Journal of Pharmaceutical and Biomedical Analysis 222 (2023) 115112

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Analysis of phospholipids and triacylglycerols in intravenous lipid emulsions

Bijay Banstola [a], Prabhath L. Gamage [a], Wenlei Jiang [b,*], Thilak Mudalige [a,*]

[a] Arkansas Laboratory, Office of Regulatory Sciences, Office of Regulatory Affairs, US Food and Drug Administration, Jefferson, AR 72079, USA
[b] Office of Research and Standards, Office of Generic Drugs, Center for Drug Evaluation and Research, US Food and Drug Administration, Silver Spring, MD 20993, USA

ARTICLE INFO

Keywords:
Intravenous lipid emulsions
UHPLC
LCMS
Phospholipids
Triacylglycerides

ABSTRACT

Intravenous lipid emulsions (ILEs) are used for parenteral nutrition, providing a vital source of essential fatty acids and concentrated energy for patients who are unable to absorb nutrients via the digestive track. They are commonly used to treat local and non-local anesthetic toxicity, and lipophilic drug overdose. ILE are composed of natural lipids, and the composition of these natural lipids can be varied based on their source. The lipids are susceptible to hydrolytic degradation with time, resulting various lipid degradation products such as Lysophosphatidylcholines (LPs), affecting the actual composition of nutrients in the formulation. As a result, the identification and quantification of lipid components, including degradation products, in ILEs are crucial in quality control. In this study, lipids from different batches of ILE Intralipid® 20%, were separated and identified using a UHPLC-ESI-QTOF system and SimLipid® high throughput lipid identification software. Out of 47 lipids identified, 34 were phospholipids (PLs) and the others were triacylglycerols (TAGs). Most of the phospholipids detected were phosphatidylcholines (PC) and Lysophosphatidylcholines (LPC). A total of 9 LPCs, 18 PCs, 6 phosphoethanolamines (PEs), and 1 sphingomyelin (SM) were identified. The LPCs concentration changed with the manufacturing date and storage time. This UHPLC method enabled the identification and quantification of lipids and their decomposition products in complex ILE emulsion mixtures on a single 20-minute chromatographic run.

1. Introduction

Lipids have multiple physiological roles and are biologically vital for an individual's growth, especially in neonates [1]. A well-balanced fatty acid supply during the neonatal period is vital for body growth and brain development [2]. Lipids are major constituents of cellular membranes, essential for the immune system, and provide a substrate for de novo biosynthesis of cholesterol and endogenous steroids [3–7]. These essential lipids are provided to our body through diet. However, for individuals who cannot get nutrition via the diet, these lipids are given through parenteral nutrition [1].

Intravenous lipid emulsions (ILEs) are one key type of parenteral nutrition, that provide essential fatty acids (FA) and concentrated energy [6,7]. ILEs are also used to treat local and non-local anesthetic toxicity, since lipid emulsions can absorb toxic hydrophobic drugs reducing bioavailability [1,8,9]. Lipomul I.V.® was the first ILE approved in the USA and was manufactured using cotton seed oil as the lipid source [10,11]. Since then, various types of plants and fish-based lipid sources have been used to develop ILEs [3,11]. Intralipid® (Baxter Healthcare Corp.) is one of the most commonly used ILEs which uses soybean oil as the lipid source [12]. This soybean oil-based ILE contains soybean oil triacylglycerols (TAG), enveloped by a phospholipid emulsifier that allows the TAG core to remain soluble in an aqueous parenteral nutrition mixture [3]. A typical soybean oil-based ILE contains 10–30% soybean oil, 1.2% egg yolk phospholipids, 2.25% glycerin, and water [3]. However, the specific components of ILEs might change based on their lipid source. In addition, lipids in ILEs are susceptible to spontaneous hydrolytic degradation to primarily free fatty acids and lysolipids during manufacturing process and storage [13]. These changes in the lipid composition might well affect the actual composition of nutrients in these ILE formulation, resulting possibly in adverse effects.

ILE use is known to provide many advantages to the patient; however, some adverse side effects have been observed in patients treated with ILEs [14,15]. The long term use of ILE has been shown to be linked with the liver disease development [3,16] and liver failure in children

* Corresponding authors.
E-mail addresses: Wenlei.Jiang@fda.hhs.gov (W. Jiang), Thilak.Mudalige@fda.hhs.gov (T. Mudalige).

https://doi.org/10.1016/j.jpba.2022.115112
Received 9 June 2022; Received in revised form 10 October 2022; Accepted 15 October 2022
Available online 18 October 2022
0731-7085/Published by Elsevier B.V.

[17,18]. Furthermore, excessive use of ILEs has resulted in plaque accumulation in heart veins, lungs, cerebrum and kidney, leading to many adverse outcomes [19,20]. In some cases, patients treated with lipid emulsions reported to be vulnerable to bacterial and fungal infections due to interactions of some poly unsaturated fatty acids leading to immune system suppression [21]. Although it is currently unclear if the degradation products of these lipids cause adverse effects, a better understanding of ILE composition including the degradation products, may help in the identification of components that lead to adverse outcomes following ILE use.

In previous studies, various methods have been used to determine the composition of lipid components in ILEs. Férézou and coworkers analyzed the lipid composition of Intralipid® using a physical fractionation method followed by NMR (nuclear magnetic resonance) analysis and studied the influence of the phospholipid/triacylglycerol (PL/TG) ratio of parenteral emulsions on physico-chemical properties. A gas chromatographic (GC) technique followed by derivatization and ^{31}P NMR analyses of these fractions indicated that at least two types of fat particles coexist in parenteral emulsions however, this technique was unable to identify and quantify the individual lipid components [22]. GC techniques have been employed by other scientists, enabling resolution and detection of most of the lipids in ILE samples; however, GC analysis of lipids requires considerable time in sample preparation and instrument time, leading to a marked reduction in throughput [23,24]. Subsequently, mass spectrometry (MS) methods using MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) and ESI (electrospray ionization) detectors were coupled with GC and emerged as powerful tools for the determination of lipids in ILE. These techniques were successful since the fundamental studies of fatty acid esters proved that MS could reveal detailed structural information from known compounds, which was highly useful in structural elucidation of unknown lipids molecules using the basic mechanisms of ion fragmentation following electron spray ionization [25]. The high sensitivity and high specificity provided by mass spectrometry have accounted for the success of MS techniques in lipid analysis. The use of liquid chromatography (LC) separation techniques along with high-resolution MS (HRMS) or tandem mass spectrometry (MS/MS) have facilitated the analysis of many different classes of lipids in complex mixtures, and enabled the determination of countless previously uncharacterized and undetected lipids [26,27].

In this study, an ultra-high-performance liquid chromatography (UHPLC) technique coupled with HRMS (QTOF) was developed as a single LC method to separate, identify and quantify the phospholipids (PLs) and triacylglycerols (TAGs) in an ILE. For this analysis, one of the commonly used soybean oil-based lipid emulsions (Intralipid® 20%) was used, and the PLs and TAG were separated into different lipid classes and quantified. Lipids were separated using reverse phased UHPLC and detected using ESI/QTOF mass spectrometer. SimLipid® software (Premier Biosoft International, San Francisco, CA USA) was used to identify the detected lipids. PLs and TAGs of several samples from two different batches of Intralipid® 20% were analyzed and reported in this manuscript.

2. Experimental

2.1. Materials and chemicals

The solvents used were either LCMS or HPLC grade. Acetonitrile (ACN), ammonium formate (AF), chloroform, isopropanol (IPA), and methanol were purchased from Fisher Chemical Co. (Fair Lawn, NJ, USA). Formic acid (LCMS grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All the phospholipid standards, including deuterated phospholipids which were used as internal standards to quantify lipids, were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). TAG standards were purchased from either Avanti Polar Lipids Inc. or Larodan (Monroe, MI, USA). One deuterated standard from each class was used as surrogate internal standard for each lipid class. This was acceptable since the lipids in each class showed close retention times. All the PL and TAG standards are listed in Supplemental Figs. S1 and S2, respectively. The internal standards used in this study, are shown in Supplemental Fig. S3. Stock solution of standards and samples were prepared and diluted using a methanol:chloroform (1:1) solution. Intralipid® 20% emulsion was purchased from WEP Clinical (Morrisville, NC, USA) and stored at room temperature and protected from ambient light.

2.2. Instrumentation

All MS and MS/MS analysis were performed using an Agilent 6550 Q-TOF MS (Agilent Corp., Santa Clara, CA, USA) coupled to an Agilent 1290 UHPLC. The LC system consisted of a binary pump, a degasser, an autosampler, and a column heater-compartment. A Kinetex® C18 column (Phenomenex® Inc., Torrance CA USA; Part #00D-4475-AN; ID 2.1 mm×length 100 mm, particle size 1.7 μm) was used at a flow rate of 400 μL/min for LC separation with methanol:water (1:1), 0.1% formic acid, and 20 mM ammonium formate as the mobile phase A and ACN: isopropanol (1:9), 0.1% formic acid, and 20 mM ammonium formate as mobile phase B. This higher concentration of ammonium formate buffer than typically used in LC was used to reduce peak broadening through ion-pair formation [28].

2.3. Method development

The UHPLC_QTOF based method was developed to separate and identify the lipids in the formulation. Since the formulation consists of lipids components having different number of fatty acids which determines the bulkiness of the lipids, e.g. Lysophospholipid (LPs) with one fatty acid, Phospholipids (PLs) with two fatty acids and Triacylglycerols (TAGs) with 3 fatty acids, a gradient eluent method was designed to separate the lipid classes according to their retention time. The lipids' retention time within a class depends on the length of fatty acid groups and the nature of unsaturation. Therefore, the gradient and flow rate were modified to get the maximum separation of the lipids within each class and same time method was developed to analyze all the lipid components in a single run. The mobile phase started at 25% B in A and was isocratic until 1.5 min, then linearly increased to 60% B in A between 1.5 min and 3.0 min. The mobile phase was kept isocratic at 60% B in A from 3.0 mins to 4.0 mins, and then linearly increased to 70% B in A at 5.0 mins. The mobile phase was isocratic at 70% B in A until 6.5 mins, and then increased linearly to 80% B in A at 8.0 mins. The mobile phase was isocratic at 80% B in A until 15 mins. Between 15.0 min and 15.1 min the mobile phase was increased to 100% Band maintained at 100% B until 16.5 min. The system was reequilibrated by changing the mobile phase to 25% B in A in 0.1 min, and maintained isocratic at 25% B in A for 5 min Supplemental Table S1 shows the details of the UHPLC gradient. The column's eluent was directly connected to the MS source, where eluents were ionized using an ESI source. Data were acquired in positive mode with sheath gas temperature of 350 °C, sheath gas flow at 11 L/min, nebulizer pressure at 60 psi, capillary voltage at 3500 V, and the fragmentor voltage at 175 V. The MS/MS data were collected in positive ion mode in the range of m/z 100–3200 at a collision energy of 30 V. Two reference ions at m/z 121.050873 and m/z 922.009798 [Agilent P/N G1969–85001] were used to calibrate the mass spectrometer, ensuring the mass error of less than five ppm during analysis. SimLipd® software was used to identify lipids based on their m/z values and fragmentation patterns. Finally, the lipid contents were quantitatively or semiquantitatively quantified.

The ILE fomulation was mixed gently and two samples were prepared by diluting the original sample 1:100 and 1:1000 in chloroform: methanol (50:50). After determining the approximate concentration of each analyte, the calibration ranges were determined for the respective standards. The detailed information for the standards and internal

standards used for each lipid component are listed in Supplemental Table S3. The intraday method validation data for all the standards are summarized in Supplemental Table S4. As shown in Supplemental Table S4, the intra-day method validation was carried out in 3 consecutive days for all the calibration standards using the optimized method for interday validation. Three different quality control concentrations were chosen for each standard depending on the calibration range (LQC, low concentration; MQC, middle concentration; HQC, high concentration) and averaged and compared among the days. Further, continuing calibration verification (CCV) standards were used to determine precisions and %-accuracy of the sample analysis. Even though the limits of quantitation were lower than the concentration of lowest calibrant for each lipid component, the concentration of lowest calibrant was used as limit of detection, and lipid samples were diluted accordingly to achieve the detected concentration within the calibration range.

2.4. Data analysis

Initial data processing was completed by Agilent MassHunter software. Premier Biosoft SimLipid® software was used for database searching to identify lipids based on MS/MS data. The mass quantitation software was then used to build calibration curves and determine the concentration of PLs and TAGs in the sample. For the lipids which the standards were not available, PLs and TAGs concentrations were semi-quantitatively determined from the sample by comparing the instrumental response to internal standards (deuterium labeled lipid internal standard) under the conditions of analysis. Application of surrogate labeled lipid internal standard in lipid analysis is a well-accepted practice in lipid quantitation in the absence of standards for all the analytes [29]. It is noteworthy to mention that the software gives the potential lipd identification according to its molecular ion and fragmentation pattern, but it cannot distinguish regiochemistry between *sn*-1 and *sn*-2 positions of a particular lipid if the isomers molecular ions and fragmentation patterns are identical. Analyte concentrations were based on six-point calibration curves, obtained from standard solutions of PLs and TAG. Each sample was spiked with an internal standard mixture. The internal standard mixture consisted of five lipid standards with different concentrations, one from each class of lipids analyzed.

2.5. Statistical analysis

The weighted least square model was used for generating calibration plots for all the standards. Limit of detection (LOD) and limit of quantification (LOQ) values were calculated using gradient (m) and standard deviation of response (Sy) from the calibration curves for each PL and TAG standard. Calibration ranges for each lipid components are listed in the Supplementary Table S3. The standard mixtures were run on 3 consecutive days (intraday method validation) to account for the variation of instrument performance. Intraday method validation data are summarized in Supplemental Table S4.

3. Results and discussion

As described in the product label, Intralipid® 20% is an aqueous emulsion composed of 20% of soybean oil, 1.2% egg yolk phospholipid, and 2.25% glycerin. The final product pH is adjusted to 8 with sodium hydroxide by the manufacturer. The soybean oil is a refined natural product, consisting of a mixture of neutral triglycerides, predominantly unsaturated fatty acids. The major fatty acid composition is given as follows: linoleic (44–62%), oleic (19–30%), palmitic (7–14%), linolenic (4–11%) and stearic (1.4–5.5%). These fatty acids can be available as phospholipids or triglycerides in the formulation.

In our study, to determine lipids present in Intralipid® 20%, the lipid classes were separated using an optimized UHPLC method and quantified with ESI QTOF/mass spectrometry. Fig. 1 shows the total ion chromatogram (TIC) of a 100-fold diluted Intralipid® 20% sample. As

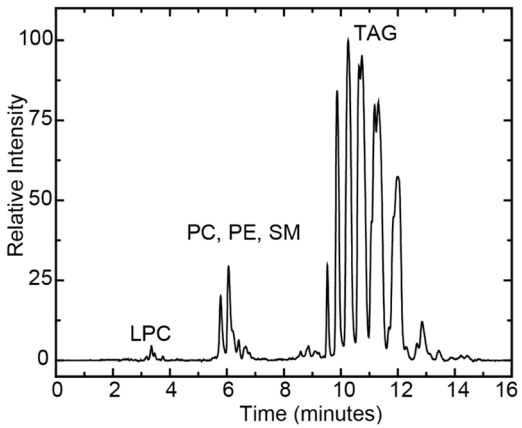

Fig. 1. Total ion chromatogram of Intralipid® 20% from UHPLC-MS analysis and peaks represent lysophosphatidylcholines (LPC), phosphatidylcholines (PC) phosphoethanolamines (PE), sphingomyelin (SM) and triacylglycerols (TAG).

shown in the figure, three different clusters of peaks corresponding to 5 major lipid classes were observed. The retention time of lipids in reverse-phased liquid chromatography depends on hydrophobic interaction between lipid and the C_{18} nonpolar stationary phase, lipid molecular structure, and lipid solubility in the mobile phase [27]. The lipids retention time within a class depends on the length of fatty acid groups and the location of double bonds within the group. Lysophospholipid (LPs) with one fatty acid group were eluting earliest between three and four minutes. LPs are degradation products of phospholipids. The phospholipids (PLs) have two fatty acids groups and eluted between five and seven minutes (Fig. 1). The cluster of high-intensity peaks eluting between approximately 9 and 14 min is from TAGs which accounts for 20% of the emulsion mixture. This method could separate isomeric species of phospholipids such as PC (16:0/20:4) and PC (18:1/18:3), but TAG isomers were not separated. Previously published data along with the fragmentation pattens were used resolve the structures of isomeric PL species [30].

The lipids identification was accomplished using Premier Biosoft SimLipid® Software, which used MS/MS fragmentation patterns to identify lipids. A total of 47 different lipids were identified in each mixture. Phospholipids were detected as $[M+H]^+$ ions while TAGs were detected as $[M+NH_4]^+$ ions [31,32]. TAGs are difficult to ionize by protonation; however, the presence of ammonium salts helped to ionize TAGs as ammonium adducts. Fig. 2 shows the extracted ion chromatogram of the identified lipids. Out of 47 lipids identified, 34 were phospholipids and the others were triacylglycerols. Most of the PLs detected were phosphatidylcholines (PC) and lysophosphatidylcholines (LPC). In this method a total of 9 LPCs, 18 PCs, 6 phosphoethanolamines (PEs), and one sphingomyelin (SM) were detected. The list of all the lipids detected along with their retention time, theoretical mass, analyzed mass, and mass error is shown in Supplemental Table S3. The identified lipid masses were further verified using mass extraction from MassHunter software and it was confirmed that majority were within 5 ppm of the actual mass [33]. Three of the LPCs (20:5, 20:4 and 20:3) had a mass differential of greater than 5 ppm, which could be due to their very low concentrations in the sample.

Supplemental Fig. S1 shows the fifteen PL standards that were used for the quantification of phospholipids while Supplemental Fig. S2 shows the seven TAG standards that were used for the quantification of TAGs. Analyte responses were referenced to six-point calibration curves obtained from standard solutions of PLs and TAGs for the quantification. Lipids for which the standards were not available, a corresponding internal standard was spiked to quantify each lipid class, which is a well-accepted practice in semiquantitative lipid analysis [29]. A total of five different internal standards were used for semiquantitative analysis, one for each class of lipids. The list of internal standards used for

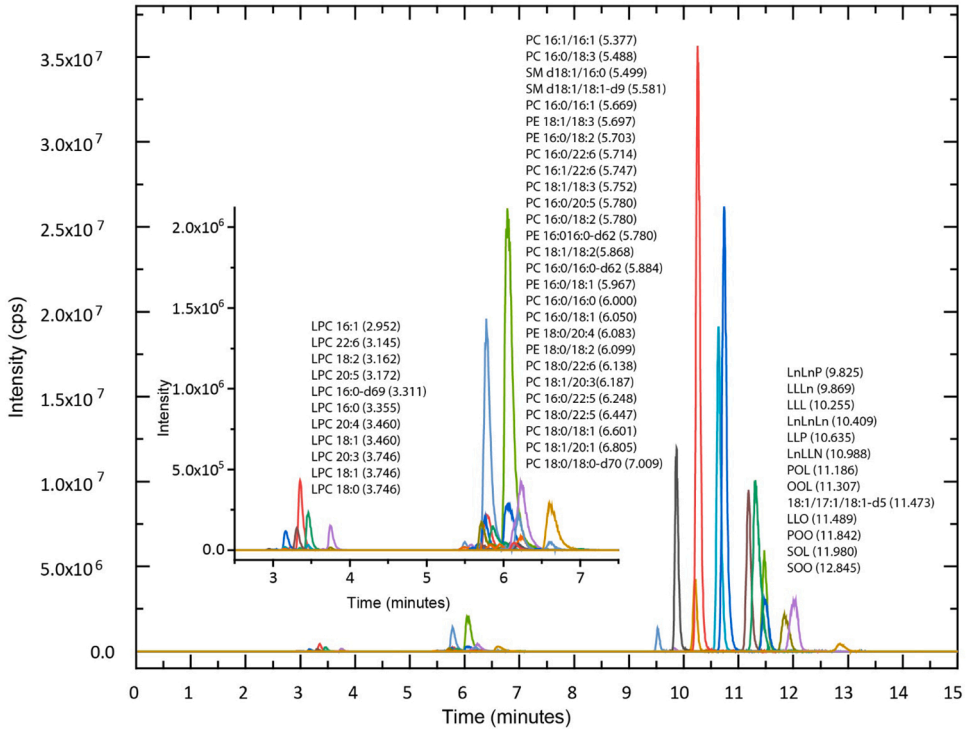

Fig. 2. Ion chromatogram of the identified lipids and lipid standards belong to lipid classes of lysophosphatidylcholines (LPC), phosphatidylcholine (PC) phosphoethanolamines (PEs), sphingomyelin (SM) and triacylglycerols (TAG). Retention time for each lipid is provided within the parentheses. (All the lipids identified are listed in Supplemental Table S2).

semiquantitative analysis is shown in Supplemental Fig. S3. In this case, PLs and TAGs concentrations were calculated from the sample by comparing response of the analyte to an internal standard. The experimental flow for lipid identification and quntitation is shown in Fig. 3.

Supplemental Table S2 shows the linear range, limit of detection (LOD), limit of quantitation (LOQ), and linear regression (R^2) for calibration of each standard. The LOD and LOQ for each compound were calculated based on (3 SD)/b or (10 SD)/b formulation, respectively. The SD is the standard deviation of y-intercept, and b is the slope of the regression line in the calibration curve. Precision and %-accuracy of the method were examined by running continuing calibration verification (CCV) standards at three different concentrations covering the calibration range, over three consecutive days. Standard deviations for intra-day precisions were less than 10% for all analytes with accuracy values in the range of 85–115%.

Two different batches of Intralipid® 20% were analyzed using the validated method. Three samples from each batch were selected randomly and each sample was analyzed three times. A total of nine injections for each batch was used to calculate the average concentration of the lipids in the sample, and Table 1 shows the concentration of lipids present in each sample. When the two batches were compared, the concentration of each lipid were found to be within ± 5% difference, with the exception of the LPCs. The concentrations were higher for LPCs in batch 1 compared to batch 2, with differences ranging from 6% to 23%. One possible reason for the higher LPC concentrations in batch1 could be due the age of the sample. Batch 1 was manufactured 7 months earlier than batch 2, and the data suggests that the phospholipids such as PCs can be hydrolyzed to lysophospholipids and further to free fatty acids during the storage as the result of exposure to moisture, light, or heat [13]. Though there was a significant difference in LPCs concentration in the emulsion mixtures, the difference in concentration of PCs between two batches was very low (Table 1). We could observe that a total of 13 out of 19 PCs had lower concentration in batch 1 compared to batch 2.

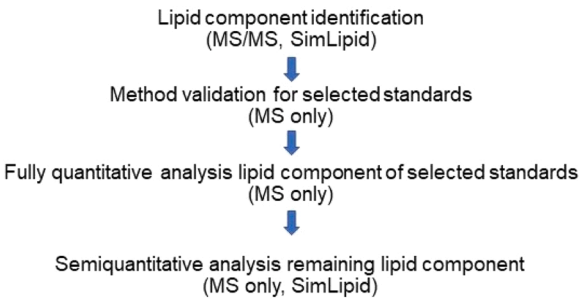

Fig. 3. Experimental flow for lipid identification and quantitation.

Egg phospholipids are the main source of phospholipids in this lipid emulsion, containing 1.2 g/dL of total egg phospholipids. In Table 2, we compared the published data [31] with our results, accounting for the concentration of egg phospholipids in Intralipid® 20%. Consistent with published reports, we determined PC was the most predominant lipid species present in egg yolk phospholipid and our quantitative analysis of Intralipid® 20% proved to be accurate and accounted for approximately 95% of PC mass listed in literature.

There are two possible reasons there was about 6.8% lower PC levels in the Intralipid® 20% we analyzed, compared to literature values for PC in egg phospholipids. First, only the major PC species were detected and semiquantified in our method. Second, there is a possibility that some of the PC species were hydrolyzed to LPC or free fatty acids during manufacturing and storage. Higher concentrations of PE and SM should be present in the egg phospholipid contribution to the Intralipid® 20% according to the literature; however, only six PE species and one SM species were detected with this method. No phosphatidylinositol and lysoPE species were detected. Negative mode ionization was carried out seeking for potential free fatty acids (FFA); however, no mass signal corresponds to free fatty acids were observed. In our study, PE and PI species were barely detected in negative mode, but positive mode peaks were used for quantification due to better sensitivity under the acid

Table 1
Lipid concentration in two separate batches of Intralipid® 20%.

Lipid	Batch 1, mg/dL ± (SD)	Batch 2, mg/dL ± SD	Average, mg/dL (% difference)[a]
LPC (16:0)	22.27 (± 0.56)	16.91 (± 0.64)	19.59 (27.36)
LPC (18:1)	14.66 (± 0.70)	9.13 (± 0.42)	11.89 (46.5)
LPC (18:0)	10.25 (± 0.33)	8.42 (± 0.31)	9.34 (19.6)
LPC (16:1)	1.48 (± 0.04)	1.28 (± 0.03)	1.38 (14.5)
LPC (22:6)	1.46 (± 0.05)	1.27 (± 0.04)	1.37 (13.92)
LPC (18:2)	7.95 (± 0.31)	5.42 (± 0.22)	6.68 (37.85)
LPC (20:5)	1.55 (± 0.04)	1.32 (± 0.05)	1.43 (16.03)
LPC (20:4)	2.48 (± 0.07)	1.71 (± 0.06)	2.10 (36.75)
LPC (20:3)	1.83 (± 0.06)	1.62 (± 0.05)	1.72 (12.17)
PC (16:0/ 22:6)	20.97 (± 0.72)	22.28 (± 0.89)	21.63 (−6.06)
PC (16:0/ 20:4)	27.67 (± 1.99)	27.25 (± 1.43)	27.46 (1.53)
PC (16:0/ 18:2)	154.93 (± 8.15)	172.55 (± 8.02)	163.74 (−10.76)
PC (16:0/ 16:0)	3.74 (± 0.15)	3.83 (± 0.15)	3.79 (−2.38)
PC (16:0/ 18:1)	250.38 (± 4.82)	252.51 (± 7.84)	251.45 (−0.85)
PC (18:0/ 22:6)	7.77 (± 0.11)	8.47 (± 0.16)	8.12 (−8.62)
PC (18:0/ 20:4)	36.74 (± 0.81)	37.85 (± 0.63)	37.30 (−2.98)
PC (18:0/ 18:2)	78.58 (± 1.66)	83.19 (± 0.88)	80.89 (−5.70)
PC (18:0/ 18:1)	78.41 (± 1.54)	78.13 (± 1.16)	78.27 (0.36)
PC (16:1/ 16:1)	1.22 (± 0.03)	1.25 (± 0.02)	1.23 (−2.43)
PC (16:0/ 18:3)	6.17 (± 0.17)	6.39 (± 0.14)	6.28 (−3.50)
PC (16:0/ 16:1)	7.40 (± 0.35)	7.15(± 0.37)	7.27 (3.44)
PC (16:1/ 22:6)	4.68 (± 0.22)	4.41 (± 0.23)	4.55 (5.94)
PC (18:1/ 18:3)	38.28 (± 1.39)	38.79 (± 1.69)	38.53 (−1.32)
PC (16:0/ 20:5)	35.93 (± 1.48)	39.42 (± 2.14)	37.67 (−9.26)
PC (18:1/ 18:2)	15.76 (± 0.33)	15.93 (± 0.53)	15.9 (−1.07)
PC (16:0/ 22:5)	3.10 (± 0.09)	3.06 (± 0.13)	3.08 (1.30)
PC (18:1/ 22:6)	6.41 (± 1.33)	6.51 (± 1.34)	6.46 (−1.55)
PC (18:1/ 22:5)	1.97 (± 0.05)	1.95 (± 0.06)	1.96 (1.02)
PE (16:0/ 18:1)	13.37 (± 0.34)	13.62 (± 0.30)	13.50 (−1.85)
PE (18:0/ 18:1)	12.65 (± 0.43)	12.71 (± 0.36)	12.68 (−0.47)
PE (18:1/ 18:3)	6.45 (± 0.19)	6.29 (± 0.17)	6.37 (2.51)
PE (18:0/ 20:4)	33.90 (± 1.14)	33.07 (± 0.69)	33.5 (2.48)
PE (18:0/ 18:2)	18.22 (± 0.46)	19.85 (± 0.65)	19.03 (−8.56)
PE (16:0/ 18:2)	7.77 (± 0.24)	8.68 (± 0.21)	8.23 (−11.06)
SM (16:0)	11.64 (± 0.41)	11.48 (± 0.28)	11.56 (1.38)
LnLnLn	17.40 (± 0.68)	19.68 (± 0.79)	18.54 (−12.30)
LnLnL	145.41 (± 12.41)	155.15 (± 10.89)	150.27 (−6.48)
LLLn, LnLnO	1097.69 (± 56.06)	1143.62 (± 43.09)	1121.65 − 4.10
LLL	7596.21 (± 446.43)	7654.00 (± 583.97)	7625.10 (−0.76)
LLP, PLnO	2173.83 (± 89.00)	2163.64 (± 103.60)	2168.73 (0.47)
LLO, OOLn, LnLS	3216.99 (± 166.48)	3167.89 (± 168.21)	3192.44 1.54
OOL, LnOS, LLS	1838.06 (± 95.02)	1768.41 (± 102.78)	1803.23 (3.86)
LnLnP	17.32 (± 0.26)	18.55 (± 0.42)	17.93 (6.86)
LLnP	462.15 (± 14.54)	479.47 (± 10.64)	470.81 (−3.68)
PLO/PLnS	1239.64 (± 24.06)	1194.50 (± 26.35)	1217.07 (3.71)
OOP/PLS	570.77 (± 10.54)	547.56 (± 8.88)	559.16 (4.15)
LOS/LnSS/ OOO	780.18 (± 17.16)	745.64 (± 15.38)	762.91 (4.53)
OOS/SSL	291.36 (± 11.03)	291.37 (± 11.17)	291.37 (0.00)

L = Linoleic acid, Ln = Linolenic acid, O = Oleic acid, P = Palmitic acid, S = Stearic acid (n = 9).

[a] The '% difference' is calculated as difference between batch 1 and 2 results divided by the mean. A negative value indicates batch 2 had a higher value.

Table 2
Phospholipids in Intralipid® 20% (this study) and egg phospholipids indicated in literature [36].

Phospholipids	Intralipid® 20% (mg/ dL) (this study)	Egg phospholipid (mg/ dL) [31]
LPC	55.5 (5.8%)	39.6 (3.3%)
PC	796 (82.7%)	853 (71.1%)
PE	93.3 (9.7%)	220 (18.3%)
LPE	ND	13.2 (1.1%)
PI	ND	46.8 (4.0%)
SM	17.3 (1.8%)	Not listed
Total mass (mg)	962 mg	1200 mg

Number in parentheses indicated percent of total lipid mass.

mobile phase conditions. In addition, the fatty acid composition of egg yolk can vary depending on animal feed composition and these variations are well documented in recent literature [34,35].

Soybean oil is the source of triacylglycerol in the emulsion mixture and 20 g of soybean oil is used per 100 mL of Intralipid® 20%. A total of eight different TAG masses were detected accounting for 19.4 g (97%) of the expected TAG. Hence the chromatographic retention of triglycerides is based on number of carbons in the three fatty acid chains of TAGs the structural isomers cannot be separated as they elute simultaneously. In addition to having identical retention times, those isomers are isobaric, thus making the differentiation by mass impossible. Furthermore, the standards are not available for each isomer. Since the isomeric triglyceride species could not be separated in this study, we separately measured total isomeric mixture to individual triglycerides based on composition of soybean oil reported in the literature [30]. The concentration of each TAG was then used to determine the fatty acid composition in each mixture. The percentage of fatty acid obtained in this study was compared with the fatty acids percentage listed in the product information and fatty acids (%) in soybean oil in literature as shown in Table 3[37]. The composition of TAG in this study was very similar to the product label and the published data for soybean oil. However, our analysis showed the percentage composition of linolenic

Table 3
Comparison of fatty acid composition in Intralipid® and soybean oils.

Fatty acid	Fatty Acids (%) in Intralipid® 20%[a] (based on the product label)	Experimental Fatty Acids (%) in Intralipid® 20% (this study)	Fatty acids (%) in soybean oil in literature [37]
18:3	4–11	12	13
18:2	44–62	52	55
18:1	19–30	23	18
18:0	1.4–5.5	4	4
16:0	7–14	9	10

[a] Data provided by the manufacturer.

acid was slightly higher than the numbers provided by the manufacturer. The presence of slightly higher linolenic acid could be due to the growth conditions of soybean plants, which affects the fatty acid composition of soybean oil, and these variations are well documented in recent literature [38]; however, without analysis of multiple manufactured lots, it is impossible to know if the value is statistically different compared to the range indicated by the manufacturer.

In summary, two batches of Intralipid® 20% were analyzed using a UHPLC-ESI-QTOF method and the data analyzed using Premier Biosoft SimLipid® software. Thirty-four phospholipids were identified, including 9 LPCs, 18 PCs, 6 PEs, and 1 SM. The detected phospholipids accounted for 80% (962 mg) out of the 1.2 g of total expected phospholipids in the tested samples. A total of 13 m/z values were observed for TAG including 26 TAG species as most of the triglycerides are isobaric isomers having similar retention times under the chromatic conditions of this study. The quantified amount of total TAG was 19.4 g, which represent 97% of expected TGA mass (20.0 g) in the emulsion mixture. A better understanding of the lipid composition could help to develop ILEs that provide optimal nutrition to patients [39]. For example, a balanced emulsion ratio prepared in a recent study by mixing soybean oil and fish oil to achieve ω-6 fatty acid to ω-3 fatty acid ratio of 2:1 compared to the typical 7:1 ratio found in soybean-based lipid emulsions, and resulted in better patient outcome in clinical studies [40]. In addition, a comprehensive lipid composition profiling could help regulatory agencies to understand the composition differences between ILE brands and generic formulations.

4. Conclusion

An UHPLC-ESI-QTOP method was developed and optimized for the detection and the quantitation of all the lipid species in Intralipids® 20% including lysophospholipid and triacylglycerols in a single analytical method with a 20-minute chromatographic run time. The method was used to analyze and compare samples from two different batches of the lipid formulation. Out of the 47 lipids identified, 34 were phospholipids (PLs) and the others were triacylglycerols. A total of 9 LPCs, 18 PCs, 6 phosphoethanolamines (PEs), and 1 sphingomyelin (SM) were detected and quantified. This study is evidence that this method can be utilized to quantify both the lipids and their degradation products in complex ILE emulsion mixtures. This analytical method may be important in monitoring the quality of commercially available ILEs. Additionally, we believe this analytical method could be useful to adopt in the evaluation of other ILE products, to determine the degradation products and their actual lipid composition. This would be beneficial in diverting and implementing new methods for the development of generic ILE manufacturing, stability and storage evaluation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

These studies were conducted using the Nanotechnology Core Facility (NanoCore) located on the U.S. Food and Drug Administration's Jefferson Laboratories campus (Jefferson, AR), which houses the FDA National Center for Toxicological Research and the FDA Office of Regulatory Affairs Arkansas Laboratory. This project was supported in part by an appointment to the Research Participation Program at the ORA, U. S. Food and Drug Administration, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and FDA. Authors would like to acknowledge Drs. Michael Wichman, Marilyn Khanna, Sean Linder, Jennifer Brzezinski, Selen Stromgren, John Shugart, and Paul Howard for their support.

Disclaimer

The views expressed in this document are those of the authors and should not be interpreted as the official opinion or policy of the U.S. Food and Drug Administration, Department of Health and Human Services, or any other agency or component of the U.S. government. The mention of trade names, commercial products, or organizations is for clarity, and should not be interpreted as an endorsement of a product or manufacturer.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2022.115112.

References

[1] G.L. Fell, P. Nandivada, K.M. Gura, M. Puder, Intravenous lipid emulsions in parenteral nutrition, Adv. Nutr. 6 (5) (2015) 600–610.
[2] S.M. Innis, Fatty acids and early human development, Early Hum. Dev. 83 (12) (2007) 761–766.
[3] M.S. Mundi, B.R. Salonen, S.L. Bonnes, R.T. Hurt, Parenteral nutrition–lipid emulsions and potential complications, Pract. Gastroenterol. 41 (8) (2017) 32.
[4] M. Raman, A. Almutairdi, L. Mulesa, C. Alberda, C. Beattie, L. Gramlich, Parenteral nutrition and lipids, Nutrients 9 (4) (2017) 388.
[5] M. Rayyan, H. Devlieger, F. Jochum, K. Allegaert, Short-term use of parenteral nutrition with a lipid emulsion containing a mixture of soybean oil, olive oil, medium-chain triglycerides, and fish oil: a randomized double-blind study in preterm infants, J. Parenter. Enter. Nutr. 36 (2012) 81S–94S.
[6] G. Deshpande, K. Simmer, Lipids for parenteral nutrition in neonates, Curr. Opin. Clin. Nutr. Metab. Care 14 (2) (2011) 145–150.
[7] Y.A. Carpentier, I.E. Dupont, Advances in intravenous lipid emulsions, World J. Surg. 24 (12) (2000) 1493–1497.
[8] L. Rothschild, S. Bern, S. Oswald, G. Weinberg, Intravenous lipid emulsion in clinical toxicology, Scand. J. Trauma Resusc. Emerg. Med. 18 (1) (2010) 51.
[9] W.S. Waring, Intravenous lipid administration for drug-induced toxicity: a critical review of the existing data, Expert Rev. Clin. Pharmacol. 5 (4) (2012) 437–444.
[10] V.E. de Meijer, K.M. Gura, H.D. Le, J.A. Meisel, M. Puder, Fish oil–based lipid emulsions prevent and reverse parenteral nutrition–associated liver disease: the Boston experience, J. Parenter. Enter. Nutr. 33 (5) (2009) 541–547.
[11] K. Hippalgaonkar, S. Majumdar, V. Kansara, Injectable lipid emulsions—advancements, opportunities and challenges, AAPS PharmSciTech 11 (4) (2010) 1526–1540.
[12] M. Rotenberg, M. Rubin, A. Bor, D. Meyuhas, Y. Talmon, D. Lichtenberg, Physicochemical characterization of Intralipid™ emulsions, Biochim. Biophys. Acta (BBA) Lipids Lipid Metab. 1086 (3) (1991) 265–272.
[13] D.A. Siriwardane, C. Wang, W. Jiang, T. Mudalige, Quantification of phospholipid degradation products in liposomal pharmaceutical formulations by ultra performance liquid chromatography-mass spectrometry (UPLC-MS), Int. J. Pharm. 578 (2020), 119077.
[14] B.D. Hayes, S. Gosselin, D.P. Calello, N. Nacca, C.J. Rollins, D. Abourbih, M. Morris, A. Nesbitt-Miller, J.A. Morais, V. Lavergne, Systematic review of clinical adverse events reported after acute intravenous lipid emulsion administration, Clin. Toxicol. 54 (5) (2016) 365–404.
[15] L.M. Haber, E.P. Hawkins, D.K. Seilheimer, A. Saleem, Fat overload syndrome: an autopsy study with evaluation of the coagulopathy, Am. J. Clin. Pathol. 90 (2) (1988) 223–227.
[16] J.A. Meisel, H.D. Le, V.E. de Meijer, V. Nose, K.M. Gura, R.V. Mulkern, M.R. A. Sharif, M. Puder, Comparison of 5 intravenous lipid emulsions and their effects on hepatic steatosis in a murine model, J. Pediatr. Surg. 46 (4) (2011) 666–673.
[17] V. Colomb, A. Jobert-Giraud, F. Lacaille, O. Goulet, J.C. Fournet, C. Ricour, Role of lipid emulsions in cholestasis associated with long-term parenteral nutrition in children, J. Parenter. Enter. Nutr. 24 (6) (2000) 345–350.
[18] P. Nandivada, G.L. Fell, K.M. Gura, M. Puder, Lipid emulsions in the treatment and prevention of parenteral nutrition–associated liver disease in infants and children1–3, Am. J. Clin. Nutr. 103 (2) (2016) 629S–634S.
[19] I. Hessov, F. Melsen, A. Haug, Postmortem findings in three patients treated with intravenous fat emulsions, Arch. Surg. 114 (1) (1979) 66–68.
[20] E.A. Klein, D.K. Montague, E. Steiger, Priapism associated with the use of intravenous fat emulsion: case reports and postulated pathogenesis, J. Urol. 133 (5) (1985) 857–859.
[21] P. Lenssen, B.A. Bruemmer, R.A. Bowden, T. Gooley, S.N. Aker, D. Mattson, Intravenous lipid dose and incidence of bacteremia and fungemia in patients

undergoing bone marrow transplantation, Am. J. Clin. Nutr. 67 (5) (1998) 927–933.
[22] J. Férézou, N.-T. Lai, C. Leray, T. Hajri, A. Frey, Y. Cabaret, J. Courtieu, C. Lutton, A. Bach, Lipid composition and structure of commercial parenteral emulsions, Biochim. Biophys. Acta (BBA) Lipids Lipid Metab. 1213 (2) (1994) 149–158.
[23] C. Hu, R. van der Heijden, M. Wang, J. van der Greef, T. Hankemeier, G. Xu, Analytical strategies in lipidomics and applications in disease biomarker discovery, J. Chromatogr. B 877 (26) (2009) 2836–2846.
[24] A. Carrasco-Pancorbo, N. Navas-Iglesias, L. Cuadros-Rodríguez, From lipid analysis towards lipidomics, a new challenge for the analytical chemistry of the 21st century. Part I: modern lipid analysis, TrAC Trends in, Anal. Chem. 28 (3) (2009) 263–278.
[25] R.C. Murphy, S.J. Gaskell, New applications of mass spectrometry in lipid analysis, J. Biol. Chem. 286 (29) (2011) 25427–25433.
[26] M.R. Wenk, Lipidomics: new tools and applications, Cell 143 (6) (2010) 888–895.
[27] N. Kothalawala, T.K. Mudalige, P. Sisco, S.W. Linder, Novel analytical methods to assess the chemical and physical properties of liposomes, J. Chromatogr. B 1091 (2018) 14–20.
[28] C. Horvath, W. Melander, I. Molnar, P. Molnar, Enhancement of retention by ion-pair formation in liquid chromatography with nonpolar stationary phases, Anal. Chem. 49 (14) (1977) 2295–2305.
[29] T. Züllig, M. Trötzmüller, H.C. Köfeler, Lipidomics from sample preparation to data analysis: a primer, Anal. Bioanal. Chem. 412 (10) (2020) 2191–2209.
[30] W.C. Byrdwell, E.A. Emken, W.E. Neff, R.O. Adlof, Quantitative analysis of triglycerides using atmospheric pressure chemical ionization-mass spectrometry, Lipids 31 (9) (1996) 919–935.
[31] S. Gerbig, Z. Takáts, Analysis of triglycerides in food items by desorption electrospray ionization mass spectrometry, Rapid Commun. Mass Spectrom. 24 (15) (2010) 2186–2192.
[32] W.W. Christie, X. Han, Lipid analysis, Trends Food Sci. Technol. 11 (7) (1996) 145.
[33] A.M. McAnoy, C.C. Wu, R.C. Murphy, Direct qualitative analysis of triacylglycerols by electrospray mass spectrometry using a linear ion trap, J. Am. Soc. Mass Spectrom. 16 (9) (2005) 1498–1509.
[34] W. Gładkowski, G. Kiełbowicz, A. Chojnacka, M. Gil, T. Trziszka, Z. Dobrzański, C. Wawrzeńczyk, Fatty acid composition of egg yolk phospholipid fractions following feed supplementation of Lohmann Brown hens with humic-fat preparations, Food Chem. 126 (3) (2011) 1013–1018.
[35] G. Cherian, A. Campbell, T. Parker, Egg quality and lipid composition of eggs from hens fed Camelina sativa, J. Appl. Poult. Res. 18 (2) (2009) 143–150.
[36] C.N. Blesso, Egg phospholipids and cardiovascular health, Nutrients 7 (4) (2015) 2731–2747.
[37] T.E. Clemente, E.B. Cahoon, Soybean oil: genetic approaches for modification of functionality and total content, Plant Physiol. 151 (3) (2009) 1030–1040.
[38] L.R. Gibson, R.E. Mullen, Soybean seed composition under high day and night growth temperatures, J. Am. Oil Chemists' Soc. 73 (6) (1996) 733–737.
[39] H. Grimm, A balanced lipid emulsion—a new concept in parenteral nutrition, Clin. Nutr. Suppl. 1 (3) (2005) 25–30.
[40] R. Grimble, Fatty acid profile of modern lipid emulsions: scientific considerations for creating the ideal composition, Clin. Nutr. Suppl. 1 (3) (2005) 9–15.