acylcarnitines. Acetylcarnitine seems to be an exception, but the slightly improved abundance of the target ion in the urinary matrix can also be caused by accompanying compounds that interfere with the ion at m/z 204.

The influence of the matrix is considerable and the problems increase with often changing compositions of urinary samples. The full-scan spectrum in Fig. 7 makes

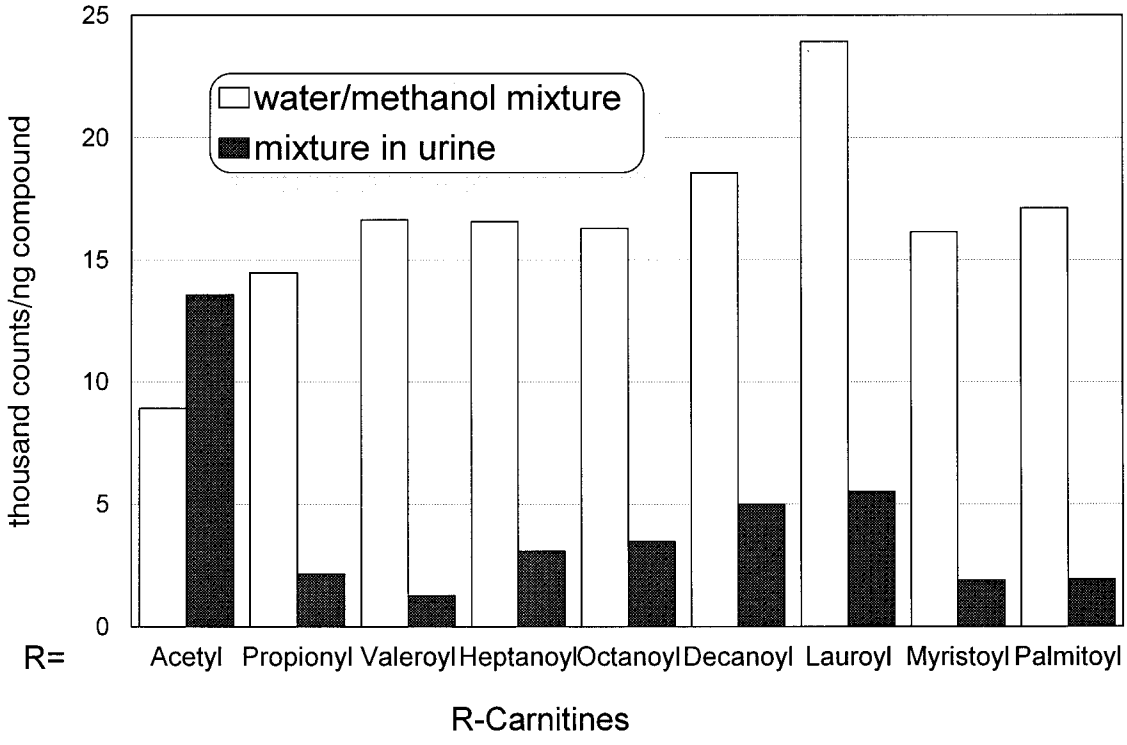


Figure 6. Comparison of the target ion abundance of an acylcarnitine mixture depending on the matrix.

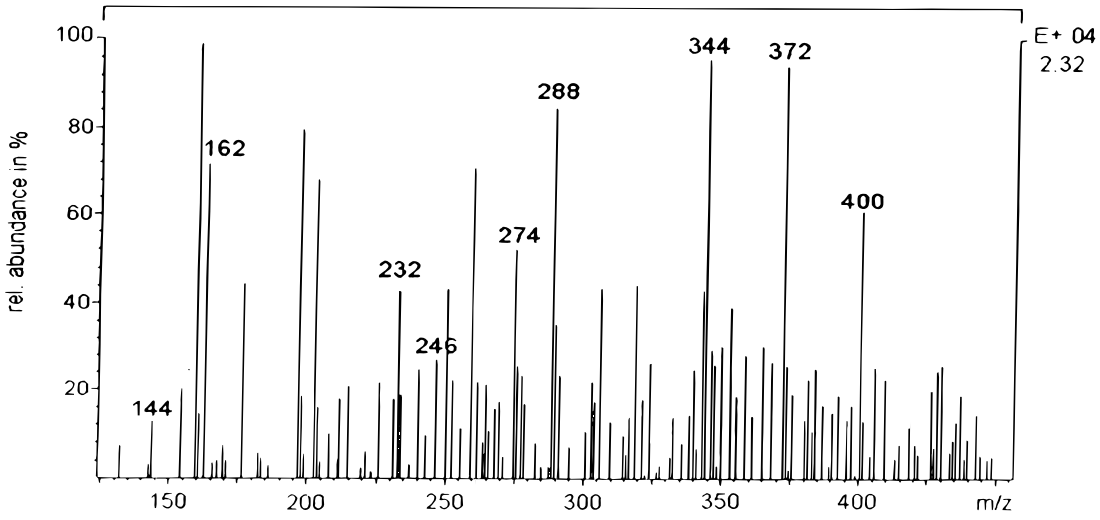


Figure 7. ESI$^+$-full scan spectrum of a urine sample spiked with a mixture of acylcarnitines.

plain the complexity of the matrix. Background subtraction can support the assignment of the target ions; nevertheless, the interfering ions could not removed completely and make interpretation and detection at trace concentration levels more difficult. Consequently, quantitation of the acylcarnitines by loop injection is only possible using standard addition or MS/MS analysis.

In this work, solid-phase microextraction was examined as an alternative method for acylcarnitine detection that avoids expensive sample preparation.

Application of SPME to urine samples

SPME, especially in combination with gas chromatography, is well known as an effective method for the determination of organics in water samples. In this work, SPME fibres coated with polymers such as Carbowax and poly(dimethylsiloxane) were used to extract and to accumulate acylcarnitines from water, urinary samples and blood plasma. The extraction with a poly(dimethylsiloxane) fibre is based on the adjustment of the partition equilibrium of an analyte between the aqueous sample and the polymer coating. The water solubility of a compound is also one of the crucial parameters that determines the extraction yield of the compound. The extraction mechanism of a Carbowax fibre includes additional adsorption effects of the coating which sometimes cause long extraction times and incomplete desorption. In the case of HPLC and ESI-MS coupling the desorption is realized by a solvent. The acylcarnitines extracted were desorbed into the ion source using acidic methanol (pH 3).

Prior to the quantitation by SPME/ESI-MS, a series of extractions were performed with the aim of optimizing the extraction times of the acylcarnitines from water solutions. With poly(dimethylsiloxane) fibre the partition equilibrium of each acylcarnitine are adjusted after 1 h, but generally the extraction yields are too low for detection at μg ml^{-1} levels. The use of a Carbowax-coated fibre is more successful and permits the detection of selected acylcarnitines at concentration levels that are of interest for clinical diagnostics. Therefore, the following discussion is focused on experiments with a Carbowax-coated SPME fibre.

Although the extraction process with a Carbowax fibre occurs very slowly and the maximum extraction yields are obtained only after 15 h, we use an extraction time of 1 h. The extension of the extraction time results in an increase in the yields of about 15% but the loss of time seems to be a greater disadvantage. Therefore, exactly 1 h was applied as the extraction time in all subsequent experiments.

A mixture of acylcarnitines at the ng ml^{-1} level was prepared with human urine.The mixture was injected first by loop injection and analysed by ESI-MS. Then, for comparison, 4 ml of the same solution were sampled with the Carbowax fibre, desorbed and analysed in the same way. The results are compared in Fig. 8. The low extraction yields of the short- and medium-chain acylcarnitines from acetyl- to heptanoylcarnitine are caused by their extremely good water solubility and related low affinities to the fibre coating.

The long-chain acyl esters from decanoyl-to palmitoylcarnitine were extracted more effectively by a factor of between 2 and 3 compared with the loop injection analysis. The lower detection limits achieved are mainly caused by the considerable accumulation of the analytes on the fibre coating. Additionally, the selectivity of the microextraction reduces the chemical noise because accompanying substances are not extracted and the interference with target ions is reduced. The mean RSD for lauroyl-, myristoyl- and palmitoylcarnitine detected from urine is ~15%. Experiments to improve the SPME yield and reproducibility are in progress, especially attempts that guarantee more complete desorption of the acylcarnitines from the fibre coating.

The special response for long-chain acylcarnitines is an advantage of this method combination. At present, the determination of individual long-chain acylarnitines at ng ml^{-1} levels is not possible without derivatization and chromatographic separation, a time- and substance-consuming procedure. However, the detection and quantitation of long-chain acylcarnitines is of value for the clinical diagnosis of special disorders such as diabetes mellitus type I.

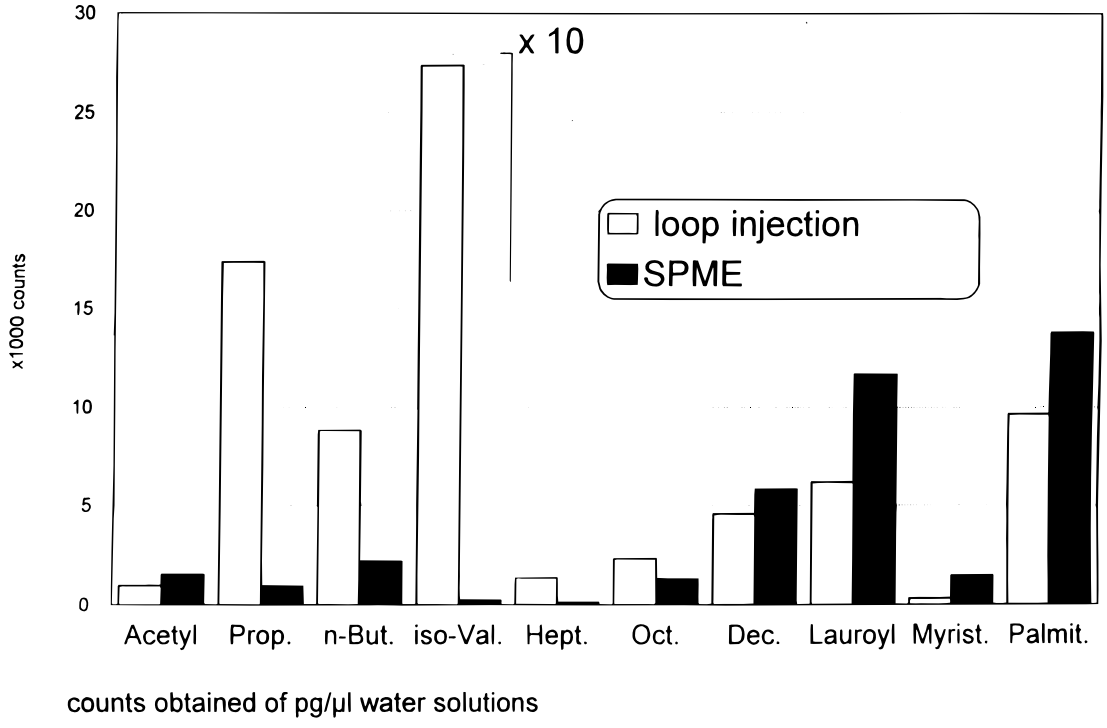


Figure 8. Comparison of acylcarnitine determinations performed by loop injection/ and SPME/ESI$^+$-MS.

A disadvantage of the method combination is the low response for carnitine and short- and medium-chain acylcarnitines. Nevertheless, the microextraction offers the possibility of monitoring acylcarnitines besides the well established methods for the determination of free and total carnitine. A comparison of SPME/ESI-MS with the commonly used enzymatic technique gave good agreement. The total carnitine content of a blood plasma sample from a healthy subject was determined as 40.39 µg l^{-1} and the concentration of free carnitine was 39.34 µg l^{-1}. The difference of 1.05 µg l^{-1} corresponds to the sum of acylcarnitines present in the plasma. The sum of long-chain acylcarnitines detected by SPME analysis was 1.02 ± 0.2 µg l^{-1} and fits well with the data obtained by enzymatic methods. Additionally, SPME supplies some more detailed information about the concentration of lauroyl-(0.32 µg l^{-1}), myristoyl-(0.47 µg l^{-1}) and palmitoylcarnitine (0.23 µg l^{-1}).

An application to the clinical diagnosis of patients suffering from ischaemic cardiomyopathy is shown in Table 3. Interpretation of these data is difficult because no comparable values are available to correlate the pattern of acylcarnitines and certain diseases with each other.

Among the carnitine contents which can be obtained by established enzymatic techniques, more detailed information is now available about individual long-chain acylcarnitines. Considering the importance of these compounds in the β-oxidation process, the data obtained can be of diagnostic value for appropriate therapeutic advice.

Table 3. Determination of selected acylcarnitines in human urine samples

Compound	Concentration (µg l^{-1}) Patient 1	Patient 2	Patient 3	Patient 4
Carnitine	31.4	50.6	38.8	27.8
Myristoylcarnitine	45	68.6	24	36.6
Palmitoylcarnitine	9.5	36.6	9.5	13.2
Crotonobetaine	1.7	3.3	68.6	1.1

CONCLUSION

ESI-MS was used to study the mass spectrometric behaviour of acylcarnitine HCl salts. The conventional ESI$^+$ mass spectra of the acylcarnitines are dominated by the quasi-molecular ion that is formed by the loss of a chloride. Only few fragmentation reactions occur and result in fragment ions of low abundance. Under CID conditions some fragmentation pathways are favoured that form ions in the lower mass range, e.g. at m/z 85. No additional ions are observed although suitable collision energy was applied and the missing fragments were those which point to the nature of the acyl moiety. Thus the quasi-molecular ion was the main feature for the determination of the particular carnitine esters. Furthermore, the results demonstrate that ESI$^-$ mass spectra are not as well suited for quantitation because of their low ion abundances.

The nature of the matrix clearly influences the ion abundance of the acylcarnitines—the more complex is the matrix, the more unfavourable are the detection limits found. The interpretation of the ESI$^+$ mass spectra of acylcarnitine mixtures directly introduced

into the mass spectrometer is sometimes difficult owing to interfering ions of matrix components.

In order to improve the detection limits of acylcarnitines, a combination of SPME and ESI-MS was examined. The acylcarnitines were extracted with a Carbowax-coated SPME fibre and desorbed into a special SPME/HPLC interface that was coupled to an ESI source. On comparing SPME with loop injection, the former gives detection limits improved by factors of 2–3, especially for the long-chain carnitine esters (decanoyl- to palmitoylcarnitine). Unfortunately, carnitine itself and the short- and medium-chain acylcarnitines are detected with lower response. Their good water solubility and small molecule size cause low affinities to the Carbowax coating used.

The possibility of distinguishing between long-chain acylcarnitines can be of value in clinical diagnostics of special carnitine disorders because only the free and total contents of carnitines are available for diagnostic purposes. The detection of isomeric acylcarnitines is another problem that still requires time-consuming prederivatization and separation procedures.

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