Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 859 (2007) 131–136
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

High-performance liquid chromatography method for quantifying sphingomyelin in rat brain

Diana J. Azzam [a,b], Julnar A. Usta [a], Youssef Mouneimne [c], Jimmy A. El Hokayem [a,b], Mohamad A. Mikati [a,b,*]

[a] Department of Biochemistry, Faculty of Medicine, American University of Beirut, Lebanon
[b] Department of Pediatrics and Adolescent Medicine, Faculty of Medicine, American University of Beirut, Lebanon
[c] Central Research Science Laboratory, American University of Beirut, Lebanon

Received 16 May 2007; accepted 5 September 2007
Available online 14 September 2007

Abstract

A rapid, reproducible and accurate high-performance liquid chromatographic (HPLC) method for the quantitative determination of sphingomyelin in rat brain was developed and validated using normal-phase silica gel column, acetonitrile–methanol–water (65:18:17 (v/v)) at a flow rate of 1 ml/min, isocratic elution, UV detection at 207 nm and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine as an internal standard. Total run time was 10.0 min. The calibration curve was linear over the range of 0.025–0.4 mg/ml sphingomyelin ($R^2 > 0.99$). The intra-day coefficient of variation ranged from 1.4% to 2.2%. The average inter-day coefficient of variation over a period of 4 days was 3.1%. The practical limit of detection was 0.005 mg/ml with a quantification limit of 0.01 mg/ml.

Keywords: HPLC; Sphingomyelin; Brain; Internal standard; Rat; Isocratic; Quantification

1. Introduction

Sphingomyelin (SM), a phosphocholine ester of ceramide, is a principal structural and functional membrane component in mammalian tissue. The SM cycle is involved in the activation of specific transduction pathways that lead to apoptosis, proliferation, or senescence [1–5]. The importance of studying changes in SM levels in animal tissues is shown in many published reports that documented the alterations in SM levels with seizures, ischemia, cell death, oxidative stress, neurodegeneration, hepatic encephalopathy and many other pathological disorders [6–11]. Thus, in order to investigate the role of SM in health and disease, it is important to have sensitive, valid and reliable methods for quantification.

Unfortunately, many existing methods for the measurement of SM have several important disadvantages. Methods based upon incorporation of radioactive isotopes in enzymatic assays are very sensitive, but these are problematic due to their costly and time-consuming experimental procedures as well as the inconvenience in manipulation and disposal of radioisotopes. Consequently, most investigators have studied changes in SM levels in biological samples using chromatography with a variety of detection methods, such as ultra-violet (UV) detection [12–17], mass spectrometry (MS) [18–21] and evaporative light scattering detector (ELSD) [22–25].

UV detection is considered to be the method of choice in the assaying of SM as it is nondestructive to phospholipids [26–30]. In addition, measurement of SM by UV detection does not include the laborious steps of isolation, scraping, and re-extraction of SM on HPTLC [6,31,32]. Thus, it simplifies sample processing and improves measurement accuracy. So far, most published HPLC methods with UV detection have been used to separate SM from other phospholipids without quantification [12,13,15,27]. Only a few methods have been reported

* Corresponding author at: Adult and Pediatric Epilepsy Program, Department of Pediatrics and Adolescent Medicine, PO Box 11-0236/B52 Riad el Solh, American University of Beirut, Beirut 1107-2020, Lebanon.
Fax: +961 1 370781.
E-mail address: mamikati@aub.edu.lb (M.A. Mikati).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.09.005

for quantification of SM and these either lack validation and/or have not used an internal standard (I.S.) [14,16,17,26,28–30]. Some of these methods also use complicated gradient elution for separation [29].

Methods using LC-MS detection mode offer higher sensitivity and selectivity compared to UV detection methods but the disadvantage is the more limited accessibility to these systems due to very high hardware costs and complex extraction procedures.

The methods that use ELSD are widespread, however, they are often cumbersome to reproduce, calibration of standards require non-linear regression analyses due to varying sensitivity of the detector and often give results with high variations compared to UV detection methods. Moreover, some of these methods involve complex ternary gradient programs.

Therefore, there is a need for a simple, quick and inexpensive method to quantitate sphingomyelin in the brain on a routine basis. In this article, we describe what we believe is such a method. This method, the first to assay SM using an I.S., was validated using 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine as the I.S. compound which does not exist endogenously in the brain.

2. Experimental

2.1. Chemicals and reagents

Sphingomyelin and L-α-phosphatidylcholine (PC) from bovine brain, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and sphingomyelinase from human placenta were obtained from Sigma Chemical Co., St. Louis, USA. Acetonitrile (HPLC grade) and methanol (HPLC grade) were also purchased from Sigma Chemical Co. Laboratory-prepared water (Milli-Q) was used throughout the study.

2.2. High-performance liquid chromatography

The HPLC system was equipped with Agilent 1100 Series system (Agilent Technology, Palo Alto, CA, USA) consisting of vacuum degasser, temperature controlled autosampler, column thermostat, quaternary pump, photo diode array detector (DAD) and a Chemstation computer software system.

Chromatographic analysis was carried out using a Zorbax Rx-SIL silica gel (25 cm × 4.6 mm i.d., 5 μm particle size, 80 Å pore size) column from Rockland Technologies, Inc., USA. The column temperature was maintained at 25 °C. The analytical column was protected with an Rx-SIL Cartridge Guard column from Rockland Technologies, Inc., USA. Mobile phase flow rate was set to 1.0 ml/min with a run time of 10 min. The DAD was operated at 207 nm with the recorder response set at 0.4 absorbance units per full scale deflection (AUFS). The injection volume was set at 20 μl. An isocratic mobile phase containing acetonitrile–methanol–water (65:18:17, v/v) was used. The samples were quantified using linear regression of response (SM/I.S. peak area ratio) versus SM concentrations. Data was collected and processed using a Chemstation software for HPLC system.

2.3. Preparation of standards

Standard stock solutions of SM (0.4 mg/ml) and I.S. (5 mg/ml) were prepared by using chloroform–methanol (2:1, v/v). Successive dilutions of SM standards were made to obtain the five levels of calibration standards having the following concentrations: 0.025, 0.05, 0.1, 0.2 and 0.4 mg/ml. To each of these standard solutions, 400 μl of I.S. (5 mg/ml) was added, obtaining a concentration of 1 mg/ml of the I.S. All the resulting working solutions were kept in the refrigerator (4 °C) during manipulation.

2.4. Rat brain tissue preparation

Sprague–Dawley rats (200–300 g) were used. These were maintained in restricted-access rooms with a controlled temperature (23 °C) and a 12 h light–dark cycle and were allowed free access to standard laboratory diet and tap water. All experimental procedures were approved by the Institutional Review Board and all attempts were made to reduce pain and discomfort to experimental animals. Animals were sacrificed and hippocampi were dissected, weighed and then homogenized with 0.8 ml of SHT buffer (0.25 M Sucrose, 10 mM Hepes, 50 mM Tris, pH 7.4).

2.5. Preparation of the samples

Three biological quality control (QC) samples of hippocampi were prepared in order to have low, medium and high concentrations of endogenous SM. These were prepared as follows:

(1) Low QC containing one hippocampus in 0.8 ml SHT buffer.
(2) Medium QC containing two hippocampi in 0.8 ml SHT buffer.
(3) High QC containing four hippocampi in 0.8 ml SHT buffer.

The exact wet weight (brain equivalents) was calculated for each QC sample and the amount of protein levels were determined using the BioRad protein determination kit. Adequate low, medium and high concentrations of proteins were prepared from the QC samples and stored at −80 °C for lipid extraction. From each QC sample, 100 μl of homogenate volume was used for lipid extraction and 200 μl of I.S. solution (5 mg/ml) was added. Lipids were extracted according to the Bligh and Dyer method [33]. Briefly, 2 ml methanol, 2 ml chloroform and 1.7 ml distilled water were, consecutively added to each sample while vortexing and the samples were then centrifuged at 1500 rpm for 15 min. The lower phase was removed, dried under N_2 gas and then resuspended in 1 ml chloroform–methanol (2:1, v/v).

2.6. Analytical method validation

The chromatographic method was validated on 4 different days, to determine the linearity, sensitivity, precision and accuracy of the present HPLC method. Biological QC samples were prepared at three concentrations spanning the concentration range. Four samples of each QC pool were analyzed along with

fresh calibration standards on each day. The precision was calculated as the coefficient of variation (CV) within a single run (intra-day) and between different assays (inter-day).

2.6.1. Calibration curves and quantitation

Five levels of SM calibration standards, containing the I.S. at a constant concentration, were used to set up calibration curves in the 0.025–0.4 mg/ml concentration range using the procedure as described above in Section 2.3. The procedure was carried out in duplicate for each concentration. Calibration curves were constructed by plotting peak area ratio (y) of SM to the I.S. versus SM concentrations (x). Linear regression was used for quantitation. Quantification of SM in brain was possible since SM standards having the same fatty acid composition as in rat brain were used [34]. These fatty acids mainly stearic acid (18:0) and nervonic acid (24:1) constitute the highest percentage of SM in bovine brain [34]. Egg SM which consists primarily of palmitic acid (16:0) also eluted in the same peak as bovine brain SM. Thus, in our method, SM elutes as a single peak regardless of the fatty acid chain length (C12–C20).

Sensitivity was determined by the limit of detection (LOD) and the limit of quantification (LOQ) which gives rise to peaks whose heights are 3 and 10 times the baseline noise, respectively.

2.6.2. Precision and extraction yield (absolute recovery)

Precision was established for SM by analyzing four batches consisting of five-point calibration curves with corresponding four sets of low, medium and high quality control samples. On each day, five different calibration curve standards (range from 0.025 to 0.4 mg/ml) of SM were prepared and concomitantly a set of previously prepared QC homogenates (each of low, medium and high), was retrieved from the deep freezer and subjected to lipid extraction as per the procedure mentioned in Section 2.5. The percentage coefficient of variation (CV%) values were obtained within the same day to evaluate intra-day precision and over four different days to evaluate inter-day precision.

In order to find the percentage extraction yield of SM and I.S., two different concentrations of SM standard solutions (having constant I.S. concentration and corresponding to the lower limit and upper limit of the calibration curve) were added to blank brain homogenate. The mixtures were subjected to lipid extraction and injected into the HPLC system. The SM/I.S. chromatographic peak area ratios obtained in this way (after extraction) were compared to those obtained from standard solutions at the same theoretical concentration (before extraction).

2.6.3. Accuracy

Two different standard SM solutions (0.15 and 0.3 mg/ml), having a constant I.S. concentration, were used to determine the closeness between the true concentration value and the experimental results. Accuracy was expressed as percentage of deviation from theory [(measured concentration − nominal concentration)/nominal concentration] × 100.

In addition, a standard addition experiment was conducted to assess the accuracy of the analysis method and to investigate the possibility of interfering matrix effects in quantification of endogenous SM. Aliquots (100 μl) of brain homogenate samples (containing 2 hippocampi) were spiked with 200 μl of I.S. (5 mg/ml) and known amounts of the standard SM to give concentrations of 0.1, 0.2 and 0.4 mg/ml. The amounts of added SM were calculated after measuring endogenous SM, to get the above mentioned final concentrations. The samples were then extracted and lipids were suspended in 1 ml of chloroform:methanol (2:1) and analyzed by HPLC, as described above.

2.6.4. Assay specificity

In order to confirm the absence of UV-absorbing contaminants that co-elute with sphingomyelin in the tissue extract, an experiment to confirm assay specificity was necessary. Two brain homogenate samples, with 100 μl of I.S. (5 mg/ml), were incubated with or without a specific sphingomyelinase from human placenta (0.1 unit of sphingomyelinase in 100 μl homogenate). Both samples were kept in a water bath at 37 °C for 2 h. After that, lipid extraction was performed and the lipids were suspended in 1 ml of chloroform:methanol (2:1) before passing through the HPLC machine.

3. Results

3.1. Method development

The aim of this work was the development of a rapid HPLC assay with a total run time < 10 min, while achieving suitable sensitivity and selectivity. The mobile phase was chosen after several trials with acetonitrile, methanol and water solutions in various proportions and at different pH values. A mobile phase consisting of acetonitrile–methanol–water (65:18:17, v/v/v) was selected to achieve maximum separation and interference-free chromatogram. Changing the mobile phase composition to 59% acetonitrile led to a better run time but SM and the I.S. were not resolved. Flow-rates between 0.5 and 2 ml/min were studied. A flow rate of 1.0 ml/min gave an optimal signal to noise ratio with a reasonable separation time. The selected chromatographic conditions provided good resolution of SM and the I.S. Identification of phospholipids was carried out by comparison with the retention time of pure standards. The average retention times for PC, I.S. and SM were observed to be 7.024, 8.223 and 9.158 min respectively (Fig. 1A). The maximum absorption of each of SM and I.S. was detected at 207 nm. Consequently, this wavelength was chosen for the analysis. Typical chromatogram of SM and I.S. is shown in (Fig. 1A). The sample was obtained from hippocampus of adult rat with 1 mg/ml of I.S. added before extraction. The chromatogram shows that SM and I.S. are separated completely and none of the brain endogenous components interfere with the assay. Only one detectable peak, that of PC, was found with retention times close to those of SM and I.S. in the lipid extract from brain tissue. In brains of both humans and laboratory animals, PC is the predominant (>95%) phospholipid class, whereas SM comprises only 1–3% of total phospholipids [35].

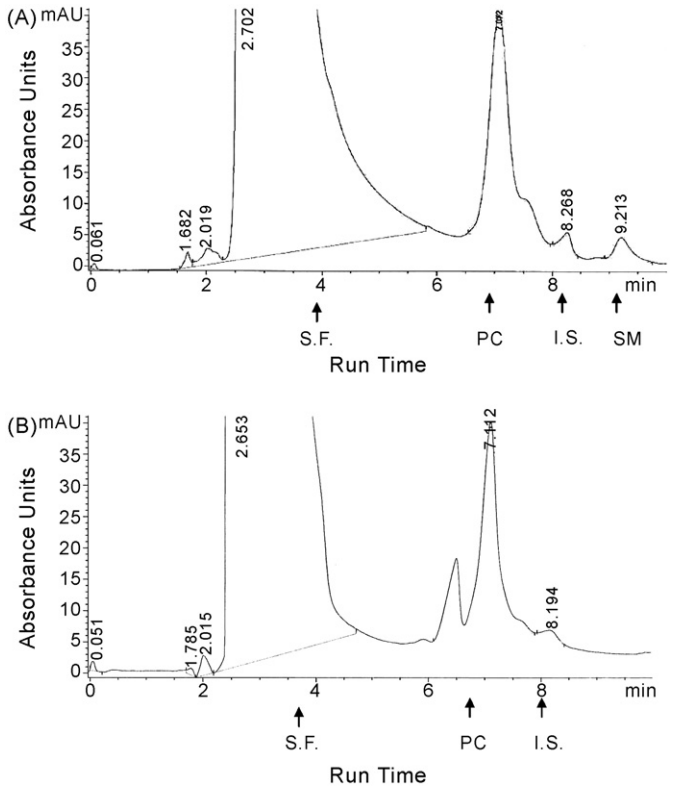

Fig. 1. (A) Representative chromatogram of lipid extract from an adult rat brain with 1 mg/ml of I.S. added before extraction. Chromatographic conditions: Zorbax Rx-SIL column (250 mm × 4.6 mm, 5 μm); mobile phase acetonitrile–methanol–water (65:18:17 (v/v)); injection volume 20 μl. (B) Chromatogram of lipid extract from brain sample with sphingomyelinase treatment as compared to (A) without sphingomyelinase treatment. Sphingomyelinase enzyme was added for 2 h at 37 °C. Note the disappearance of the SM peak after treatment with the enzyme. S.F.: solvent front; PC: phosphatidylcholine; I.S.: internal standard; SM: sphingomyelin.

3.2. Linearity and range

The linearity of the method was found in the 0.025–0.4 mg/ml concentration range for SM. Correlation coefficients (r) of standard curves, constructed during the course of validation (4 days) were found to be consistently greater than 0.999 for SM; the corresponding regression equation was $y = 31.398x - 0.2044$, where y is the SM/I.S. peak area ratio and x is the SM concentration (mg/ml). The linear curves in this range of concentration and the correlations make it suitable for quantitation.

3.3. Sensitivity

The LOD and the LOQ, experimentally verified by six injections of SM at the LOD and LOQ concentrations, were 0.005 mg/ml (6.839 μM) and 0.01 mg/ml (13.679 μM) respectively. Since endogenous SM concentrations in one hippocampus of rat brain, have been reported in the millimolar range (aproximately 0.56 mM) [36], our method is validated to be sufficiently sensitive to detect endogenous SM concentrations.

3.4. Precision and extraction yield

Results of precision assays are shown in Table 1 and expressed as CV (%). The coefficient of variation ranged from 1.4% to 2.2% for within-day while the inter-day precision was lower than 3.7% which indicates that the method is precise.

Extraction yield assays led to mean absolute recovery of SM to be 90.69% while a mean absolute recovery of I.S. to be 89.07%. All the values obtained were within the limits required for biological samples, 100 ± 15% for absolute recovery (both the analyte and I.S.) and less than 15% for CV [37].

3.5. Accuracy

To assess the accuracy of our analytical method, a standard addition experiment was conducted. As described above, this experiment was performed by spiking 100 μl aliquot of a brain homogenate sample with internal standard and known amounts of exogenous SM to give concentrations of 0.1, 0.2 and 0.4 mg/ml. Analysis by HPLC yielded the standard addition curve for SM ($R^2 = 0.994$) with corresponding regression equation: $y = 38.437x - 0.4372$ where y is the SM/I.S. peak area ratio and x is the SM concentration (mg/ml). Extrapolation to $y = 0$ yielded an endogenous SM concentration of 0.045 mg/ml. In addition, various concentrations of the standard SM and a fixed concentration of the I.S. were analyzed. A calibration

Table 1
Precision results of biological QC samples with I.S.

Sample concentration	Intra-day		
	Concentration of SM measured (mg/ml) (mean ± S.E.)	Spiked I.S. concentration (mg/ml)	Coefficient of variation (%)
Low[a]	0.0236 ± 0.00023	1	1.43
Medium[a]	0.0469 ± 0.00058	1	1.76
High[a]	0.1026 ± 0.00158	1	2.22
	Inter-day		
Low[b]	0.0239 ± 0.00028	1	2.29
Medium[b]	0.0463 ± 0.00056	1	2.39
High[b]	0.0995 ± 0.00162	1	3.67

[a] Four different samples of each concentration were extracted on the same day.
[b] The assay was performed over 4 days.

Table 2
Summary of method accuracy results

Sample	Theoretical concentration (mg/ml)	Measured concentration[a] (mg/ml)	Deviation from theory (%)	Analytical recovery[a] (%)
SM	0.15	0.148	−1.33	98.95
	0.30	0.295	−1.67	98.46

[a] Based on three data points.

curve ($R^2 = 1$) was derived with corresponding regression equation $y = 23.753x + 1.0751$. A duplicate sample was prepared and analyzed by using the calibration curve which gave an endogenous SM concentration of 0.047 mg/ml. These results indicate that the extraction and quantitation steps used in the analysis method are accurate and reproducible.

Table 2 summarizes the results of determining the closeness between the true concentration values of SM standard and the measured concentration by HPLC.

3.6. Specificity

The specificity of the method was demonstrated by comparing retention time and UV spectra of the SM peak obtained in the lipid extract with the Sphingomyelin standard.

No chromatographic interferences were found with tested molecules such as 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine and Ceramide Phosphoryl-ethanolamine. The peak observed at 7.00 min was found to be PC. Lipid extract only, without addition of I.S., showed only two peaks within the run time, specifically the SM and PC peaks.

When biological sample was treated with specific sphingomyelinase enzyme, all of the UV absorbing compounds in the SM region of the chromatogram disappeared as shown in Fig. 1B. This demonstrates that the method is specific for SM and no other UV-absorbing contaminant co-eluted with the SM peak. The figure also shows a split in the PC peak, after treatment with sphingomyelinase, probably due to the presence of a PC with a new fatty acid chain formed as a result of SM breakdown.

4. Discussion

Based on the growing interest of studying the association of SM levels with disease states, we have developed a validated method to determine SM concentration in brain tissue, which is suitable for assessing large number of samples.

Previously published methods in the field used external calibration alone [16,28–30,38] or extinction coefficient of the material analyzed [26] for quantitation and have not used an I.S. because, presumably of the difficulty in selecting a specific I.S. that does not interfere with other peaks. The novelty of our study is the use of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine as I.S., i.e., a molecule, which has not been detected to occur endogenously in the brain**.** Several advantages, in the absolute quantification of SM, are associated with the inclusion of an I.S. It allows tracking and controlling analyte recovery before and during analysis. It also allows correction for sampling errors and uncontrollable changes between runs. In addition, it compensates for inadvertent losses during the extraction process and variation in other aspects of the HPLC assay such as minor differences in sample injection volume or detector sensitivity.

The method described here is a novel approach that has several advantages over earlier HPLC published methods of separation and quantitation of sphingomyelin. First, our method is the first that provides within-day and between-day validation of endogenous SM measurements in the brain after multiple daily and between day lipid extractions performed on each of low, medium and high endogenous SM concentrations. Second, our method has the practical advantage of a short chromatographic run time and of simple sample preparation, as lipid extraction is quicker and simpler than derivatization procedures used in many previous methods. Third, our method has a high accuracy, comparable to the best of the previously described methods, due in part to the direct injection of the extraction mixtures. Fourth, and probably most importantly, our assay introduces, for the first time, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine as a reliable I.S. to be used in other SM assays and possibly in assays for other products of the ceramide pathway. This assay complies with proposed FDA guidelines for the validation of a bioanalytical method in terms of linearity, precision, accuracy of the method and stability for standards and QC samples in biological samples [37].

5. Conclusion

In summary, we have developed an HPLC method for the quantification of SM in rat brain using the 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine as an internal standard which allows rapid measurement of absolute quantities in validated manner. It has the advantage of being rapid, facile, sensitive and selective similar to previously described HPLC methods and of being the only one that uses an internal standard and that provides intra-day and inter-day validation of SM measurements.

Acknowledgements

This study was supported by an institutional grant from the American University of Beirut and from the Lebanese National Council for Scientific Research.

References

[1] Y.A. Hannun, R.M. Bell, Science 243 (1989) 500.
[2] R.N. Kolesnick, Prog. Lipid Res. 30 (1991) 1.
[3] Y.A. Hannun, J. Biol. Chem. 269 (1994) 3125.
[4] T. Okazaki, R.M. Bell, Y.A. Hannun, J. Biol. Chem. 264 (1989) 19076.

[5] T. Levade, N. Andrieu-Abadie, B. Segui, N. Auge, M. Chatelut, J.P. Jaffrezou, R. Salvayre, Chem. Phys. Lipids 102 (1999) 167.
[6] M. Nakane, M. Kubota, T. Nakagomi, A. Tamura, H. Hisaki, H. Shimasaki, N. Ueta, Neurosci. Lett. 296 (2000) 89.
[7] I. Swapna, K.V. Kumar, P.V. Reddy, C.R. Murthy, P. Reddanna, B. Senthilkumaran, Neurochem. Int. (2006).
[8] I. Swapna, K.V. Sathya Sai Kumar, R. Murthy Ch, B. Senthilkumaran, Neurotoxicology 27 (2006) 402.
[9] A. Drgova, K. Likavcanova, D. Dobrota, Gen. Physiol. Biophys. 23 (2004) 337.
[10] T.P. Kulagina, N.A. Shevchenko, V.I. Arkhipov, Biochemistry (Mosc) 69 (2004) 1143.
[11] A.V. Alessenko, A.E. Bugrova, L.B. Dudnik, Biochem. Soc. Trans. 32 (2004) 144.
[12] S. Mawatari, K. Saito, K. Murakami, T. Fujino, Metabolism 53 (2004) 123.
[13] Q.F. Jiang, Z.S. Luo, X.F. Li, L.L. Jiang, Se Pu 18 (2000) 142.
[14] C.P. Nibbering, M.C. Carey, J. Membr. Biol. 167 (1999) 165.
[15] S. Mawatari, K. Murakami, Anal. Biochem. 264 (1998) 118.
[16] R. Shafiq ur, J. Chromatogr. 567 (1991) 29.
[17] A. Rodriguez-Bernaldo de Quiros, J. Lopez-Hernandez, J. Simal-Lozano, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 770 (2002) 71.
[18] D. Pacetti, E. Boselli, H.W. Hulan, N.G. Frega, J. Chromatogr. A 1097 (2005) 66.
[19] S. Uran, A. Larsen, P.B. Jacobsen, T. Skotland, J. Chromatogr. B Biomed. Sci. Appl. 758 (2001) 265.
[20] A.A. Karlsson, P. Michelsen, G. Odham, J. Mass Spectrom. 33 (1998) 1192.
[21] W.C. Byrdwell, D. Borchman, Ophthalmic Res. 29 (1997) 191.
[22] A. Sala-Vila, A.I. Castellote, M. Rodriguez-Palmero, C. Campoy, M.C. Lopez-Sabater, Nutrition 21 (2005) 467.
[23] R. Rombaut, J.V. Camp, K. Dewettinck, J. Dairy Sci. 88 (2005) 482.
[24] A. Avalli, G. Contarini, J. Chromatogr. A 1071 (2005) 185.
[25] B.J. Stith, J. Hall, P. Ayres, L. Waggoner, J.D. Moore, W.A. Shaw, J. Lipid Res. 41 (2000) 1448.
[26] F.B. Jungalwala, J.E. Evans, R.H. McCluer, Biochem. J. 155 (1976) 55.
[27] S.F. Chen, P.H. Chan, J. Chromatogr. 344 (1985) 297.
[28] O. Itasaka, T. Hori, K. Sasahara, Y. Wakabayashi, F. Takahashi, H. Rhee, J. Biochem. (Tokyo) 95 (1984) 1671.
[29] R.L. Briand, S. Harold, K.G. Blass, J. Chromatogr. 223 (1981) 277.
[30] S.S. Chen, A.Y. Kou, J. Chromatogr. 227 (1982) 25.
[31] L. Barrier, S. Ingrand, A. Piriou, A. Touzalin, B. Fauconneau, Neurosci. Lett. 385 (2005) 224.
[32] J.H. Zhong, Q.Y. Guo, R.G. Ye, B. Lindholm, T. Wang, Adv. Perit. Dial. 16 (2000) 36.
[33] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 37 (1959) 911.
[34] B. Ramstedt, P. Leppimaki, M. Axberg, J.P. Slotte, Eur. J. Biochem. 266 (1999) 997.
[35] L.J. Macala, R.K. Yu, S. Ando, J. Lipid Res. 24 (1983) 1243.
[36] M. Chavko, E.M. Nemoto, Stroke 23 (1992) 1000.
[37] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, et al., Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 249.
[38] J. Boswart, T. Schmidt, P. Kostiuk, V. Pacakova, K. Stulik, J. Chromatogr. 495 (1989) 61.