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Quantitative determination of fat emulsion using 1,6-diphenyl-1,3,5-hexatriene as fluorescence probe: application to the compounding of all-in-one parenteral nutrition admixtures

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Abstract

The potential contamination of binary bags by traces of fat emulsion stemmed from ternary bags prepared just before, led us to determine traces of lipids into the line set of the automated compounder MM23®. Diphenylhexatriene (DPH) was chosen as fluorescence probe due to its strong fluorescence enhancement in a lipid environment. Optimization of experimental conditions (i.e. DPH amounts, pH of fat emulsion samples, ultrasounds use, light, temperature and contact duration) for fluorescence measurement and validation of analytical method were performed. This method was linear over 0.5–8.0 mg l^{-1} ($r = 0.999$) of fat emulsion. The intra-day and inter-day precisions were inferior to 2% for the 2.0 and 8.0 mg l^{-1} standards. Under optimized conditions, the detection limit and quantitation limit were 0.10 and 0.29 mg l^{-1} of lipids respectively. Compared to the colorimetric method using sodium dichromate, it is at least 100 times more sensitive. The proposed method permitted to rapidly measure fat emulsion traces in automated compounder line set for parenteral nutrition solutions and thus, to assess the risk of contamination of binary bags by lipids. At last, this method was shown to be conveniently applied to the analysis of fat emulsion in the final total parenteral nutrition bag.

Keywords: Total parenteral nutrition; Fat emulsion measurement; 1,6-Diphenyl-1,3,5-hexatriene; Fluorescence probe

1. Introduction

In the Pharmacy unit of Robert Debré Hospital (Paris, France), an automated compounder (MicroMacroCompounder® 23) has been daily used for the preparation of binary (glucose and

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amino acids) and ternary (glucose, amino acids and lipids) parenteral nutrition solutions for several years [1]. So far, as the flushing process of the main line set of the automated compounder after lipid pumping is not validated, all binary bags must be manufactured before ternary (all-in-one) bags. This allows to avoid the cross contamination of binary bags by lipid traces, as it could affect the physico-chemical and microbiological stability of binary mixtures [2]. The contamination hazard of binary bags by lipids, stemmed from ternary bags, must be assessed in order to randomly compound either binary or all-in-one bags. The aim of this work was to look for a rapid and sensitive method to determine residual lipid concentration into the line set, prior binary bags preparation, and also to quantify the fat emulsion in the final parenteral nutrition bags.

Up to now, numerous methods have been developed to quantify lipids. Among them, the chromatographic techniques (Thin Layer Chromatography and High Performance Liquid Chromatography) are the most popular for lipid analysis [3–6]. However, these methods are time consuming or require relatively complex and expensive instrumentation. For the daily quality control of parenteral nutritive solutions, a simple and rapid method is necessary. For this purpose, a simple colorimetric lipid assay using sodium dichromate could be used [7]. However, the poor sensitivity of this method do not allow lipid traces determination in the line set of automated compounder system.

In order to increase sensitivity, a direct simple and rapid spectrofluorimetric method is suggested using 1,6-diphenyl-1,3,5-hexatriene (DPH) as fluorescence probe, which has been used for post-column fluorescence detection of ceramides by some of us [8] and for phospholipid analysis [9–12]. Fluorescence probes may be defined as fluorophores which undergo changes in one or more of their fluorescence properties (e.g. quantum yield of fluorescence: Φ_F) as a result of non-covalent interaction(s) with a selected molecular specie or upon a change of their molecular environment. Among the tested probes, DPH appears as one of the most suitable for fluorimetric assay of lipids because of its low background fluorescence in water and its increased Φ_F in hydrophobic medium (i.e. lipid environment). In this work, this property is applied to determine lipid content. Optimization of experimental conditions and validation of analytical method are presented. Some results obtained for real life samples regarding lipid quantitation in total parenteral nutrition (TPN) bags will also be performed to illustrate the practical availability of the proposed technique.

2. Materials and methods

2.1. Reagents

1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Sigma (St. Quentin-Fallavier, France). Working solution of 0.4 mM DPH was made by weighing appropriate amount of DPH, then diluted in tetrahydrofurane (THF). This solution was kept at -20°C for two weeks. Non stabilized THF was supplied by Fisher Scientific (Elancourt, France) and sterile water for injection by Fresenius (Louviers, France). Fat emulsion used for experiments was Ivelip® 20% (Clintec Nutrition, Sèvres, France) and was considered as the stock solution. Working solution of 8.0 mg l^{-1} fat emulsion was freshly prepared by diluting Ivelip® 20% with sterile water for injection. All solvents were carefully checked for the absence of fluorescence prior to use. Phosphate buffers with pH varying from 2.2 to 7.4 were obtained by mixing different volumes of disodium phosphate solution (0.2 M) and citric acid solution (0.1 M).

2.2. Apparatus

A Perkin Elmer LS5 spectrofluorimeter (Courtaboeuf, France) was used for routine measurement. Excitation and emission wavelengths were set at 352 and 426 nm respectively. Excitation and emission slits were set at 5 nm initially and optimized by measuring the signal to noise ratio. Fluorescence spectra of DPH were recorded with a Perkin Elmer LS50B spectrofluorimeter for Fig. 1.

MicroMacroCompounder® 23 was bought from Cair LGL (Civrieux d'Azergues, France).

2.3. General procedure

After the determination of optimal conditions, the usual procedure for lipid analysis was as follows: an aliquot volume of lipid equivalent to 0.5–8.0 mg from working solution of fat emulsion was transferred into a glass tube and adjusted to 1 ml with sterile water for injection. Just prior to fluorescence measurement, 10 μl of 0.4 mM DPH solution were added and the mixture was thoroughly vortexed for 5 s. Fluorescence was measured at 426 nm, and the blank (10 μl of 0.4 mM DPH solution mixed with 1 ml of sterile water for injection) was subtracted systematically from the sample measurement.

2.4. Study of optimal conditions for fluorescence

The parameters (i.e. DPH amounts, pH of fat emulsion samples, ultrasounds use, light and temperature and duration contact), which could possibly affect the incorporation of DPH into lipid bilayers were successively studied.

2.4.1. Effect of ultrasounds

Two milligram per liter fat emulsion contained standards were incubated with 4 nmol DPH during 60 min in an ultrasounds bath (Touzart Matignon, Courtaboeuf, France). Samples were protected from light. Fluorescence measurements were performed at time 0, 15, 30, 45 and 60 min.

2.4.2. Effect of light and temperature

Prior to fluorescence measurements, the mixtures of DPH (4 nmol) and fat emulsion standards (2.0 and 8.0 mg l^{-1}) were incubated in the dark or at day light for 60 min, at ambient temperature (20°C) or in refrigerator (2–6°C).

2.4.3. Effect of pH

Fluorescence was measured in 2.0 mg l^{-1} fat emulsion diluted in phosphate buffer (pH varying from 2.2 to 7.4). A 2.0 mg l^{-1} fat emulsion diluted in water was used as control.

2.4.4. Effect of time

The effect of time was studied with six fat emulsion contained standards (i.e. 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 mg l^{-1}).

2.5. Analytical validation

The study of the linear analytical response curve included six fat emulsion standards (i.e. 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 mg l^{-1}). Each curve was repeated six times. The method was validated according to SFSTP (Société Française des Sciences et Techniques Pharmaceutiques) guidelines [13]. A one way analysis of variance ANOVA test was performed, and the intercept of the linear regression equation was verified to be not significantly different from zero (Student test). The level of significance for all comparisons was set at $P < 0.05$. Precision of the method was evaluated by calculating intra-day and inter-day precisions. The analysis was performed six times for two different concentrations. Statistical treatment of data was done using Regress® and Excel® softwares.

2.6. Procedure for determination of lipid traces in total parenteral nutrition line set

TPN bags were prepared using a MM23® Compounder. To assess the minimal volume of flushing, all-in-one TPN bags (glucose, lipids, amino acids, electrolytes, vitamins, trace elements) were compounded. Thereafter, flushes of different volumes of water for injection were processed. After each flush a 100 ml bag of water was made. Samples were collected from these bags. To 1 ml of these different samples, poured into glass tube, 10 μl of 0.4 mM DPH reagent were added. Thus, the glass tubes were vortexed for 5 s and content transferred into the spectrofluorimeter quartz cuvett for measurement.

The lipid traces threshold was held to be 1.0 mg l^{-1} which is the usual value considered in the Pharmaceutical Industry as significant [Baxter, Research and Development, Parenteral Nutrition, Internal report dated on 1999/01/22].

3. Results and discussion

3.1. Optimal conditions for the fluorescence determination

The addition of DPH to water solutions caused a dramatic quenching of DPH fluorescence. Conversely, when DPH was added to diluted fat emulsion, fluorescence was observed (Fig. 1). This result could be closely related to incorporation of DPH into phospholipid bilayers in which DPH is generally oriented along the lipid acyl chain axis. Consequently, the DPH fluorescence was increased in absence of rotational motion [14].

First, the excitation and emission slits of spectrofluorimeter were chosen by measurement of signal to background ratio. The best signal was obtained when excitation and emission slits were set at 5 nm. All further measurements were done using these conditions.

3.1.1. Effect of 1,6-diphenyl-1,3,5-hexatriene concentration

Fig. 2 depicts the different amounts of DPH tested to get a linear curve in the range tested (i.e. 1.0–8.0 mg l^{-1} of lipids). As illustrated, the fluorescence signal decreased with amount of DPH $\geqslant$ 16 nmol. In these samples, particles in suspension were observed. This quenching effect was attributed to an inner filter effect induced by a too high DPH concentration in the sample and/or probably to the poor solubility of DPH in water. By contrast, with

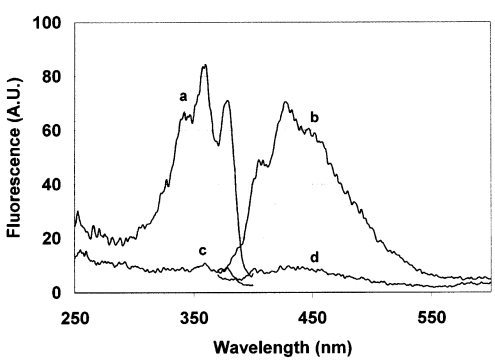

Fig. 1. Fluorescence spectra of 4 nmol ml^{-1} DPH solution: excitation (a) and emission (b) in 2 mg l^{-1} fat emulsion; excitation (c) and emission (d) in water.

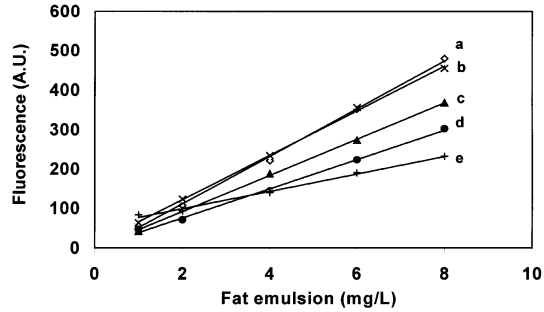

Fig. 2. Effect of DPH amount on linearity: (a) 4 nmol DPH; (b) 8 nmol DPH; (c) 16 nmol DPH; (d) 32 nmol DPH; (e) 64 nmol DPH.

about 4 nmol of DPH added, maximal luminescence was obtained. For this DPH quantity, fluorescence increased linearly with lipid concentration.

3.1.2. Effect of the ultrasounds on the assembly

It was reported in the literature, that ultrasounds could favour the incorporation of DPH into phospholipid bilayers [9]. From our experiments, no increase of the fluorescence was observed whatever the duration of the ultrasounds. By contrast, after 60 min, a decrease of the solution fluorescence (-26%) was noticed likely due to a certain DPH degradation.

3.1.3. Effect of light and temperature

The formation of the fluorescent assemblies between DPH and lipids could be influenced by light and temperature [9]. The inhibitory effect of low temperature (2–6°C) on fluorescence was observed (-30%), likely due to reduced diffusion of DPH in the lipid bilayers. On the other way, day light did not influence fluorescence signal in our case; at ambient temperature, after one hour of incubation at day light or in the dark, similar quenching effect was observed (-18%), likely due to the DPH degradation.

3.1.4. Effect of pH

Fluorescence obtained did not significantly change in 2.0 mg l^{-1} fat emulsion, diluted in phosphate buffer, while pH varied from 2.2 to 7.4. This suggested that pH did not affect DPH inclusion, thus fluorescence. However, in these tested fat

emulsions a quenching of about 30% of the signal compared to control, diluted in water at the same pH, was observed (Fig. 3). This observation strongly suggested that the presence of salts was likely to cause the decrease of the luminescence. Cations, which interact with negative phospholipids, decrease TPN emulsion stability [15] or prevent inclusion of DPH into phospholipid bilayer. This phenomena could be responsible of fluorescence decrease, as observed previously by other authors [9].

3.1.5. Effect of time

A prolonged contact between DPH and lipids is assumed to favor the penetration of DPH into phospholipid bilayers [9]. The Fig. 4 shows that fluorescence intensity increased slightly up to 90 s after the addition of DPH, reaching a plateau until 180 s. Then, from 180 s, intensity slightly decreased. It seemed that a certain time of 90 s was needed to form optimal assemblies between DPH and lipids. Then, the very slow quenching recorded could be related to photo-degradation of fluorescence probe DPH.

3.2. Analytical validation

A linear relation was obtained between the fluorescence intensity, corrected from the blank, and lipid concentration in the range 0.5–8.0 mg l^{-1}. The linear equation was $\Delta F = 64.72c + 3.66$ (where ΔF stands for relative fluorescence intensity and c represents lipid concentration with a linear correlation coefficient r of 0.999). Standard errors for slope and intercept were 0.41 and 1.80, respectively. The detection limit (LOD) (signal/background ratio = 3) was 0.10 mg l^{-1} of lipids. The quantitation limit (LOQ) (signal/background ratio = 10) was 0.29 mg l^{-1}. The validation includes statistical assessment of the linear regression. F value (Fisher test) of 24594 ($F_{\text{theoretical}(a=0.05)} = 4.13$) indicated that the independent variable can be used to predict the dependent variable whereas t value (Student test) of 1.97 ($t_{\text{theoretical}(a=0.05)} = 2.03$) verified that the intercept was not different from the origin.

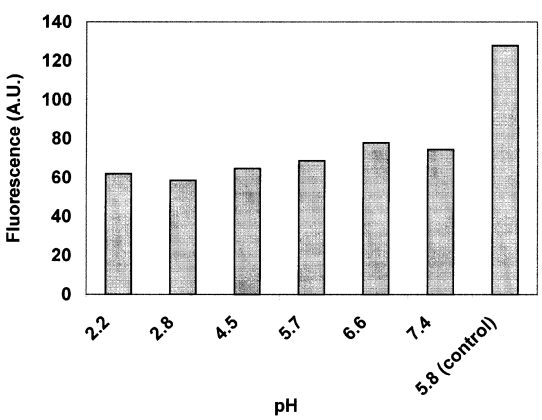

Fig. 3. Effect of pH on the DPH fluorescence. pH was measured directly in each sample.

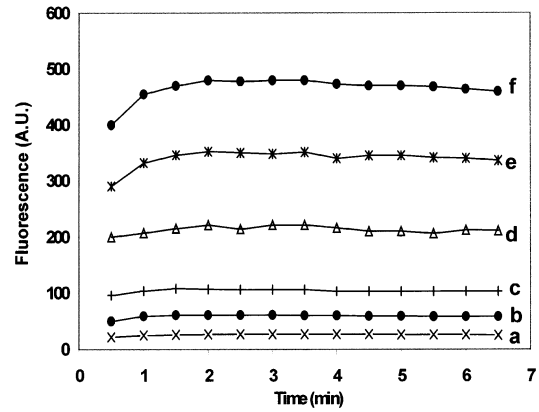

Fig. 4. Effect of contact duration between DPH solution and fat emulsion sample: (a) 0.5 mg l^{-1} fat emulsion; (b) 1 mg l^{-1} fat emulsion; (c) 2 mg l^{-1} fat emulsion; (d) 4 mg l^{-1} fat emulsion; (e) 6 mg l^{-1} fat emulsion; (f) 8 mg l^{-1} fat emulsion.

Intra-day and inter-day coefficients of variation for the standard solutions were determined to be, respectively, 1.74% and 1.44%, for a lipid concentration equal to 2.0 mg l^{-1}; 1.31% and 1.53% for 8.0 mg l^{-1}.

3.3. Application to total parenteral nutrition compounding

The assessment of the flushing volume necessary to rinse the line is particularly critical as it allows not to systematically have to compound the binary bags before the fat emulsion contained bags. Therefore, we looked at the minimal volume

Table 1
Analytical recoveries of lipid contents in total parenteral nutrition bags

Patient	Lipids added[a](g l^{-1})	Lipids measured[b](g l^{-1})	Mean recovery[b](%)
A	27.0	26.2	97 ± 8
B	27.0	27.1	100 ± 10
C	17.0	17.1	101 ± 9
D	27.0	28.0	104 ± 7

[a] Added into bags using MM23® driven by TPN Baxa® Software.
[b] Average of three measurements.

necessary to flush the line set between the two different bag types. To assess the risk of contamination of binary bags by lipid traces, the lipid concentration, after flushing volumes, was measured from 100 ml bags of water (which corresponds to the minimal volume of binary bags that we prepare). Flushing process with increasing volumes of sterile water for injection showed that 500 ml was the minimal volume to avoid lipid contamination. In these conditions, less than 0.29 mg l^{-1} of lipids were detected in the 100 ml bag of water, prepared after connection to the main line set of the automated compounder. This value, which is the LOQ of our validated method, was inferior to the lipid traces threshold considered as significant in the Pharmaceutical Industry i.e., 1.0 mg l^{-1}. This means that an amount less than 0.03 mg could be introduced into a binary PN bag. We think that it is a quantified, known and admitted risk in paediatric hospital. Moreover in most cases, children receive fat emulsion, perfused separately via an Y connector on the catheter simultaneously with the binary admixture, at a dose of 1–3 g kg^{-1} per day.

The proposed method was also used to determine lipid content into our TPN bags. As shown in Table 1, recoveries were satisfactory ranging from 97% to 104%.

4. Conclusion

This fluorimetric method for the determination of fat emulsion concentration in aqueous samples, using DPH as reagent, proved to be simple, rapid and sensitive. The LOD (0.1 mg l^{-1}) is particularly attractive and dramatically decreased compared with those obtained by visual inspection of sample turbidity or with other direct method [7]. In particular, the latter is at least 100 times less sensitive than ours using DPH.

The determination of minimal volume to rinse the final line set of automated compounder after ternary bags manufacturing allowed subsequent production of binary bags. This permitted to reorganize daily schedule of preparation and to get more flexibility.

Measurement of lipid contained samples in all-in-one TPN bags could be implemented as part of Quality Assurance System of our TPN compounding unit.

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