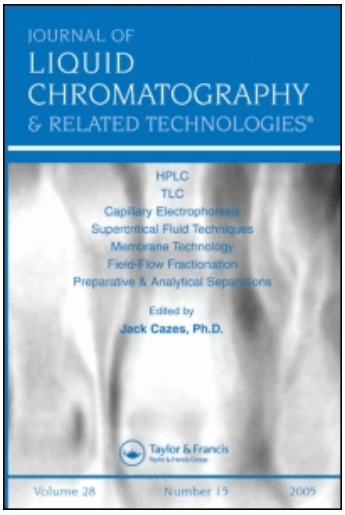

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LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY DETERMINATION OF FREE FATTY ACIDS IN PHOSPHOLIPID-BASED FORMULATIONS

Alain Carrier* and Josée Parent

RTP Pharma Inc., Ile des Soeurs, Québec, Canada, H3E 1A8

ABSTRACT

A method for the analysis of free fatty acids resulting from the degradation of phospholipids was developed using high-performance liquid chromatography-mass spectrometry with an electrospray ionization source. Separation of fatty acid molecular species was achieved using an isocratic acidified mixture of acetonitrile/water (70:30) on a reversed phased column (C_8). Optimization of the mass spectrometer conditions has allowed the method to separate and detect the fatty acids mainly as deprotonated molecular species. Separation of all major fatty acids was achieved in less than 6 min. The fatty acids studied provide a linear response over 3 orders of magnitude. Data supporting the validation of this method are also presented.

*Corresponding author.

INTRODUCTION

Free fatty acids are an important class of naturally occurring compounds. These long-chain aliphatic carboxylic acids are essential parts of most living cells and cellular fluids and are present as free fatty acids, esters, mono-, di-, or triglycerides, or phospholipids. The most common saturated fatty acids are lauric, myristic, palmitic, and stearic acid, while the most important unsaturated fatty acids have 18 carbon atoms, with one or more double bonds; the most common ones are oleic, linoleic, and linolenic acid. Fatty acids are the basic building blocks of all phospholipids. The fatty acid components of a phospholipid determine, to a large extent, its physical and biological properties.

RTP Pharma Inc., in its proprietary technology (IDD) for delivery of water-insoluble drugs, uses phospholipids as surface-modifying and stabilizing agents (1–3). This approach involves the formation of micron or submicron sized particles of a solid drug stabilized through the use of a variety of phospholipids. The phospholipids primarily used are synthetic, or lipids derived from egg or soybean. Another variation of IDD technology uses submicron sized oily droplets in which a drug is dissolved. These droplets are then stabilized from coalescence by coating with phospholipids. Typically, with the sources of phospholipids used in the IDD technology, the major fatty acids that can be found are the following: linoleic, linolenic, oleic acid, palmitic, and stearic acids. The amount of each fatty acid present will vary, depending on the source and age of phospholipid used.

In aqueous dispersions of phospholipid, under certain conditions, the phospholipids can undergo hydrolysis to free fatty acids and lysophospholipids (4). Further hydrolysis of the lysophospholipids results in glycerophospho compounds. To assess the stability of the phospholipid formulation and to characterize their free fatty acid content, it is essential to have a simple, rapid, and quantitative method of analysis.

Numerous methods using gas chromatography (5–7), high-performance liquid chromatography (8,9), electrophoresis (10,11), and enzymatic assays have been reported in the literature (12,13). Usually, the characterization of free fatty acids is typically achieved by gas chromatography, analyzing their esterified derivatives using standard detectors or mass spectrometry; other techniques include silver ion liquid chromatography (14,15). Silver ion liquid chromatography is of special value when it is used to complement other methods of separation (16). Analytical profiles obtained with this type of chromatography are easier to interpret and are more suited for preliminary simplification of complex mixtures.

Introduction of liquid chromatography (LC)/mass spectrometry (MS) techniques for the analysis of fatty acids content allows the separation and structural characterization of intact fatty acids. This technique enables the elimination of the derivatization step involved in gas chromatography analysis. Although LC/MS

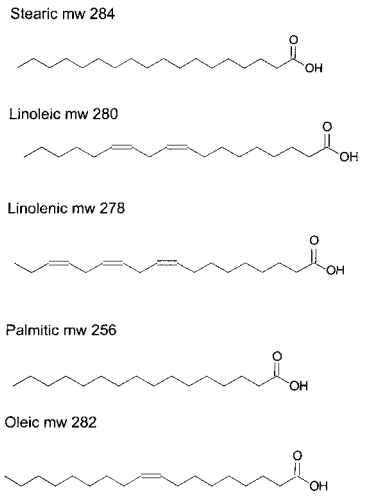

Figure 1. Chemical structures of fatty acids studied. MW, molecular weight.

work was reported (17–20), the work done with this technique was for the structural characterization and identification of fatty acids or their metabolites.

To rapidly quantitate and characterize the contents of common free fatty acids present in phospholipid-based IDD formulations and various raw materials, an LC/electrospray ionization source/MS method using negative ionization was developed. The present work describes the determination of oleic, linoleic, linolenic, palmitic, and stearic acid in various phospholipid formulations and raw material. Figure 1 gives the chemical structures of the fatty acids studied.

EXPERIMENTAL

Chemicals

The following reference free fatty acids were purchased from Sigma (Oakville, Ontario, Canada): linolenic acid, linoleic acid, palmitic acid, oleic acid, and stearic acid. The purity of all the acids used was >99%. All these standards were used without further purification.

Acetonitrile (ACN), methanol, and tetrahydrofuran were high-performance liquid chromatography (HPLC) grade and were purchased from J. T. Baker (Phillipsburg, NJ, USA).

Glass-distilled water (>18 MΩ), available in-house, was filtered before use through a 0.2-μm filter. Acetic acid was American Chemical Society grade and was purchased from Sigma.

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Chromatographic System and Conditions

HPLC-MS analysis was carried out using an HP 1100 (Agilent Technologies, Canada) system composed of the following units: a solvent delivery module, an automatic sample injector (100-well capacity), a controller module, a column oven, and a model series 1100MSD mass spectrometer. The analysis was performed on a Zorbax stablebond SB C_8 column (3.3 cm $\times$ 0.46 cm inside diameter, 3.5 μm particle size; Agilent Technologies), maintained at 25°C.

The mobile phase consisted of an isocratic 70:30 mixture of 0.15% acetic acid in acetonitrile and 0.15% acetic acid in water. The mobile phase was filtered through a 0.45-μm polypropylene filter and degassed under vacuum. The mobile phase was pumped at 1.0 mL/min. The injection volume was 5 μL.

Mass Spectrometric Conditions

Mass spectrometry was carried out on the HP 1100 mass spectrometer (Agilent Technologies), equipped with the electrospray ionization source used in the negative ionization mode. Data handling was performed with a Chemstation V. A.06.03 data handling system. Full scan spectra were obtained by scanning masses between m/z 200 and 300.

Nitrogen was used both as a drying gas (6 L/h) and as a nebulizing gas (20 psi). Quantitative analysis of different fatty acids were conducted in single-ion monitoring mode (SIM) scanning the quasimolecular ions $[M\text{-}H]^-$ with a dwell time of 100 ms.

Sample Preparation

Samples were prepared by diluting the different drug formulations (containing phospholipids, a drug, and other excipients) or raw material to solutions of approximately 50–100 μg/mL with methanol or using a mixture of methanol/chloroform (typically 90:10).

RESULTS AND DISCUSSION

Initially, standard solutions of fatty acids were prepared at 100 μg/mL and injected directly onto the mass spectrometer to study their mass spectra profiles. Figures 2A to 2E show typical spectra obtained for the different fatty acids studied, linolenic, linoleic, palmitic, oleic, and stearic acid, using the electrospray ionization technique in the negative ionization mode. Analyses were performed using a

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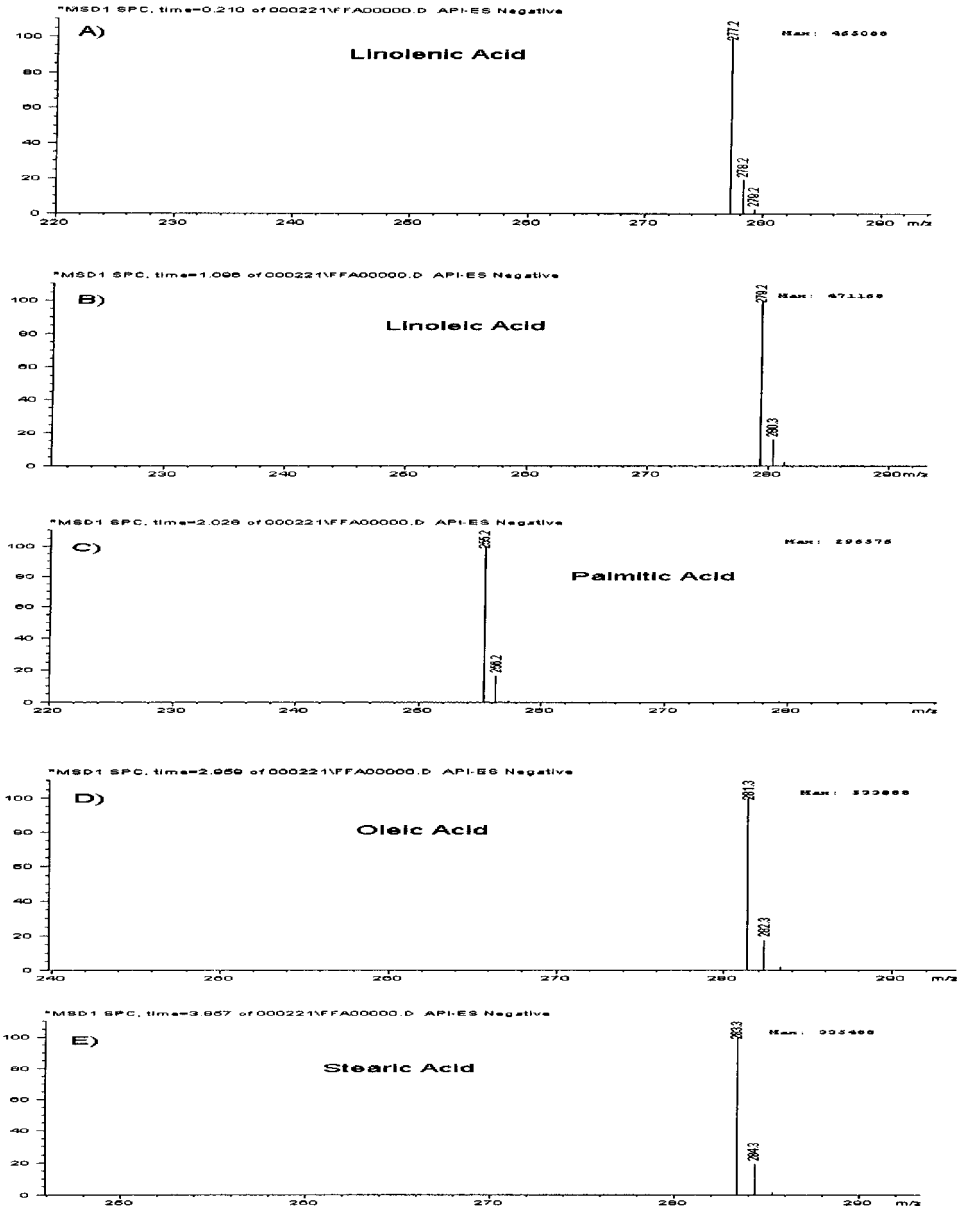

Figure 2. Negative ion electrospray ionization mass spectra of A) linolenic acid, B) linoleic acid, C) palmitic acid, D) oleic acid, and E) stearic acid. Fragmentor voltage 100 V, scanning range from m/z 200 to 300, deprotonated ion $(M\text{-}H)^-$.

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fragmentor voltage of 100 V. Results obtained indicate only the presence of the deprotonated ions $[M\text{-}H]^-$. Negative ion electrospray ionization of the fatty acids studied here showed practically no significant fragmentation for all compounds tested.

Optimization of the fragmentor voltage used for the characterization of the different fatty acids was performed; the fragmentor voltage was varied between 10 and 200 V. Results obtained indicate that no significant changes were observed in the mass spectral profile between the voltage range of 50 to 130 V. The major ions remained the deprotonated ions $[M\text{-}H]^-$; no fragments were observed for the range of voltage studied. However, as for the intensity, our results demonstrated that the abundance of the molecular ions reached a maximum for voltages between 90–110 V. From these results, a fragmentor voltage of 100 V was selected.

The objective of this work was to develop a fast, reliable, stability-indicating method to characterize and quantitate free fatty acid content in phospholipid-based pharmaceutical formulations. To achieve these objectives, a reversed-phase LC (C_8) separation assay, combined with mass spectrometry detection, was developed.

The separation obtained for a standard mixture of linolenic, linoleic, palmitic, oleic, and stearic acids by the use of the above-mentioned chromatographic system is illustrated in Figure 3. This chromatogram was obtained using the selected ion monitoring mode; the deprotonated ion of each acid was scanned: linolenic acid, m/z 277; linoleic acid, m/z 279; palmitic acid, m/z 255; oleic acid, m/z 281; and stearic acid, m/z 283. The profile corresponds to the total ion chromatogram and the mass chromatogram obtained for each fatty acid.

Under these conditions, retention times of the different acids were 1.6 min for linolenic acid, 2.1 min for linoleic acid, 2.7 min for palmitic acid, 3.1 min for oleic acid, and 4.9 min for stearic acid. As mentioned above, despite the mass spectrometry experiments previously reported for fatty acid analysis, no LC/MS method that can resolve the free fatty acids present in phospholipid-based formulations has been reported. Using the method described in this communication, a complete analysis of these free fatty acids could be done in less than 6 min.

To verify the linearity of the method in the SIM mode, mixtures containing all the fatty acids in the range of 5–1000 μg/mL were prepared and injected in triplicate. The deprotonated ions $[M\text{-}H]^-$ were monitored for each fatty acid studied. Eight standard solutions containing from 3.4 to 870 μg/mL for linolenic acid, from 4.7 to 1200 μg/mL for linoleic acid, from 14.6 to 932 μg/mL for palmitic acid, from 4.9 to 1258 μg/mL for oleic acid, and from 7.1 to 908 μg/mL for stearic acid were prepared and analyzed in triplicate.

The corresponding peak areas were fitted to a straight line using a logarithmic scale. Linearity was observed for the different fatty acids studied over 3 orders of magnitude. Correlation coefficients for the calibration curves were greater than or equal to 0.99 over the specified range studied.

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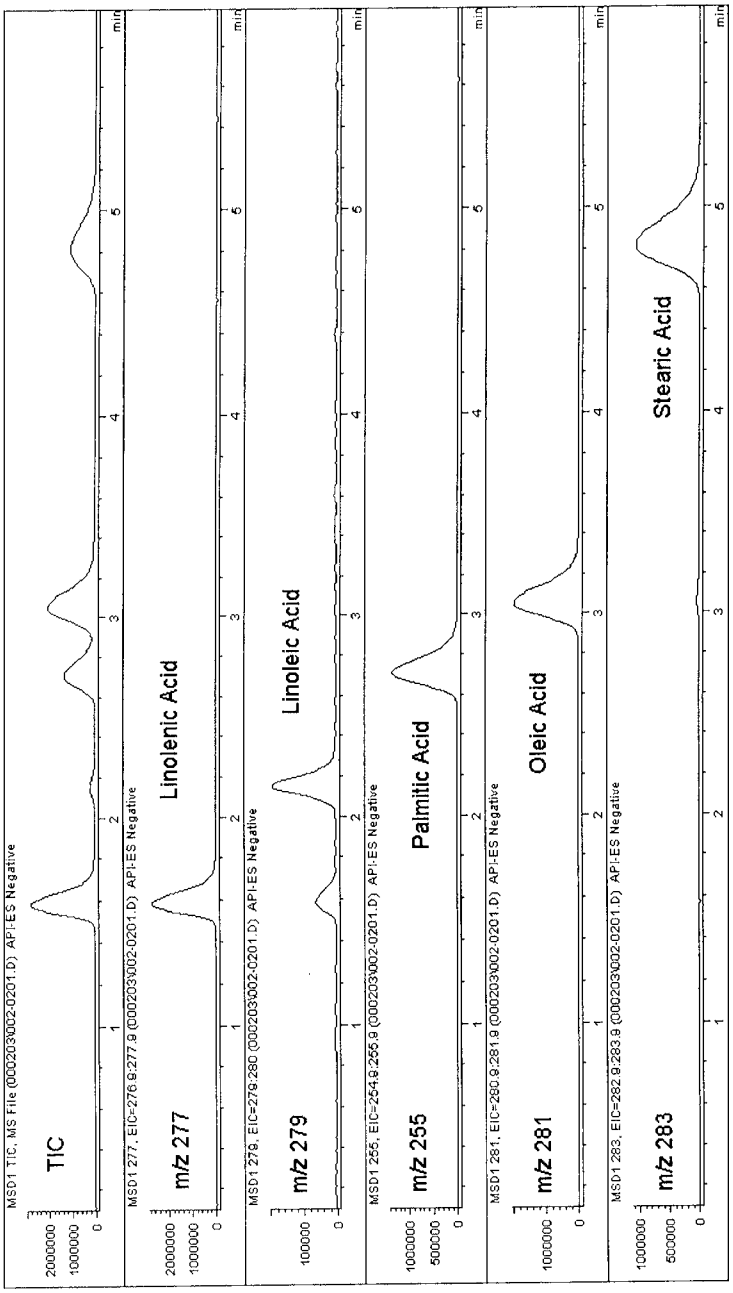

Figure 3. Typical LC-MS separation obtained for a standard mixture in methanol injected with approximately 500 ng of different fatty acids: linolenic acid (535 ng), linoleic acid (395 ng), palmitic acid (383 ng), oleic acid (495 ng), and stearic acid (373 ng).

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The limit of quantitation was determined by preparing solutions ranging from 1 to 15 μg/mL. These solutions were injected in triplicate, and the acceptance criterion was set to a relative standard deviation of less than 5%. Limits of quantitation found were 2.99 μg/mL for linolenic acid, 2.75 μg/mL for linoleic acid, 11.48 μg/mL for palmitic acid, 2.94 μg/mL for oleic acid, and 2.29 μg/mL for stearic acid. The limit of detection was determined graphically based on the signal to noise ratio. A signal to noise ratio of 3:1 was defined as acceptable. Graphical evaluation of the chromatograms obtained for the standard fatty acid solutions indicated that the limits of detection were 1.50 μg/mL for linolenic acid, 1.38 μg/mL for linoleic acid, 2.30 μg/mL for palmitic acid, 1.47 μg/mL for oleic acid, and 1.14 μg/mL for stearic acid.

For accuracy, samples were spiked at three levels (75, 100, and 125%) of the working concentration (200 μg/mL) for each fatty acid studied. The three concentrations tested were 150, 200, and 250 μg/mL. Individual solutions of each fatty acid were prepared and injected in triplicate. The average recoveries obtained for the different fatty acids at the three levels tested were 99 $\pm$ 1% for linolenic acid, 100 $\pm$ 2% for linoleic acid, 101 $\pm$ 1% for palmitic acid, 101 $\pm$ 2% for oleic acid, and 99 $\pm$ 1% for stearic acid.

For the evaluation of the precision of the method, samples were prepared at 200 μg/mL concentration (100%) and injected in triplicate on three different days, giving the following recoveries: 98% for linolenic acid, 99% for linoleic acid, 101% for palmitic acid, 101% for oleic acid, and 99% for stearic acid.

The robustness of the method was evaluated by verifying the sensitivity of the method to three minor independent changes in operational parameters. These parameters were column temperature (20 and 30°C), flow rate (0.8 and 1.2 mL/min), and mobile phase composition (65:35 and 75:25 0.15% acetic acid in ACN/0.15% acetic acid in water). The evaluation of the robustness was done by examining the retention time and the resolution. The results obtained indicate that minor changes in operational conditions do not significantly affect the results. Even though the resolution between palmitic acid (m/z 255) and oleic acid (m/z 281) is less than 1.5 for two conditions studied, 1.2 mL/min and 75:25 0.15% acetic acid in ACN/0.15% acetic acid in water, the specificity of the mass spectrometer allows the separation of these two fatty acids from each other by extracting their respective masses. Resolutions between the other fatty acids were always greater than 1.5 ($R > 1.5$).

The method developed has been applied to various phospholipid-based formulations containing a variety of drugs and is routinely used in our laboratory to assess the stability of the different phospholipids used in both raw material and finished product. Table 1 gives the amount of free fatty acid obtained at different time points for an egg phospholipid-based injectable formulation kept at 40°C for 6 months.

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Table 1. Free Fatty Acid Content of a Phospholipid-Based Formulation Kept at 40°C for 6 Months

	Stability Time Points				
Fatty Acid Content	Initial	1 Month	2 Months	3 Months	6 Months
Linolenic acid (μg/g)	Not detected	4.0	6.0	12	31
Linoleic acid (μg/g)	70.0	440	540	760	1740
Palmitic acid (μg/g)	0.2	12	19.5	26.0	90
Oleic acid (μg/g)	3.0	5.0	10.2	13.0	50
Stearic acid (μg/g)	0.1	0.1	22.4	45.0	100

As can be seen from the results presented, initially ($T = 0$) the content of free fatty acids was not significant, with the exception of linoleic acid, which was approximately 74 μg/g of formulation; the concentrations of the other free fatty acids were less than 1 μg/g. However, as reflected in the table, significant increases in free fatty acid contents were observed over the 6-month time. At the end of that period the contents of linoleic acid and linolenic acid were 1–2 mg/g of formulation.

These significant increases in free fatty acid contents indicated that a degradation of the phospholipids is occurring. Determination of the phospholipid content revealed that important reductions in phosphatidylcholine and phosphatidylethanolamine contents were observed and that an increase in the lysophospholipid content was also observed. These results confirmed the potential of the method to detect the free fatty acids generated and, at the same time, characterized the degradation of the phospholipids used in pharmaceutical formulation.

The same procedure was used to assess the initial content of free fatty acids present in egg lecithin raw material. Typically, egg lecithin will contain, initially, less than 500 μg/g of free fatty acids per gram of material. Results obtained indicate that, initially, the free fatty acid content of the egg lecithin was 212 μg/g of formulation. Specifically, concentrations obtained were as follows: linoleic acid, 181 μg/g; palmitic acid, 8 μg/g; oleic acid, 17 μg/g; and stearic acid, 6 μg/g. These results were in agreement with the certificate of analysis supplied with the material by the supplier, which states that not more than 500 μg/g of total free fatty acids per gram of material should be present.

CONCLUSION

A rapid and simple LC-MS method for the determination of free fatty acid molecular species of linolenic acid, linoleic acid, palmitic acid, oleic acid,

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and stearic acid was developed. The method described is useful for the characterization of free fatty acids present in phospholipid-based formulations using natural egg lecithin or other types of phospholipids. The LC-MS assay allows reproducible identification and quantitative analysis of all relevant acids. The mass specificity associated with the mass spectrometer is also another advantage. The assay was successfully used to assess the stability of potential phospholipid-based formulations and raw material exposed to various stress conditions.

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