ELSEVIER

Journal of Chromatography B, 744 (2000) 407–413

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Quantitative determination of dipalmitoylphosphatidylcholine and palmitic acid in porcine lung surfactants used in the treatment of respiratory distress syndrome

C.S. Gross[a], P.J. Simms[b,*], R.W. Towne[c], R.E. Miller[c]

[a]*Biosource Technologies, Inc., 3333 Vaca Valley Pkwy, suite 1000, Vacaville, CA 95688, USA*
[b]*Chugai Biopharmaceuticals, Inc., 6275 Nancy Ridge Rd., San Diego, CA 92121, USA*
[c]*Dey Laboratories, Inc., 2751 Napa Valley Corporate Dr., Napa, CA 94558, USA*

Received 21 September 1999; received in revised form 8 May 2000; accepted 8 May 2000

Abstract

A high-performance liquid chromatography (HPLC) method was developed that can separate and quantify dipalmitoylphosphatidylcholine and its degradation product, palmitic acid from various phospholipids contained in a porcine lung surfactant used in the treatment of respiratory distress syndrome, which was recently approved for use by the FDA. The method used a C_8 reversed-phase HPLC column with a (50:45:10) acetonitrile/methanol/acetic acid mobile phase, and refractive index detection. The active component of the lung surfactant, dipalmitoylphosphatidylcholine (DPPC) and palmitic acid (PA), could be quantified following a liquid-liquid extraction procedure along with an internal standard, dimyristoylphosphatidylcholine (DMPC). The assay was validated for linearity, accuracy, precision, reproducibility and ruggedness. Column stability was measured by performing the assay over time and monitoring the system suitability parameters. The extraction procedure has a 90% recovery and the assay is linear over a range of 5 μg/ml to 300 μg/ml. The assay is used to release commercial product and monitor stability of existing lots of material.

Keywords: Phospholipids; Dipalmitoylphosphatidylcholine; α-Lysophosphatidylcholine; Sphingomyelin; Phosphatidylcholine; Palmitic acid

1. Introduction

Respiratory Distress Syndrome (RDS) is the most common pulmonary disorder in premature infants. Avery and Mead were the first to demonstrate that a deficiency in lung surfactant is the major cause of RDS and that such deficiency results in high surface tension on the alevolii [1]. Later studies confirmed a correlation between deficiency in the synthesis of lung surfactant and a high rate of RDS [2]. Various natural and synthetic lung surfactants have been identified for the purpose of treating this syndrome [3–6]. Early results using aerosol preparations containing dipalmitoylphosphatidylcholine (DPPC), the major component identified in lung surfactants, were not encouraging [7]. Synthetic surfactants containing only DPPC as the primary active component had very low activity. Enhorning and Robertson demon-

*Corresponding author. Tel.: +1-858-535-5993; fax: 1-858-546-5977.

E-mail address: psimms@chugaibio.com (P.J. Simms)

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00276-0

strated, however, that crude natural surfactant, applied to the airways, protects against respiratory complications resulting from a surfactant deficiency [8]. It was later determined that a mixture of phospholipids and lipid-bound surfactant proteins are necessary to give the lung surfactant its full activity. There are four surfactant proteins that provide activity to the lung surfactant, surfactant protein A, B, C, and D [9–14]. Without the surfactant proteins available to serve a defense function, premature infants often succumb to infection at the same time, the phospholipid mixture in the surfactant act to reduce surface tension on the lung alveoli [16–18]. Currently, two surfactants have been approved for widespread clinical use in the United States. Exosurf is a protein free surfactant wherein hexadecanol and tyloxapol assume some of the functions of the surfactant proteins in the native surfactant [19]. Survanta, derived from bovine lung, contains only two of the four known surfactant proteins. Although lung surfactant proteins account for less than 10% by weight of pulmonary surfactant, they are known to be important for optimizing surfactant function in a variety of ways [11,20–22]. The porcine lung surfactant, which has been approved by the FDA, contains all four surfactant proteins and is more active. To accurately characterize the surfactant mixtures, we developed an HPLC method that can separate the various phospholipids, and quantifies the phospholipid most related to surfactant activity.

The majority of HPLC separations for phospholipids have been carried out using C_{18} reversed-phase stationary phases [23–29]. These separations involved several different types of mobile phases and detectors. Isocratic separations have been carried out using UV detectors at 200–210 nm, or using refractive index detection [30–34]. With UV detection, however, low sensitivity and variation in response factors make quantifying of lipids difficult. Universal detectors such as evaporative light scattering detectors (ELSD) and mass spectrometry are useful for quantification, but ELSD requires elevated temperatures, which lead to rapid sample degradation, and MS can be expensive.

We now report on a sensitive HPLC assay for quantification of DPPC and its degradation component, palmitic acid, in porcine lung surfactant that uses a C_8 reversed-phase column along with refractive index detection.

2. Experimental

2.1. Materials

The C_8 and C_{18} Inertsil columns 150×4.6 mm was purchased from MetaChem Technologies (Torrance, CA, USA). Dipalmitoylphosphatidylcholine (DPPC 99% purity), α-Lysophosphatidylcholine (L-PC, 99% purity), sphingomyelin (Sph, 99% purity), phosphatidylcholine (PC, 99% purity), Dimyristoylphosphatidylchloline (DMPC, 99% purity) and palmitic acid (PA, 99% purity) standards were purchased from Sigma Chemical Co. (St Louis, MO, USA). Porcine lung surfactant was obtained from Chiesi Pharmaceuticals, Inc. (Milan, Italy). All reagents used were purchased from Fisher Scientific Co. The 0.2 μm nylon filters were purchased from Alltech Associates (Deerfield, IL, USA).

The HPLC system used was a Hitachi system equipped with an L-7300 column oven, L-7200 autosampler, L-7100 pump and a D-7000 interface. Samples were detected on an L-3350 refractometer or a Waters 410 differential refractometer.

2.2. Preparation of mobile phase

The mobile phase was prepared by combining 500 ml acetonitrile, 450 ml methanol and 100 ml 50 m*M* acetic acid so that the final ratio was 50:45:10. It was filtered through a 0.22 μm filter and allowed to warm to room temperature. The mobile phase was degassed using an online degasser and the flow-rate was 1 ml/min. A 100 μl injection was used for the standards and the samples.

2.3. Preparation of samples

Samples of standard lipids and samples of the porcine lung surfactant (0.5 mg/ml) were dissolved in the mobile phase (50:45:10) acetonitrile/methanol/acetic acid 10 m*M*. This was diluted 1:1 with chloroform/methanol (2:1). DMPC was dissolved in chloroform/methanol at a final concentration of 5 mg/ml and this was used as the internal standard. 40 μl of a stock solution DMPC was added to each sample, resulting in a final concentration of 200 μg per sample. Samples were evaporated to dryness under N_2 at 37°C for 30 min. The dry lipids were then reconstituted in 200 μl of mobile phase and

filtered through a 0.2 μm nylon filter. 100 μl injection volume was used for the analysis.

The Folch extraction procedure was performed by taking the dissolved sample and adding it to a chloroform/methanol/50 m*M* acetic acid solution (8:4:3). This was centrifuged for 5 min at 9000 *g*. The organic layer was collected and the samples were evaporated to dryness under N_2 at 37°C for 30 min [17,34].

The Schmid extraction procedure was performed by taking the dissolved sample and adding it to a chloroform/methanol/50 m*M* acetic acid solution (12:6:1). This solution had to maintain an organic/aqueous ratio of 17:1 to ensure a single phase. The resulting sample was evaporated to dryness under N_2 at 37°C for 30 min [17].

Recovery studies were performed by mixing the various lipids together at 100 μg/ml performing the appropriate extraction, and comparing the peak areas of the extracted samples with neat samples. The recovery studies were performed in triplicate to ensure accuracy of the extraction. Since the lung surfactant already contained DPPC it was not possible to spike DPPC into the mixture and determine the percent recovered. Therefore, the percent recovery was determined by comparing the two extraction peak areas to one another.

2.4. Validation of the assay

The assay was validated for dipalmitoylphosphatidylcholine (DPPC) and palmitic acid (PA) over a range of 5–300 μg/ml. Intra-day variability and linearity were examined by analyzing three standard curves, and precision was determined by making five replicate injections using separately prepared concentrations at levels near the low, middle and high points of the standard curves. The lower limit used was ten times the limit-of-detection (0.5 μg/ml), which is commonly used for quantification limits. Above 300 μg/ml the assay started to deviate from linearity. Inter-day variability was examined by repeating the analysis on a second day. Reproducibility and ruggedness were measured by having a second analyst repeat the analysis on two different days. The limit of detection was determined by diluting stock solutions of the lipids until a signal-to-noise ratio of 3:1 was obtained.

3. Results and discussion

3.1. Sample preparation

Porcine lung surfactant is composed of several phospholipids and the known amount of each phospholipid found in porcine lung surfactant is listed in Table 1. The lung surfactant contained salts that were incompatible with the HPLC mobile phase and often precipitated on the column during the analysis. In addition, the added water in the sample would often cause negative peaks to occur in the chromatogram due to the difference in refractive index. These negative peaks would often interfere with the peaks that eluting at the same time. In order to avoid this an extraction procedure were used. Two extraction procedures were tried: one involving the Schmid extraction solvent (chloroform/methanol/acetic acid 12:6:1) and Folch extraction solvent, which was 8:4:3 chloroform/methanol/acetic acid [17]. The Schmid extraction solvent involved a one phase system and in order to achieve this the ratio of chloroform and methanol to water must be 17:1 or greater. The Schmid extraction allowed the phospholipid to remain solvated and stay in the single

Table 1
Composition of the natural porcine lung surfactant

Components	Concentration (mg/ml)
Total phospholipids	74.4
Phosphatidylcholine (PC)	49.0
Dipalmitoylphosphatidylcholine (DPPC)	26
Acidic phospholipids	
Phosphatidylserine (PS)	0.97
Phosphatidylinositol (PI)	3.27
Phosphatidylglycerol (PG)	2.16
Other phospholipids	
Sphingomyelin (SM)	9.15
Phosphatidylethanolamine (PE)	7.3
Lysophosphatidylcholine (L-PC)	1
Fatty acids	0.55
Triglycerides	0.1
Cholesterol	0.02
Total surfactant protein	1
Cetyl alcohol	Not present
Tyloxapol	Not present
Tripalmitin	Impurity
Palmitic acid	Impurity

phase. The Folch extraction involved a two phase system. In this system the aqueous portion should contain saline and acid to prevent sample degradation. The Folch extraction had an addition step that called for centrifugation to ensure complete separation. A comparison of the methods was carried out using the standard samples and the percent recovery was used to determine extraction efficiency. When the samples were extracted using the two extraction conditions, the Folch extraction procedure gave the best results. Table 2 shows an 88% or higher recovery for all the major lipids. The single phase extraction only gave good recovery of the internal standard and L-PC (Table 2).

3.2. Chromatography

HPLC was performed on the major lipid components of the lung surfactant. Other lipids listed in the Table 1 were only found in trace amounts and were not analyzed by this method. Analysis of purchased phospholipid standards revealed that they were heterogeneous mixtures, with the exception on DPPC, DMPC and palmitic acid (data not shown). Multiple peaks arising in the chromatogram were given the same abbreviation. This allowed us to determine how certain peaks in the sample were eluting when the conditions were changed.

The mobile phase was allowed to warm to room temperature after preparation. Freshly prepared mobile phase is cold, and caused the RI detector baseline to drift considerably during the analysis. The column temperature control was unable to completely prevent baseline instability, unless the solvent temperature was permitted to equilibrate to ambient room temperature. The assay time was reduced by increasing the column temperature to 45°C. On a C_{18} column this made the total analysis time around 40 min. When the column was switched to a C_8 column the analysis time was reduced to less than 30 min. Under these conditions DPPC eluted in approximately 17 min compared to 24 min on the C_{18} column. There was little loss in resolution when the column was switched from a C_{18} column to a C_8 column.

Triethylamine was tried, in place of acetic acid, as the aqueous portion of the mobile phase. This resulted in increased peak tailing of the phospholipids. In addition, there was increased hydrolysis of some of the phospholipids at the elevated temperatures. When acetic acid is used, the peaks are sharper and there is no on-column degradation of the sample.

The separation is best using a C_8 reversed-phase column with a 50:45:10 (v/v/v) acetonitrile/methanol/acetic acid 50 m*M* pH 3.0 mobile phase at 45°C (Fig. 1). A C_8 column allows much faster analysis time than a C_{18} column. There was little loss in

Table 2
Percent recovery of phospholipids after sample extraction[a]

Component	Schmid extraction (%)	Folch extraction (%)
PC	65	95.4
DPPC	78.3	93.3
L-PC	93.3	91.3
PA	62.8	92.8
Sph	42.8	88.3
DMPC	100	100

[a] The recovery samples were analyzed on a C_8 Inertsil 150×4.6 mm column using an 50:45:10 acetonitrile/methanol/50 m*M* acetic acid mobile phase at 1 ml/min and 45°C with refractive index detection. L-PC: α-Lysophosphatidylcholine; PA: Palmitic acid; Sph: Sphingomyelin; DPPC: Dipalmitoylphosphatidylcholine; PC: Phosphatidylcholine and DMPC: Dimyristoylphosphatidylcholine.

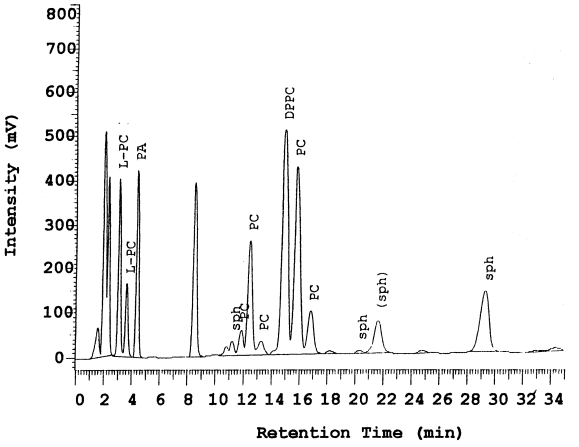

Fig. 1. A chromatogram of a mixture of standard phospholipids on a C_8 Inertsil 150×4.6 mm column using an 50:45:10 acetonitrile/methanol/50 m*M* acetic acid mobile phase at 1 ml/min and 45°C with refractive index detection. L-PC: α-Lysophosphatidylcholine; PA: Palmitic acid; Sph: Sphingomyelin; DPPC: Dipalmitoylphosphatidylcholine; PC: Phosphatidylcholine and DMPC: Dimyristoylphosphatidylcholine.

resolution when going from the C_{18} to the C_8 column. L-PC and PA eluted close to each other; however, the resolution between the two was good enough (resolution of greater than 1.5) to quantitate the amount of PA present in the sample. The resolution (resolution of 0.9) between DPPC and one peak from PC was good enough to calculate the concentration of DPPC accurately. System suitability test performed on the system should that the column was good to approximately 300 injections. During that time there was little loss in resolution. Afterward, the early eluting peaks started to co-elute with the void.

When porcine lung surfactant is analyzed under optimized conditions, the chromatograms show all phospholipids are well resolved, and there is no interference from negative peaks due to excess water in the sample (Fig. 2). In addition, there were no peaks that eluted at the same retention time as the internal standard. Chromatograms of partially degraded lung surfactant samples demonstrated that L-PC and PA are well resolved from and the void volume peak and were easy to quantify. The peak shapes were good and there were no interfering peaks from salts or water in the sample. The extraction procedure decreased the limit of detection to 0.5 μg/ml for DPPC and PA.

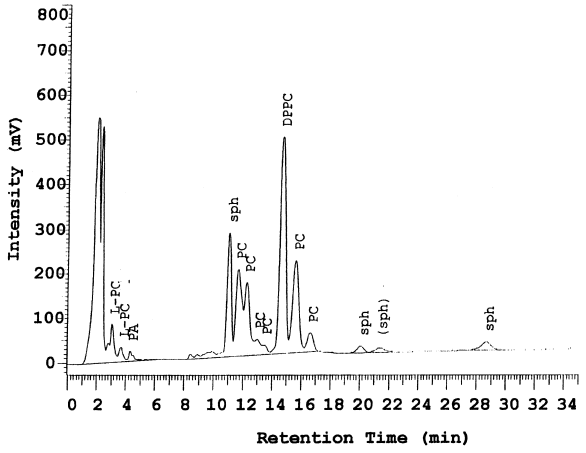

Fig. 2. A chromatogram of the lipid compounds of a porcine lung surfactant on a C_8 Inertsil 150×4.6 mm column using a 50:45:10 acetonitrile/methanol/50 m*M* acetic acid mobile phase at 1 ml/min and 45°C with refractive index detection. See Fig. 1 for lipid abbreviations.

3.3. Assay validation

The assay was validated for the major active component DPPC and its degradation product PA, which is produced as the lung surfactant ages. L-PC is another degradation product, but considering the heterogeneity of the sample the amounts of this was not determined. Other lipids were considered impurities and had little effect on the overall activity of the surfactant. Therefore, the other phospholipids are only separated from the phospholipids of interest and not quantified. However, these phospholipids were spiked into the samples to ensure accuracy and precision could be obtained. The assay was validated over a range of 5–300 μg/ml and both compounds had an r^2 value of greater than 0.99. Precision and accuracy were determined by making five replicate injections at concentration levels of 20 μg/ml, 125 μg/ml and 275 μg/ml, and the experimental amounts were compared to the theoretical amounts. The data is tabulated in Table 3. Even though DPPC eluted close to a peak from PC, the concentration of DPPC could still be accurately determined as seen in Table 3. The method is linear and precise over this range and good for quantitative use. Data tabulated in Table 4 showed the amounts of DPPC and PA that were determined to be present in some lots of the porcine lung surfactant. There were only trace amounts of PA present in the active lots. This assay could be used to monitor stability of lung surfactants used in the treatment of R.D.S. The assay was used to monitor stability and to release the lung surfactant for clinical studies, as well as, commercial use. In addition, the concentration of the active component, DPPC, could be monitored, as well as its degradation product PA. An increase in PA concentration would decrease the potency of the lung surfactant for treating R.D.S. This test along with a test that measures lung recoil in premature rabbits was used to monitor product stability and set expiration dates.

4. Conclusion

The concentration of palmitic acid and dipalmitoylphosphatidylcholine could be determined simultaneously by this assay. This was a nice alternative for determining fatty acid concentration to

Table 3
Precision and accuracy determination for DPPC and PA[a]

	20 μg/ml		125 μg/ml		275 μg/ml	
	DPPC	PA	DPPC	PA	DPPC	PA
Analyst 1						
	21.51	19.84	125.65	125.44	273.91	273.56
	21.43	19.41	125.76	125.10	274.44	270.83
	21.25	19.61	126.26	124.66	275.39	270.19
	20.90	19.79	126.34	127.41	274.92	272.01
	20.98	19.85	125.43	125.35	277.00	275.65
Av.	21.21	19.7	125.89	125.59	275.13	272.45
S.D.	0.27	0.19	0.40	1.06	1.18	2.20
R.S.D. (%)	1.27	0.96	0.31	0.84	0.43	0.81
Analyst 2						
	21.54	21.44	125.10	124.04	275.19	274.06
	21.43	21.61	124.96	124.31	275.56	275.88
	21.43	21.18	125.23	124.19	275.47	274.79
	21.95	21.30	125.08	124.19	277.88	275.58
	21.63	21.29	125.42	124.52	277.30	273.90
Av.	21.59	21.36	125.16	124.25	276.26	274.84
S.D.	0.21	0.17	0.18	0.18	1.19	0.88
R.S.D. (%)	1.00	0.78	0.14	0.14	0.43	0.32

[a] Data was collected on a C_8 Inertsil 150×4.6 mm column using an 50:45:10 acetonitrile/methanol/50 m*M* acetic acid mobile phase at 1 ml/min and 45°C with refractive index detection.

current techniques like GC, which require derivatization prior to sample analysis and an improvement over previously developed HPLC assays used to analyze phospholipids in lung surfactants [35]. This assay has the advantage of having a linear detection system and used an isocratic mobile phase. In addition, it allowed for the fatty acids of corresponding phospholipids to be determined in the same assay. Modifications in the mobile phase should allow for the concentration of other fatty acids and their corresponding phospholipids to be determined, using HPLC. Shorter chain fatty acids might require less methanol in the mobile phase to ensure separation from the void volume. The sensitivity of the method was improved by concentration of the samples prior to injection, allowing for a detection limit of 0.5 μg/ml. This assay is linear over a range of 5–300 μg/ml, with and r^2 value of greater than 0.99 for DPPC and PA. Recovery studies showed that the Folch extraction method gave the best recovery and resolution, with all the lipids being recovered at approximately 90 percent. The improved limit of detection allows for trace analysis. These values can be improved upon when a more selective detection system is used. This assay was limited by the analysis time, which arises from the need to separate so many components. The 30 min analysis time limited the number of lots of material that could be released in a given day. Since the time was needed to ensure good resolution, shortening the assay further might require using smaller bore chromatography columns. This would improve the resolution and allow for a shorter analysis time. Another important consideration with this assay was column perform-

Table 4
DPPC and PA levels in the porcine lung surfactant[a]

Lot number	DPPC Surfactant (μg/mg)	PA Surfactant (μg/mg)
94/0026	22.55	1.19
	23.96	1.21
94/0032	17.73	1.65
	17.17	1.93
	18.70	1.94

[a] The lots were analyzed on a C_8 Inertsil 150×4.6 mm column using an acetonitrile/methanol/50 m*M* acetic acid 50:45:10 mobile phase at 1 ml/min and 45°C with refractive index detection.

ance. Since some peaks elute close to another, the column age had a big effect on the assay. Resolution, capacity factor, retention times and theoretical plates were monitored closely to ensure adequate separation and accuracy. For example, when the resolution was below 0.7, the column was replaced. Below this value it was was difficult to quantitate low levels of DPPC. We found that more than 300 injections could be made on the column. The Intersil column was used because that was readily available in our laboratory. Columns by other manufacturers that have similar characteristics to the Intersil should provide similar separation. This assay could be used to separate phospholipids from other systems such as tissue samples.

References

[1] M.E. Avery, J. Mead, Am. J. Dis. Child. 97 (1959) 517.
[2] M. Skegami, A. Jobe, Am. Rev. Resp. Dis. 136 (1987) 1256.
[3] W. Oh, P. Boivin, F. Puleo, L. Waute, Hosp. Formul. 28 (1993) 49.
[4] H.L. Halliday, W.O. Larnow-Morid, J.D. Corcoran, C.C. Patterson, Arch. Dis. Child 69 (1993) 276.
[5] J.D. Horbar, E.C. Wright, L. Onstad, Pediatrics 92 (1993) 191.
[6] Y. Tanaka, T. Takii, T. Aiba, K. Maruda, A. Kiuchi, I. Fujiwara, J. Lipid Res. 27 (1986) 475.
[7] J.T. Chu, J.A. Clements, E.K. Cotton, M.H. Klaus, A.Y. Sweet, W.H. Looley, Pediatrics 40 (1967) 709.
[8] G. Enhorning, B. Robertson, Pediatrics 50 (1972) 58.
[9] K. Shiffer, S. Hawgood, H.P. Haagsman, B. Benson, J. Clements, J. Goerke, Biochemistry 33 (1993) 590.
[10] M.R. Morrow, J. Pérex-Gil, G. Simatos, J. Stewart, D. Absolom, V.K. Sarvin, K.M.W. Keough, Biochemistry 32 (16) (1993) 4397.
[11] S.H. Yu, F. Possmayer, Biochim. Biophys. Acta 1046 (1990) 233.
[12] W. Seeger, G. Stohr, H. Wolf, H. Neuhof, J. Appl. Physiol. 58 (1985) 326.
[13] A.M. Cockshutt, J. Wietz, F. Possmayer, Biochemistry 29 (1990) 8424.
[14] F.R. Poulain, A. Lennell, M.C. Williams, R.L. Hamilton, S. Hawgood, Am. J. Physiol. 262 (1992) L730.
[15] W.R. Perkins, R.B. Duare, R.A. Parenta, S.R. Minchey, K.C. Neuman, S.M. Gruner, T.F. Taraschi, A.S. Janoff, Science 273 (1996) 330.
[16] T. Kobayashi, B. Robertson, G. Grossmann, K. Nitta, T. Curstedt, Y. Suzuki, Acta Pediatrics 81 (1992) 665.
[17] Y.S.H. Possmayer, Biochim. Biophys. Acta 1046 (1990) 233.
[18] B. Robertson, H.W. Taeusch, Lung Biol. Health Dis. 686 (1995).
[19] W. Lamm, R. Albert, Am. Rev. Respir. Dis. 142 (1990) 1279.
[20] A. Charon, H. Taeusch, C. Fitzgibbons, G. Smith, S. Treves, D. Phelps, Pediatrie 83 (1989) 348.
[21] A. Waring, H.W. Taeush, R. Brunin, J. Amirkhanian, B. Fan, R. Stevens, J. Young, Peptide Res. 2 (1989) 308.
[22] M. Smith, F.B. Jungalwala, J. Lipid Res. 22 (1981) 697.
[23] J.K.-Y. Hsiek, D.K. Welch, J.G. Turcotte, J. Chromatogr. 208 (1981) 398.
[24] A. Carafora, A. DiBiase, D. Alvaro, M. Angelico, M. Marin, A.F. Attili, Clinica Chimica Acta 134 (1983) 281.
[25] W.W. Christi, M.L. Hunter, J. Chromatogr. 325 (1985) 473.
[26] B.J. Compton, W.C. Purdy, J. Liq. Chromatogr. 38 (1980) 1183.
[27] W.W. Christie, High Performance Liquid Chromatography of Lipids, Pergamon Press, New York, 1992.
[28] F.B. Jungalwala, V. Hayssen, J.M. Pasquini, R.H. McCluer, J. Lipid Res. 20 (1979) 479.
[29] J.Y.-K. Hsiek, D.K. Welch, J.G. Turcotte, Lipids 16 (1981) 761.
[30] W.W. Christi, M.L. Hunter, J. Chromatogr. 294 (1984) 489.
[31] M.L. Blank, M. Robinson, V. Fitzgerald, F. Snyder, J. Chromatogr. 298 (1984) 473.
[32] M. D'Costa, R. Dassin, H. Ryan, P. Jousti, Clin. Biochem. 18 (1985) 27.
[33] K. Stoh, A. Suzuki, Y. Kuoki, T. Akino, Lipids 20 (1985) 611.
[34] H. Bünger, U. Psion, J. Chromatogr. B 672 (1995) 25.