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Determination of long-chain fatty acid acyl-coenzyme A compounds using liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

Acyl-CoAs have important role in fat and glucose metabolism of the cells. In this study we have developed an on-line HPLC–ESI-MS/MS method for determination of long-chain acyl-CoA compounds in rat liver samples. Six long-chain acyl-CoAs (C16:0, C16:1, C18:0, C18:1, C20:0 and C20:4) were separated with a C_4 reversed-phase column using triethylamine acetate and acetonitrile gradient. Negative electrospray ionization is very suitable for acyl-CoA compounds and excellent MS/MS spectra for long-chain acyl-CoAs can be obtained. MS/MS method with an ion trap mass spectrometer makes it possible to identify and quantitate individual acyl-CoAs simultaneously. The method proved to be sensitive enough for determination of all compounds of interest using 0.4–0.7 g of tissue and was validated in the range of 0.1–15.0 pmol/μl.

Keywords: Long-chain acyl-coenzyme A compounds

1. Introduction

Acyl-CoAs are active intermediates involved in lipid metabolism converted by acyl-coenzyme synthetase from free fatty acids [1]. However, the role of acyl-CoAs in cellular metabolism is much broader than just part of fat metabolism. Recently, acyl-CoAs have been found to have an important function in the regulation of intermediary metabolism and gene expression [2]. The objective to determine the physiological function of acyl-CoAs has lead to development quantitative methods from biological samples.

The extraction procedure from biological samples is essential for successful analysis of acyl-CoAs. Different extraction methods have been extensively described in numerous studies [3–7]. In these methods mainly solid-phase and liquid–liquid phase extraction were used.

Several methods have been reported for determination of long-chain acyl-CoA compounds in tissue samples using high-performance liquid chromatography (HPLC). Corkey [5] described a HPLC method with UV detection for acyl-CoA from biological samples. Mangino et al. [6] reported a reversed-phase HPLC method with solid-phase extraction (SPE) procedure for the determination of acyl-CoA esters from liver tissue samples. More recently, Shrago and Woldegiorgis [8] reported a quite similar method for analysis of acyl-CoA compounds from tissue samples. DeMar and Andersson [9] reported a HPLC method using liquid–liquid extraction procedure for acyl-CoA compounds from bovine heart, liver and retina. The major disadvantages of all these methods are quite low or unreproducible recoveries.

In contrast, HPLC–tandem mass spectrometry is well suited for biological analysis [10]. The high selectivity of mass spectrometry makes it a powerful tool for acyl-CoA determination from biological samples. Methods based on

Abbreviations: LC–ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; HPLC, high-performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry; MRM, multiple reaction monitoring; SIM, selected ion monitoring; R.S.D., relative standard deviation; LOQ, limit of quantitation

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mass spectrometric detection have been reported previously. Zirrolli et al. [11] described the detection of long-chain acyl-CoAs by negative ion fast-atom bombardment mass spectrometry. Wolf et al. [12] reported a long-chain acyl-CoAs determination by gas chromatography–mass spectrometry (GC–MS). Kalderon et al. [13] presented mass spectrometric method using electrospray ionization without chromatographic separation. Most recently, Kasuya et al. [14] described a LC–ESI-MS method for medium-chain acyl-CoAs using positive ionization mode. However, LC–MS methods have not been described in the analysis of long-chain acyl-CoAs in biological samples.

In this study we have developed an on-line HPLC–ESI-MS/MS method for determination of long-chain acyl-CoA compounds in rat liver samples. The method is based on separation of the compounds by reversed-phase chromatography using triethylamine acetate and acetonitrile gradient and detection with MS/MS method.

2. Material and methods

2.1. Chemicals

Long-chain acyl-CoA standards were purchased from Sigma Chemicals. Acetonitrile (HPLC grade) was purchased from Rathburn (Walkerburn, Scotland) and triethylamine from Sigma. Water was distilled and further purified with Millipore-Q UF Plus apparatus (Molsheim, France).

2.2. Sample preparation

Tissue (0.4–0.7 g) from the rat liver (Wistar rats; 350–450 g) was powdered on dry ice under liquid nitrogen and 5 nmol of heptadecanoyl-CoA (C17:0) (Sigma) was added as a internal standard according to a modified protocol described by DeMar and Andersson [9]. Tissue was homogenized in 5 ml ice cold isopropanol–potassium hydrogenophosphate solution (1:1, v/v, pH 7.2) using Ultra-Turrax. Homogenate were acidified with 100 μl glacial acetic acid and washed with 4 ml of hexane–isopropanol (2:1, v/v) and 3 ml of hexane. Upper hexane phase was discarded and proteins in lower phase were precipitated with 200 μl of saturated ammonium sulphate and 9 ml of methanol–chloroform (2:1, v/v) solution. After 10 min the precipitate was centrifuged at 4000 rpm for 8 min at 4 °C. The protein pellet was washed with 4 ml of methanol–chloroform–water (5:2.5:1, v/v/v) solution and centrifuged at 4000 rpm for 10 min. Supernatants were combined and 4 ml of water was added. The upper aqueous phase was washed twice with 3 ml of chloroform. The chloroform phases were combined with the lower phase and washed with 2 ml of water. Aqueous phases from the extractions were then pooled. The final sample volume was 15 ml. For the analysis 2 ml of sample was evaporated under nitrogen and the residue dissolved in 100 μl of 5% methanol–water prior to analysis.

2.3. Chromatographic conditions

Optimization of chromatographic separation was performed by testing Genesis C_4 (2.1 mm × 50 mm, particle size 4 μm) and Genesis C_{18} (2.1 mm × 50 mm, particle size 4 μm) reversed-phase columns.

The compounds were separated with both mentioned columns using Ultimate HPLC pump (LC Packings, Netherlands) with 10 mM triethylamine acetate (buffer A, pH 7.0) and acetonitrile (buffer B) gradient. The gradient profile was 7–100% B in 9 min, 100% B for 3 min and 100–7% B in 2 min. The flow was diverted to waste for first three min preventing early eluting impurities entering the mass spectrometer. The flow was set to 120 μl/min and the sample volume was 30 μl.

2.4. MS analysis

The system used for LC–MS/MS analysis was a Finnigan LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with electrospray ion source. In negative ionization mode electrospray was stabilized using nitrogen sheath gas (flow 100 AU) and the spray needle voltage was set at −4 kV. The inlet capillary was heated to 225 °C and the tube lens offset was set to 15 V. The full scan data was obtained by scanning from *m*/*z* 200 to 1200 and the full scan MS/MS spectra were measured using 300 ms for collection of the ions in the trap. Tandem mass spectrometry and multiple reaction monitoring (MRM) were used for the quantitative determination of acyl-CoAs from the samples. The quantitation was based on monitoring of typical MS/MS fragment ion for each acyl-CoA chain length and double bound number. The quantitation was based on comparing peak area ratio of each analytes and internal standard vs. concentration. The ions recorded were 1002 → 655.3, 1004 → 657.3, 1031 → 683.3, 1033 → 685.3, 1052 → 705.5 and 1060 → 713.3 for acyl-CoAs C16:1, C16:0, C18:1, C18:0, C20:4 and C20:0, respectively. Following parameters were used: Isolation width 6 mass units, collision energy 35% and scan range *m*/*z* 280–1200. The wide isolation width was used to record simultaneously ions from the compounds containing different amount of double bonds.

2.5. Calibration standards and quality control samples (QC)

The 5 nmol/μl stock solutions of acyl-CoAs were prepared in water solution and stored frozen until used (−20 °C). Calibration standard mixtures containing six acyl-CoAs; palmitoyl-CoA (C16:0), palmitoleoyl-CoA (C16:1), stearoyl-CoA (C18:0), oleoyl-CoA (C18:1), arachidoyl-CoA (C20:0) and arachidonoyl-CoA (C20:4) (Sigma) were prepared at concentrations of 0.1, 0.5, 1.0, 5.0, 9.0 and 15.0 pmol/μl. Equal amount of internal standard (C17:0) was added in each mixture to achieve the concentration of 2.0 pmol/μl. Standard mixtures were stored at −20 °C

until used. Standard curves were obtained by plotting the peak area ratios versus internal standard concentrations.

A 5 nmol/μl stock solution was used to prepare quality control (QC) samples containing 0.67 and 3.3 pmol/μl of each acyl-CoA compounds. Internal standard concentration used was 2 pmol/μl. The QC samples were stored at −20 °C until used.

2.6. Recovery

The recovery was tested using two methods. First, the standard addition method [16] was used because it was not possible to prepare a blank sample without presence of acyl-CoAs. Secondly, recovery was also tested by comparing peak areas obtained when samples were analyzed by adding the internal standard (C17:0) and C16:0 standard in the extract prior to and after extraction procedure. For recovery evaluation sample loss during evaporation was estimated by adding internal standard (C17:0) to samples after evaporation. Obtained signal was compared to a control sample with C17:0 addition before evaporation.

2.7. Precision and accuracy

For evaluation of extraction procedure accuracy and precision four liver tissue samples were spiked with 5 pmol/μl of C16:0 prior extraction. The internal standard was added normally. Concentration of C16:0 in spiked samples were compared with four samples without C16:0 addition and the accuracy and precision were calculated.

The reproducibility of the quantitation method was determined by analyzing same sample prepared according to extraction method described earlier in ten consecutive runs. The concentration of all analytes was assessed and standard deviations were calculated (n = 10). For further evaluation of the precision and accuracy of the method QC samples were analyzed at two different concentration (0.67 and 3.3 pmol/μl) in four separate runs. The precision and accuracy were determined for each analyte by calculating the deviations of predicted concentration from their nominal values.

3. Results and discussion

3.1. LC–MS analysis

The aim of the present study was to develop and validate the method for analysis of long-chain acyl-CoA compounds in rat liver tissue samples using HPLC–MS/MS. HPLC was used to separate analytes from early eluting impurities. A chromatographic run was completed in 15 min and initial conditions were restored in 20 min. The chromatographic separation in Genesis C_4 and C_{18} columns was evaluated (data not shown). Peak broadening occurred with

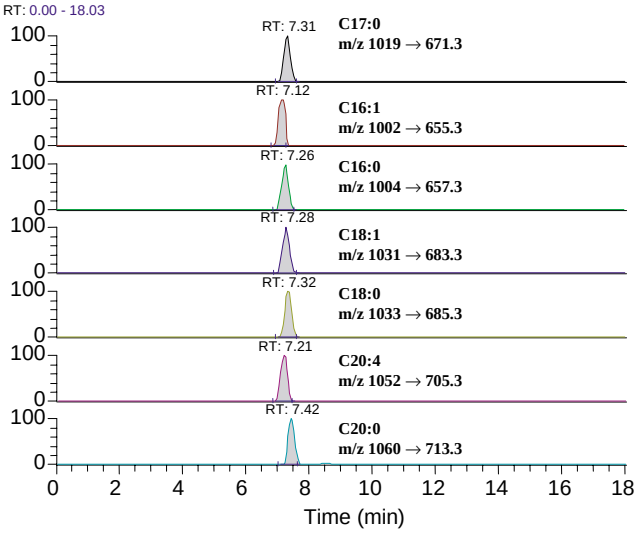

Fig. 1. The multiple reaction monitoring chromatograms for acyl-CoAs measured. No additional peaks due the matrix were observed in the ion channels used.

C_{18} whereas analysis in C_4 column produced narrow and symmetric peaks with satisfying chromatographic separation from early eluting interfering compounds. Therefore, for later analysis, Genesis C_4 column was chosen. No additional peaks due the background were observed in the ion channels used. Each analyte gave a sharp and narrow peak with good signal-to-noise ratio (Fig. 1). Blank samples injected after standard mixtures or biological samples did not present any traces of carry-over. Triethylamine acetate (10 mM) did not cause signal suppression.

The negative ion full scan spectrum shows deprotonated molecular ions for the acyl-CoA standards (Fig. 2). In real biological samples also other long-chain acyl-CoA compounds were identified based on their molecular weights and MS/MS. However, the quantitation analysis of other acyl-CoAs was abandoned because lack of standards.

MS/MS method with an ion trap mass spectrometer makes it possible to identify and quantitate individual acyl-CoAs simultaneously. In negative ion mode MS/MS analysis of the deprotonated molecules gave a good fragment ion spectra (Fig. 3) with a good signal-to-noise ratio (>100). The ion which retained the phosphoadenosine group was observed at *m/z* 426 and corresponding $-H_2O$ ion at *m/z* 408 for each compound measured as described also in earlier studies [14]. The observed fragmentation is typical for the phosphoadenosine containing compounds [15]. The cleavage of phosphoadenosine was the major fragmentation pathway for all compounds producing ion 671.3 for acyl-CoA C17:0 in Fig. 3. The ion containing the indicative acyl chain were used for quantitation of the compounds. The ions recorded were 1004 → 657.3, 1002 → 655.3, 1033 → 685.3, 1031 → 683.3, 1060 → 713.3 and 1052 → 705.3 for acyl-CoAs C16:0, C16:1, C18:0 C18:1, C20:0 and C20:4, respectively. The identity of compounds was verified with full MS/MS.

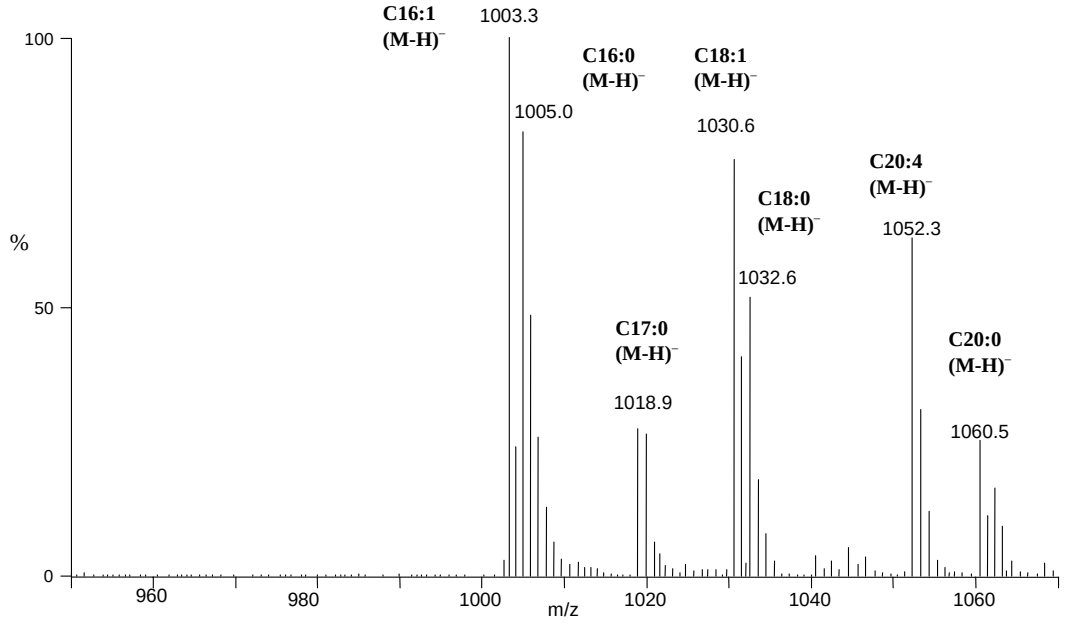

Fig. 2. The negative ion full scan spectrum (*m*/*z* 950–1070) obtained by HPLC–MS from a standard sample. Spectrum shows deprotonated molecular ions $(M - H)^-$ for acyl-CoAs.

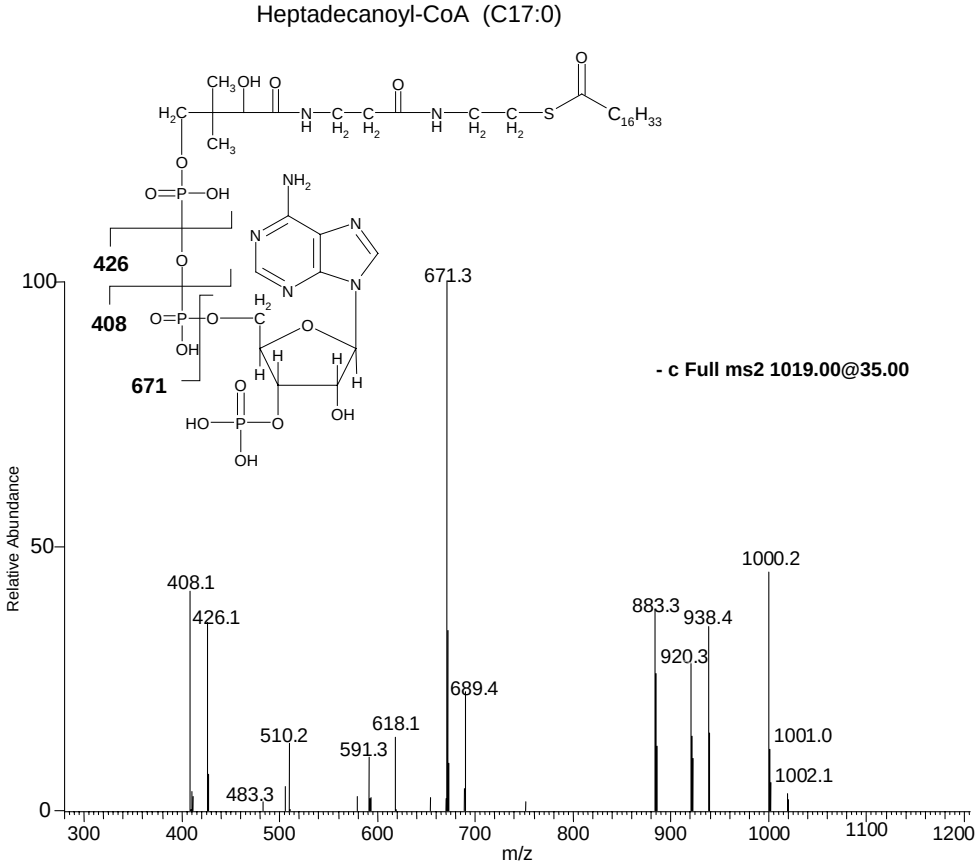

Fig. 3. The MS/MS fragmentation spectrum of deprotonated acyl-CoA C17:0 used as internal standard with negative ionization mode. The cleavage of phosphoadenosine (*m*/*z* 671.3) was the major fragmentation ion. The quantitation was based on monitoring of typical MS/MS fragment ion for each acyl-CoA chain length and double bond number. The fragment ion retained the phosphoadenosine group was observed at *m*/*z* 426 and its respective $-H_2O$ peak at *m*/*z* 408.

Table 1
Method calibration; slopes and intercepts obtained from three replicate analysis

Compound	Calibration line, slope[a]	Calibration line, intercept[a]	Correlation coefficient (r^2)[a]
C16:0	1.3651 ± 0.2357	0.2954 ± 0.1112	0.9956 ± 0.0032
C16:1	1.2267 ± 0.1436	0.1018 ± 0.1991	0.9877 ± 0.0126
C18:0	1.0546 ± 0.0066	−0.1642 ± 0.0124	0.9974 ± 0.0020
C18:1	1.0809 ± 0.0584	0.0217 ± 0.0116	0.9968 ± 0.0014
C20:0	1.1410 ± 0.1137	−0.3904 ± 0.0814	0.9850 ± 0.0050
C20:4	0.5453 ± 0.0264	0.0603 ± 0.0349	0.9980 ± 0.0020

The quantitation was based on peak area ratios of analytes and internal standard.
[a] Mean ± S.D. of three replicates.

Table 2
Extraction procedure precicion and accuracy obtained for C16:0

Compound	*n*	Calculated addition (pmol/μl)	Estimated addition mean ± S.D. (pmol/μl)	Precision (R.S.D.)	Accuracy (percent error)
C16:0	4	2.0	2.118 ± 0.265	12.5	5.9

As known by the MS/MS data the compounds were identified and number of double bonds specified but no position of the double bond could be determined. No significant interfering peaks from the tissue were found at the retention time and in the ion channels of the internal standard or the analytes. Very recently, Kasuya et al. [14] described a quite similar method for medium-chain acyl-CoAs determination based on selected ion monitoring (SIM) with positive ionization mode. They used longer analysis time and achieved a better chromatographic separation than in our method. However, less interfering peaks occurred using multiple reaction monitoring in real biological samples.

3.2. Linearity and limit of quantitation

Linear calibration curves were obtained for the compounds of interest with correlation coefficient 0.99 or higher (Table 1). On the base of correlation coefficient and visual examination of the slopes method was estimated linear in the range of 0.1–15 pmol/μl. The linearity range of the method was considered adequate for the purpose of the biological sample analysis.

The lowest data point used for standard curve (0.1 pmol/μl) was well above the criteria for LOQ [16]. The signal-to-noise ratio was over 20 and the precision (R.S.D.) was under 10% for all compounds. Acyl-CoA levels in biological samples studied were above lowest data point.

3.3. Recovery

Recovery was tested by spiking samples and control samples with internal standard and C16:0 standard before and after extraction. The signals obtained from samples were compared and recovery was calculated. Liquid–liquid phase extraction procedure was found to be suitable technique for sample preparation resulting in satisfying recoveries. The recovery obtained after extraction was 60 ± 5%. The calculated recovery of the method is average when compared to the described procedures. Shrago et al. [8] reported extraction procedure with recovery over 80% but the results could not be repeated by others using the methodology described [3]. Additional studies found similar or lower recovery levels than obtained in our study [6–9]. The sample loss during evaporation was assessed insignificant.

3.4. Precision and accuracy

The extraction procedure precision and accuracy obtained for C16:0 is shown in Table 2. For precision and accuracy the amount of added C16:0 was estimated and compared to known added amount. The deviation (S.D.) and R.S.D. were calculated.

Ten replicate measurements were analyzed to test for reproducibility and the results are shown in Table 3. The estimated concentrations were within 15% for all compounds and Table 4 shows the summary of the individual QC data obtained from four separate runs used for validation of the method. In each run the deviations of the predicted concentrations were within ±15% for at least 95% of the QC samples as required by Food and Drug Administration [17].

Table 3
The results obtained from ten replicate runs for reproducibility (intra-day) test

Compound	Concentration mean ± S.D. (pmol/μl)	Precision (R.S.D.)
C16:0	0.735 ± 0.074	10.0
C16:1	0.302 ± 0.044	14.5
C18:0	0.975 ± 0.066	6.8
C18:1	1.432 ± 0.148	10.3
C20:0	0.153 ± 0.026	14.8
C20:4	0.806 ± 0.071	8.9

Table 4
Inter-day precision and accuracy obtained for acyl-CoAs

Compound	*n*	Concentration (pmol/μl)	Estimated mean ± S.D. (pmol/μl)	Precision (R.S.D.)	Accuracy (percent error)
C16:0	4	3.3	3.840 ± 0.265	6.9	16.4
	4	0.67	0.690 ± 0.075	10.9	3.0
C16:1	4	3.3	3.273 ± 0.385	11.8	0.8
	4	0.67	0.715 ± 0.061	11.7	6.7
C18:0	4	3.3	3.060 ± 0.269	8.8	7.3
	4	0.67	0.578 ± 0.010	1.7	13.8
C18:1	4	3.3	3.230 ± 0.376	11.6	2.1
	4	0.67	0.603 ± 0.017	2.8	10.1
C20:0	4	3.3	3.010 ± 0.081	2.7	8.8
	4	0.67	0.768 ± 0.015	2.0	14.6
C20:4	4	3.3	3.195 ± 0.310	9.7	3.2
	4	0.67	0.730 ± 0.048	6.6	9.0

Table 5
Long-chain acyl-CoA concentrations in rat liver and brain tissues

Acyl-CoA	Liver (pmol/μl)[a]	Brain (pmol/μl)[a]
C16:0	0.83 ± 0.34	2.86 ± 0.46
C16:1	0.38 ± 0.16	0.16 ± 0.03
C18:0	2.17 ± 0.51	1.44 ± 0.23
C18:1	1.70 ± 0.58	2.63 ± 0.44
C20:0	0.82 ± 0.05	0.15 ± 0.03
C20:4	1.96 ± 0.57	0.81 ± 0.07

Results are obtained from six animals.
[a] Mean ± S.D. from six animals.

3.5. Application of the method

The method described is applied to long-chain acyl-CoA determination from rat liver and brain tissues. The measured acyl-CoA levels in these tissues from six animals are listed in Table 5. No significant interfering peaks from the tissue were found at the retention time and in the ion channels of the internal standard or analytes. If necessary, the volume of used samples (now 2 ml of 15 ml) could be increased to improve sensitivity of the method for biological samples.

4. Conclusions

LC–MS/MS method in negative ionization mode is very suitable method for analyzing long-chain fatty acid acyl-CoA compounds in rat liver samples with good specificity and reliability for routine use. Using a C_4 reversed-phase column gave satisfactory chromatographic separation from early eluting impurities. Negative electrospray ionization is suitable for acyl-CoA compounds and excellent MS/MS spectra for long-chain acyl-CoAs can be obtained. The method proved to be sensitive enough for determination of all compounds of interest using 0.4–0.7 g of tissue and was validated in the range of 0.1–15.0 pmol/μl.

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