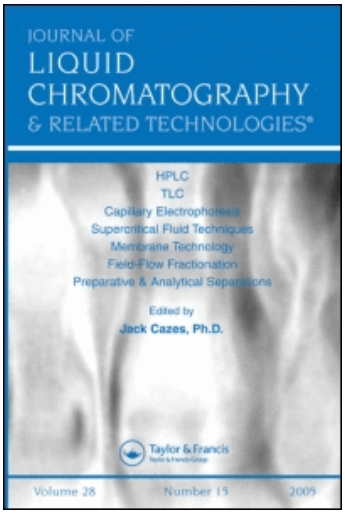

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ASSAY OF ANALYTES IN COMPLEX MATRICES. I. HPLC ASSAY OF GLYCERIN IN INTRAVENOUS FAT EMULSIONS

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ABSTRACT

An unexpected chromatographic behavior of fat globules has led to a simple HPLC assay for glycerin in the complex matrix of fat emulsions intended for intravenous injection. When a combination of a Hamilton PRP-1 (Polymer Reverse-phase) Column, a Biorad Microguard Carbo C Guard Column, and a Biorad HPLC Carbohydrate Analysis Column connected in series in the order listed was used for the separation of glycerin, with water as the mobile phase, the fat globules eluted unretained. The glycerin, which eluted by aqueous size exclusion, was detected and quantitated using a refractive index detector.

INTRODUCTION

Since their commercial introduction in the early seventies, intravenous fat emulsions have assumed the role of the major caloric component in parenteral nutritional admixtures. IV fat emulsions have given physicians an unsurpassed

capability to plan and manage the nutritional and caloric needs of chronically ill patients, patients with temporary as well as permanent gastrointestinal system impairments, and neonates and premature babies born with very low birth weights. Fat emulsions are also indicated for the treatment of essential fatty acid deficiencies. All commercially available fat emulsions are highly micronized oil-in-water dispersions of soy bean and/or safflower oil. Their state of dispersion is such that the globule-size range for the oil droplets (globules) in these emulsions are typically 0.2 to 0.8 μm. Soy lecithin or egg-yolk phospholipids are used as the emulsifying agent. Glycerin is added to the fat emulsions to make them isotonic. Glycerin is also believed to have a stabilizing influence on the fat globules in the emulsion. The complexity of the emulsion matrix imposes limitations on the approaches to quantitation of any of its individual constituents. The HPLC assay procedure described below achieves, under very mild operating conditions, both the separation and quantitation of glycerin in a complex matrix such as an IV fat emulsion.

EXPERIMENTAL

Equipment and Chromatographic Conditions

a) The liquid chromatographic system used in generating data reported here included a Model 510 Pump and a Model 710B autosampler (both from Waters Associates, Milford, MA), a column oven (DuPont Instruments, Wilmington, DE), a refractive index detector (Knauer differential refractometer, Rainin Instruments, Emeryville, CA), and a Chromatopac CR3 A integrator equipped with an FDD1A dual floppy disk drive and a monochrome monitor (Shimadzu, Kyoto, Japan). The procedure has since been performed on instrumentation from numerous other vendors.

b) Columns: Hamilton PRP-1 Column (15 cm x 4.5 mm, Hamilton Part No. 79425), Biorad Microguard Carbo C Guard Column (Biorad Part No. 125-0128), and Biorad HPLC Carbohydrate Analysis Column (Biorad Part No. 125-0095), connected in series in the order listed, with the latter (Biorad) columns kept in a column oven maintained at 80°C.

c) Mobile Phase: Nanopure water, twice filtered through a 0.4 μm polycarbonate membrane and kept degassed at 60°C.

d) Injection Volume: 50 μL; Detector Range: 16 X; Mobile Phase Flow Rate: 0.8 mL per minute.

Note: Because of the potential for high backpressure, the flow rate was initially set at 0.2 mL per minute. As the column assembly equilibrated at the oven temperature, the flow rate was incrementally increased to the indicated flow rate.

Materials

Nanopure water was used in the preparation of the mobile phase as well as in the standard and sample preparations. Abbott Laboratories Reference Standard Glycerin was used in the Standard Preparation. The glycerin used for addition/recovery studies as well as for manufacturing the fat emulsions for precision studies was tested and approved per the specifications and testing methodology for Glycerin USP. A placebo emulsion containing 40% (w/v) of a 50:50 mixture of safflower oil and soybean oil and 24 mg/mL of egg-yolk phosphatides was prepared by the Solutions Technology Pilot Plant of the Hospital Products Division of Abbott Laboratories using a proprietary manufacturing procedure. This placebo emulsion was used to prepare the spike/recovery samples used for establishing accuracy of the assay. The safflower oil, soybean oil, and egg-yolk phosphatides were tested and approved for human use by Abbott Laboratories' Quality Assurance Department against a set of FDA-approved proprietary specifications and testing methodology.

Standard, Sample, and System Suitability Preparations

The standard preparation contained 5.0 mg/mL of Abbott Laboratories' Glycerin Reference Standard in water. Samples of Liposyn were quantitatively diluted to an expected glycerin concentration of 5.0 mg/mL with water. The system-suitability preparation was an aqueous solution containing glycerin at 5 mg/mL and isopropyl alcohol at 6mg/mL.

System Suitability

With all system components in place, the column assembly was equilibrated at 80°C at a mobile-phase flow rate of 0.8 mL/minute for 30 minutes or until a steady baseline was obtained and two successive 50 μL injections of the standard gave peak responses within 1% of each other. A subsequent 50 μL injection of the system suitability preparation gave two well-resolved peaks with a resolution factor greater than 3.0. The tailing factor for glycerin was less than 1.5. Validation data presented here were generated using a pair of dedicated column assemblies.

Procedure

The standard and sample preparations were injected (injection volume = 50 μL) in duplicate. The respective average peak area values (A_s and A_u) were calculated. Using these average A_s and A_u values the sample assay results were calculated as:

$$(A_u/A_s) \times \text{Glycerin Conc'n in the Standard (mg/mL)} \times \text{Sample Dilution Factor} = \text{Glycerin Conc'n in the Sample (mg/mL)}$$

A reduced mobile-phase flow was maintained during the cool down prior to disassembly of the column ensemble for short-term storage (two to five days). For long-term storage after disassembly, 100 mL isopropyl alcohol followed by 100 mL water were pumped through the PRP-1 column. A mixture of acetonitrile and water (15:85) was pumped at a flow rate of 0.6 mL/min through the combination of Microguard Carbo C Guard and Carbohydrate Analysis columns for four hours, followed by water at 0.6mL/min for four hours with the column assembly maintained at 80°C for the whole duration.

Linearity, Accuracy, and Short-Term Precision

Averaged peak-area responses from duplicate injections of each of five solutions of glycerin reference standard ranging in concentrations from about 1mg/mL to 10 mg/mL were plotted against the respective concentrations. Accuracy of the procedure was established by adding known amounts of a glycerin reference standard into a placebo emulsion preparation and subjecting the spiked preparations to the assay procedure. These preparations contained 2, 15, and 30% (w/v) of fat and 20, 25, and 30 mg/mL of glycerin at each level of fat content. Short-term precision of the assay was established by replicate analyses of the same two lots IV fat emulsions over a period of four days by two analysts.

RESULTS AND DISCUSSION

Fat emulsions marketed by Abbott Laboratories under the Liposyn registration mark contain 100 or 200 mg/mL of safflower and/or soybean oil, 6 to 12 mg/mL of egg-yolk phosphatides, and 25 mg/mL of glycerin. The glycerin concentration must be controlled within 90 and 110% of the label claim (22.5 and 27.5 mg/mL, respectively), necessitating a precise, accurate, and specific assay procedure for glycerin. Until the recent advent of polymeric sulfonated styrene-divinylbenzene cation ion-exchange columns, assay procedures for glycerin were based on its facile and quantitative oxidation and subsequent quantitation of the products of oxidation. The primary step in the

assay procedure described in the United States Pharmacopoeia for glycerin by itself or in dosage forms such as Glycerin Ophthalmic Solution, Glycerin Oral Solution, and Glycerin Otic Solution involves its oxidation with an acidic solution of periodate.[1] Oxidation results in the formation of one mole of formic acid and two moles of formaldehyde for each mole of glycerin reacting. Quantitation of glycerin is accomplished by either an iodimetric titration of the excess oxidant or by titration of the formic acid produced in the reaction with sodium hydroxide.

A procedure that is widely used for the determination of serum triglycerides entails saponification of the triglycerides and oxidation with the periodic acid of the resulting glycerin. However, instead of quantitating the formic acid or the excess oxidant, the formaldehyde produced during the oxidation is subsequently reacted with a 1,3-dicarbonyl compound (e.g., acetylacetone) in the presence of ammonium ions (Hantzsch Reaction or Hantzsch Pyridine synthesis) to give a lutidine derivative. The lutidine concentration, which is spectrophotometrically determinable, is then correlated to the starting triglyceride levels. Saponification of triglycerides followed by enzymatic oxidation of glycerin is another approach adapted for triglyceride assay in clinical laboratories. This would suggest clinical triglyceride assays could be adapted for glycerin assay in intravenous fat emulsions, if the initial saponification step were avoided.

Because of the ubiquitous presence of free fatty acids in fat emulsions, titrimetric estimation of formic acid can lead to erroneous results. The unsaturated fatty acid residues that constitute a major complement of most triglycerides cannot be considered impervious to oxidation by periodate. Therefore, an assay based on quantitation of oxidant consumed can also lead to erroneous results. Hence, the adaptation of either variant of the USP procedure for the assay of glycerin in IV fat emulsions presented potential uncertainties about accuracy. Formaldehyde formed in the initial oxidation step of glycerin can also not be considered immune to further oxidation with periodate. Because of the stringent levels of accuracy and precision desired for assays of drugs in pharmaceutical formulations, we decided that none of the oxidation products of glycerin could be used as means for the accurate assay of glycerin in a matrix such as an IV fat emulsion.

Triglycerides and egg-yolk phospholipids can be considered as prodrugs for glycerin since hydrolysis of either can result in the formation of additional glycerin. Hence, assay procedure specific to glycerin in intravenous fat emulsions predicated a prior quantitative removal of both the triglycerides and the egg-yolk phospholipids by an operation under mild non-hydrolytic conditions. The neutral aqueous mobile phase used in the procedure discussed above avoids stressing the emulsion system to hydrolysis or oxidation.

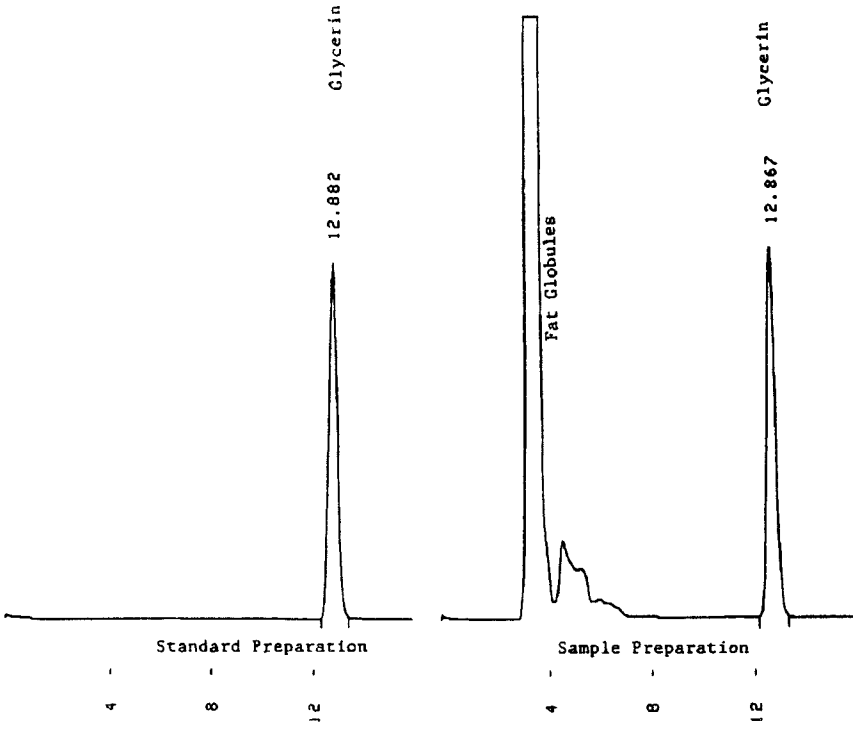

Figure 1. Chromatogram of the glycerin standard and a fat emulsion sample preparation.

Breslin and Stephenson have reported a reverse phase HPLC procedure for the determination of glycerin in 18 pharmaceutical preparations, based on a SPHERISORB S5-NH2 column and a mixture of acetonitrile and water as the mobile phase.[2] HPLC columns packed with sulfonated styrene-divinylbenzene copolymer packing material and variants of this packing material in other ionic forms (e.g., H+, Ag^+, Pb^+) provide separation of low molecular-weight polar molecules such as sugars, oligosaccharides, and alcohols and low molecular-weight organic acids by aqueous size exclusion. There have been a number of reports on the use of this class of stationary phases in the separation and quantitation of glycerin in several diverse but primarily aqueous matrices such as fermentation media, beers, wines, and grapes.[3-7] Columns packed with sulfonated styrene-divinylbenzene packing material lose their efficiencies very quickly when contaminated with hydrophobic materials such as triglycerides and phospholipids, limiting their potential adaptation for use in the assay of glycerin in fat emulsions.

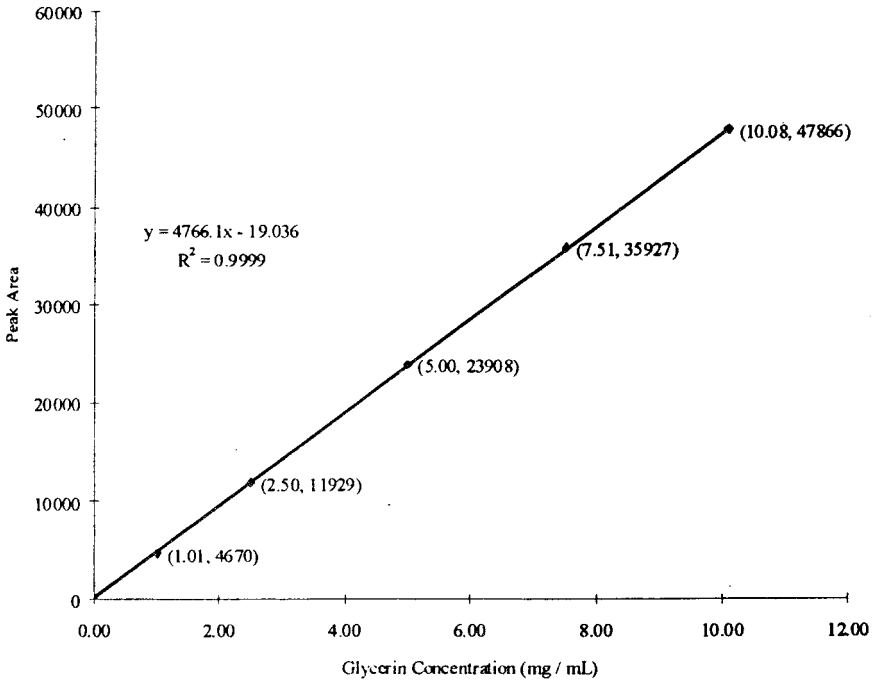

Figure 2. Plot of peak area versus glycerin concentration (mg/mL).

The packing material used in the PRP-1 column is a highly cross-linked copolymer of styrene and divinylbenzene. PRP columns can tolerate both acidic and basic pHs. These columns also tolerate a wide range of aqueous, aqueous-organic, and organic mobile phases. PRP columns have very high retention of lipophylic analytes and in general behave as reverse-phase columns with very high capacity factors for lipophylic analytes. Since both the triglycerides and egg-yolk phospholipids are non-polar and hydrophobic, it was assumed that they would be quantitatively retained on the PRP column when a primarily aqueous mobile phase was employed in the chromatography. It was felt that when a sample of emulsion was injected on a tandem combination of PRP and carbohydrate analysis columns, the PRP column would act as a scavenger column, retaining the fat globules (with their full complement of vegetable oil and egg-yolk phospholipids). At the same time, only glycerin would enter the carbohydrate analysis column for chromatography by size exclusion, thus permitting a mass selective detection by techniques such as differential refractometry.

Table 1

Addition/Recovery of Glycerin in Fat Emulsion Placebos

Analyst	Fat Content (% w/v)	Glycerin (mg/mL) Added	Recovered	% Recovery
A	2	20.15	20.32	100.8
B	2	20.24	20.37	100.6
A	15	20.15	20.26	100.5
B	15	20.24	20.20	99.8
A	30	20.15	20.17	100.1
B	30	20.24	20.26	100.1
A	2	25.18	25.46	101.1
B	2	25.30	25.39	100.4
A	15	25.18	25.27	100.4
B	15	25.30	25.31	100.0
A	30	25.18	25.25	100.3
B	30	25.30	25.33	100.1
A	2	30.22	30.45	100.8
B	2	30.36	30.38	100.1
A	15	30.22	30.25	100.1
B	15	30.36	30.34	99.9
A	30	30.22	30.07	99.5
B	30	30.36	30.51	100.5

Mean Recovery 100.3%
Standard Deviation ± 0.4
Standard Relative Deviation ± 0.4%

Typical chromatograms of a glycerin standard preparation and a fat emulsion sample preparation are shown in Figure 1. Contrary to our expectations, the fat globules went through the column combination unretained and eluted practically at the void volume as intact globules. This confirmed that chromatographic conditions do not impose any stress on the emulsion system. This chromatographic behavior, though unexpected, was not totally surprising. The PRP material requires a certain minimum percentage of organic solvent in the mobile phase to provide adequate wetting by reduced surface tension before its lipophylic nature can be brought into play. Such wetting is expected to be a prerequisite for the partitioning of the fat between the aqueous mobile phase and the lipophylic stationary phase surface. Typically an organic content of at least 5% (v/v) is needed for the polymer surface wetting before PRP columns can

Table 2

Assay of Glycerin in IV Fat Emulsions: Precision Data

	Glycerin (mg/mL)	
Day	**Lot A**	**Lot B**
1	26.25	25.74
2	26.35	25.68
3	26.26	25.66
4	26.37	25.79
Mean	26.31	25.72
Standard Deviation	± 0.06	± 0.06
Relative Standard Deviation	± 0.2%	± 0.02%

behave as true HPLC reverse phase columns. In the absence of surface wetting, the column is forced to behave like a filter bed, with no retention of the submicron sized fat globules. As mentioned earlier, the separation on the carbohydrate analysis column is by size exclusion. Fat globules with a typical diameter range of 0.1 to 0.5 μm can be considered as molecules with a molecular-weight range of 300 million to about 40 billion Daltons and can be expected to elute at the void volume in all commercially available Size Exclusion Chromatography columns.

A typical plot of peak area against glycerin concentration is shown in Figure 2. A linear response with an intercept practically at the origin and a correlation coefficient of unity is routinely obtained for this assay. Commercially available fat emulsions are formulated at 10 and 20% fat-content levels. Recovery studies were performed using emulsions containing 2%, 15%, and 30% (w/v) of fat and glycerin at 20, 25, and 30 mg/mL for each level of fat content. The recovery results that establish accuracy are shown in Table 1. Two analysts obtained a mean recovery of 100.3% ±0.4% at the three levels of glycerin addition to each of the three percentages of fat content. Short-term precision data were obtained over four days for two lots of Liposyn. The data are shown in Table 2. The observed relative standard deviation (RSD) of ±0.2% is unrealistically low for an HPLC assay (typical values are about ±1 to ±2%). These data are testimonials to the excellent validity of the assay procedure. The simplicity of the sample preparation procedure (just a simple dilution with water) and the chromatographic conditions (use of just water as the mobile phase) are both expected to provide an assurance of long-term robustness to the assay.

We feel that only intact submicron sized fat globules will traverse the length of the column assembly. The sample preparation can potentially contain trace levels of free fatty acids as well as tiny oil droplets. Both of these will be trapped on the PRP column. Contamination of the carbohydrate analysis column can have detrimental effects on its performance. Therefore, even though it does not appear to be necessary, the use of a PRP column and a guard column ahead of the carbohydrate analysis column is still recommended.

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