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Solid-phase extraction of phospholipids from hemoglobin solutions using Empore styrene–divinylbenzene disks

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Abstract

Styrene–divinylbenzene Empore disks were investigated for the extraction of phospholipids from red blood cells or aqueous solutions of hemoglobin as a means to reduce the time and solvent use required in sample preparation. Red blood cells are the source for hemoglobin used in the preparation of a hemoglobin-based oxygen carrier which is being developed to replace blood in transfusion therapy. Phospholipids are a major component of the membrane of red blood cells, and are toxic when administered directly into the vasculature. Sensitive analytical methods are required to detect phospholipids to ensure that concentrations in purified hemoglobin are well below toxic levels. This requires isolation from large volumes of purified hemoglobin solutions. The method described utilizes Empore disks to extract phospholipids from 30 ml of stroma free Hb preparations. Phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine and sphingomyelin were recovered with an average of 92% yield. The recovery of phosphatidylserine was 65%. The use of solvent and time required for sample preparation were reduced by an average of 80% relative to liquid–liquid extraction. The capacity of the 47-mm disk for the total of five phospholipids exceeds 0.3 mg. The method has been used for quantitation of phospholipids in red blood cells and stroma free hemoglobin solutions. © 1997 Elsevier Science B.V.

Keywords: Hemoglobin; Phospholipids; Styrene–divinylbenzene disks

1. Introduction

Hemoglobin based oxygen carriers (HBOCs) are a class of therapeutic products being developed for use as replacement for blood and red blood cells (RBCs) during surgery or in the emergency room [1]. Potential advantages of these products include a greater than one year shelf life, elimination of the need for typing and cross-matching, and inactivation of blood-borne viral contaminants. The hemoglobin (Hb) used in the preparation of an HBOC can be obtained from outdated RBCs. The phospholipid components of the RBCs are potential toxins if present in the product when it is administered by intravenous infusion. For example the inner membrane phospholipids phosphatidylethanolamine (PE) and/or phosphatidylserine (PS) can activate complement, initiate intravascular coagulation, induce thrombocytopenia and leukopenia and facilitate fibrinolysis [2] when administered at 0.05 mg kg^{-1} body weight to rabbits. Such effects can be lethal and so it is essential that the manufacturing process reduce phospholipid concentrations to below toxic levels.

Phospholipids are separated from Hb in several steps of the production process. After lysing of the

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RBCs the phospholipids are separated from Hb by a microfilter. Subsequent to pasteurization, which inactivates or destroys viruses, the Hb is separated from other proteins or contaminants by chromatographic steps [3]. These steps have also been found to further decrease the concentration of phospholipids in Hb solution. As a result phospholipids are present in very low concentrations in the final product.

Since phospholipids are present only at trace levels in the final purified Hb, large sample volumes (30 ml of 8 g dl^{-1} Hb) need to be extracted for quantitation. The classical liquid–liquid extraction (LLE) methods of Bligh and Dyer [4], Folch et al. [5] or Rose and Oklander [6] require 300 ml of a mixture of chloroform and methanol to extract 30 ml of 8 g dl^{-1} of Hb. With the LLE methods, multiple extractions are necessary for maximal recovery, enhancing the risk of operator error and material loss. In addition, any impurity present in the solvent is concentrated by such a procedure and increased detector background noise results. There are also attendant health risks and disposal costs.

The use of styrene–divinylbenzene (SDB) macroreticular resins offers an approach to improving methods. These resins adsorb amphipathic molecules from aqueous environments [7] primarily through Van der Waals forces. Applications include the simultaneous extraction and derivatization of organic acids from water [8] and microbial fatty acids from body fluids [9], and the extraction of phospholipids [10] and prostaglandins [11] from plasma. Recently, an extraction disk made from styrene–divinylbenzene has been developed by 3M. SDB Empore extraction disks [12] combine the adsorbing qualities of the SDB macroreticular resin in a convenient disk form. The disk has small SDB particles (10 μm) enmeshed in a polytetraflouroethylene (PTFE) backbone. The uniform and densely packed particle distribution, combined with a high surface area, permits efficient extraction at rapid flow-rates.

The use of SDB Empore disks to extract phospholipids from RBCs or hemoglobin solutions is reported in this paper. The separation, identification, and quantification of the individual phospholipids using a high-performance liquid chromatography (HPLC) separation method with laser light scattering detection is also presented [13–16].

2. Experimental

2.1. Materials and samples

2.1.1. Chemicals and reagents

Solvents and reagents were purchased from Caledon Labs (Georgetown, Canada). The chloroform and methanol were HPLC grade and the ammonium hydroxide was reagent grade.

Lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA) as 10 or 25 mg ml^{-1} solutions in chloroform. The chemical name and abbreviation of the lipids used routinely are: PE, 1,2 dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1 fatty acid); PI, l-α-phosphatidylinositol; PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (9-*cis*-octadecanoic or 18:1 fatty acid); PS, 1,2- dioleoyl-*sn*-glycero-3-phospho-l-serine (18:1 fatty acid); SM, sphingomyelin. The PE, PC and PS were synthetic, SM was isolated from chicken egg and PI from bovine liver; PI and SM were not available as synthetic products. Human sources for phospholipids were not available, however the 18:1 fatty acid version of the synthetic lipids were chosen to represent phospholipid from human RBCs.

2.1.2. Extraction disks

Extractions were performed using Empore extraction disks made with styrene–divinylbenzene (SDB), (J.T. Baker, Phillipsburg, NJ, USA).

2.1.3. Process samples extracted

Outdated human RBCs (Canadian Red Cross) are the raw material used for the Hb based products at Hemosol since they are available in large quantities.

To prepare the raw material RBCs for analysis of phospholipid content, one ml of packed RBCs was mixed with 2 volumes of 50 m*M* Tris [hydroxymethyl]-aminomethane hydrochloride (Tris) buffer (pH 8.9) and the mixture vortexed briefly. The sample was extracted as described in Section 2.2.4.

To purify Hb from stroma on a production scale, the RBCs were lysed and the membrane-associated phospholipids were separated from Hb by a microfilter. A sample of the stroma free Hb was removed and analyzed directly for phospholipids by the SDB method as described in Section 2.2.3.

The stroma free Hb solution was then pasteurized

to further reduce the risk of viral contamination and two subsequent chromatography steps removed basic and acidic impurities. The final purity of the hemoglobin A_0 was >99.5% by an anion exchange purity assay [3]. This purified Hb was tested for phospholipid content by the analytical procedure described in Section 2.2.2.

2.2. Procedures

2.2.1. Treatment of glassware

The cleaning procedure for the glass test tubes used in sample preparation involved washes with methanol and chloroform and a final rinse with methanol.

2.2.2. SDB extraction method applied to purified hemoglobin samples

A 30-ml sample of an 8 g dl^{-1} solution of purified Hb in 50 m*M* bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane (Bistris), pH 6.5 were transferred into cleaned borosilicate glass test tubes. The extractions were performed on a six-port solid-phase extraction apparatus, 47 mm, from Millipore (Mississauga, Canada). The SDB disks were placed on the filtration base of each port and the funnels were attached. Each disk was activated with 25 ml of methanol which wets the disk and enhances the interaction between the disk and the aqueous phase. Without letting the disk dry, each disk was washed with 50 ml of deionized water. The methanol and water were directed to a waste container. The hemoglobin sample was filtered through the disk; phospholipids present in the Hb solution adsorb to the disk and the filtered Hb was directed into a waste container. The disk was washed with 50–100 ml of distilled water to remove residual hemoglobin and was allowed to dry by pulling air through. In the elution step, a clean empty borosilicate test tube was placed underneath the disk and 5 ml of methanol was added to the disk. The disk was soaked in methanol for 15 min to ensure complete elution of the lipids; after 15 min, the methanol was filtered through the disk into the test tube and was followed by a 3-ml methanol rinse. This elution step was repeated with solvent A instead of methanol. [Solvent A is a mixture of chloroform–methanol–ammonium hydroxide (80:19.5:0.5, v/v)]. The methanol and solvent A were combined in the test tube and were evaporated under a gentle stream of nitrogen using a Pierce Reacti-Therm 3 Evaporator (Rockford, IL, USA) at 30–40°C. The residue was reconstituted in 4 ml of solvent A and filtered through a 0.22-μm PTFE filter to remove insoluble materials. The filtered eluent was evaporated and reconstituted in a small volume of solvent A (usually 0.4 ml) for the final analysis by HPLC.

2.2.3. SDB extraction method applied to stroma free hemoglobin samples

The SDB extraction of stroma free Hb samples was performed in exactly the same way as purified stroma free Hb samples. The only notable difference was the volume of stroma free Hb sample used, which varied up to 90 ml. The other elution volumes described in Section 2.2.2 remained the same.

2.2.4. SDB extraction method applied to red blood cells

The extraction disk was activated and washed as described in Section 2.2.2. The RBCs in Tris buffer were filtered directly through the activated disk. To improve the time of filtration, a Glass Microfibre Filter of 1- or 2-μm pore size was placed directly on top of the SDB disk (GMF, Whatman, Maidstone, UK). The two sets of disks were activated with methanol, washed with water and the RBCs were filtered through both sets of filters. Both sets of filters were eluted as described in Section 2.2.2.

2.3. Determination of recoveries by method of standard addition (MOSA) [17]

Phospholipid standards in chloroform (26 or 5 μg of each phospholipid) were added to a clean, empty tube and dried under a stream of nitrogen. The hemoglobin test sample was added to the tube (30 ml of 8 g dl^{-1} solution of Hb in 50 m*M* Bistris, pH 6.5), the mixture solubilized by sonication for 15 min, and then vortexed briefly. The extraction proceeded as described in Section 2.2.2.

2.4. Chromatography

2.4.1. Instrumentation

HPLC analysis was performed using a Beckman system (Beckman Instruments, Mississauga, Canada). Detection was performed with a Varex ELSD 2a evaporative light scattering detector (Varex, Burtonsville, MD, USA). The detector was set to the following conditions: N_2 gas outlet on regulator set to 80 p.s.i., gas inlet pressure 28 p.s.i., flow meter set at 45 mm, drift tube 116°C.

2.4.2. Column

The HPLC column used was a Spherisorb silica 3 μm, 150×4.6 mm I.D. from Alltech (Deerfield, IL, USA).

2.4.3. Mobile phase and gradient

The solvent mixtures A and B for the binary gradient system were similar to those of Becart et al. [13]. The gradient used and a chromatogram of the standards are shown in Fig. 1. Becart used a single linear gradient from 100% A to 100% B over 14 min [13]. In our method, two linear gradients were used and are shown in Fig. 1. All standards were resolved, and sphingomyelin gave two bands as previously documented by other researchers [16,18]. The solvents are solvent A, chloroform–methanol–ammonium hydroxide (80:19.5:0.5, v/v); solvent B, chloroform–methanol–water–ammonium hydroxide (60:34:5.5:0.5, v/v). The flow-rate was 1 ml min^{-1}.

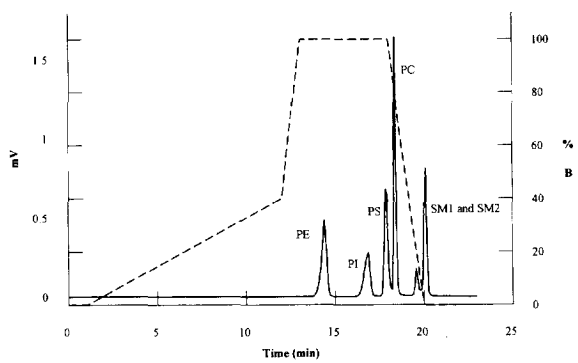

Fig. 1. Chromatogram of standards. Lipids are (1) phosphatidylethanolamine (PE), (2) phosphatidylinositol (PI), (3) phosphatdiylserine (PS), (4) phosphatidylcholine (PC) and (5) sphingomyelin (SM1 and SM2). Modified binary gradient is shown.

2.4.4. Calculations

A standard curve of the logarithm of area detector response versus the logarithm of μg lipid standard for concentrations of phospholipid up to 45 μg was used, where the response of the detector was linear. Standard curves were prepared for each of the five lipids. All standard curves had correlation coefficients of 0.999±0.0004 (mean±standard deviation, $n=5$, 11 point curve). Concentrations were calculated by substituting the peak area into the standard curve. For wider ranges of concentration, for example up to 300 μg, the detector response was not linear and a polynomial fit was used for the calculations. The concentration of the extracted phospholipids was calculated from the standard curve, in micrograms and the percentage recovery was calculated by:

$$\%\ \text{Recovery} = \frac{\mu\text{g calculated (standard curve)}}{\mu\text{g theoretical (manufacturer of lipid)}} \times 100$$

2.5. Determination of accuracy

2.5.1. Comparison of LLE with SDB for purified Hb samples

For the LLE method, four 5-ml samples of an approximate 8 g dl^{-1} solution of Hb were spiked with 26 μg of each of the five lipids tested. The 5-ml aqueous sample was extracted with a solution consisting of 12 ml of methanol and 6 ml of chloroform and the mixture was vortexed. The four tubes were centrifuged for 20 min at 2240 *g* and 4°C, using a Beckman Model J2-21 centrifuge and JA-20 rotor (Beckman Instruments). This separated a liquid phase and a protein pellet. The supernatant from the samples was decanted into four clean tubes. The protein pellets were re-extracted with 8 ml of chloroform–methanol (1:1, v/v) per sample. The mixture was vortexed and centrifuged. The supernatant was decanted into the liquid phase. In the final step, the water and organic layer were separated by adding 6 ml of chloroform and 10 ml of 0.1 *M* NaCl. The separation of water and organic layers required at least 1 h. The organic layer was removed (32 ml), was evaporated to dryness under nitrogen, reconstituted with solvent A and filtered through a 0.22-μm PTFE filter to remove particles. The eluent was

evaporated and reconstituted in 400 μl of solvent A for analysis.

For the SDB method, four samples each consisting of 30 ml of 8 g dl^{-1} solution of Hb spiked with 26 μg of the five lipids were analyzed as described in Section 2.2.2.

The eight samples from the two methods were analyzed by the normal phase HPLC method and quantitation was done using a standard curve as described in Section 2.4.4.

2.5.2. Comparison of LLE versus SDB for RBCs samples

The LLE of RBCs samples was similar to the purified Hb samples, except 1-ml samples of RBCs were extracted. The amounts of methanol and chloroform used for the extraction were decreased by a factor of 3.

2.6. Peak identification

A large volume of non-spiked purified hemoglobin (190 ml of an 8 g dl^{-1} solution), and a spiked sample of 8 g dl^{-1} Hb (26 μg of the 5 lipid standards spiked into 30 ml of Hb) were extracted using the SDB method. The extracts were injected onto the HPLC, the peaks collected, dried under nitrogen, reconstituted in methanol (15–50 μl), and then analyzed by a Sciex API3 Biomolecular Mass Analyzer (Sciex, Toronto, Canada). Direct injection (ionspray) was used with methanol as carrier solvent in a 50-μm silica capillary. The injection was 2 μl in a Rheodyne 20-μl loop. The methanol flow-rate was 20 μl min^{-1} using a LKB Bromma 2150 HPLC pump.

2.7. Determination of capacity of SDB disks

Two sets of spiking and extraction studies were performed with and without Hb present, at phospholipid concentrations of 2, 9, 19, 38, and 75 μg of PL.

In the first set of experiments, no hemoglobin was present, and the SDB disks were spiked directly with the 5 phospholipids. Each disk was washed with 100 ml of water which was directed to waste. The disks were dried by pulling air through for 30 min, and were then extracted by elution with methanol and solvent A as described in the elution step of Section 2.2.2.

In the second set of experiments, Hb was spiked with the 5 phospholipids as in the MOSA. The spiked Hb was then extracted as described in Section 2.2.2.

3. Results and discussion

Process and quality control for the production of an HBOC requires the determination of phospholipids in the RBCs and after each step of the purification process. LLE is routinely used as a standard method for extraction of phospholipids from RBCs or stroma free Hb solutions. It is an expensive and labour intensive method. SPE offers an attractive alternative; however there are no literature methods available for the extraction of phospholipids from RBCs or Hb by SPE. Several solid-phase extractions were tried including those using fumed silica and XAD-2 resin. The silica suffered from poor extraction efficiency; the XAD-2 resin gave good recovery, but could not be validated due to the co-adsorption of phospholipid onto the glass or PTFE frits used to isolate the resin. In addition the XAD-2 resin required extensive cleaning before use.

The SDB disks offer an alternative SPE method. These disks are manufactured under tight quality control conditions, are free of interferences and can be used under fast flow-rates. The SDB analysis of phospholipids from RBCs indicated a total concentration of 344 μg in 1 ml of packed RBCs (or 3185 μg g^{-1} Hb, $n=4$). A representative chromatogram of the extract is given in Fig. 2a. These results were comparable to the analysis of RBCs by LLE, which yielded a total of 305 μg phospholipid in 1 ml packed RBCs ($n=4$).

The drawback to the analysis of RBCs with the SDB method was the time required to filter the RBCs sample; more than 1 h was required for filtration, presumably due to the occlusion of the disk with fibrin or cellular debris. To improve filtration time, a pre-filter consisting of glass microfibre filters of 1- or 2-μm pore size was placed on top of the SDB disk [19]. The RBCs were filtered through both disks. The use of the pre-filter reduced the filtration time to

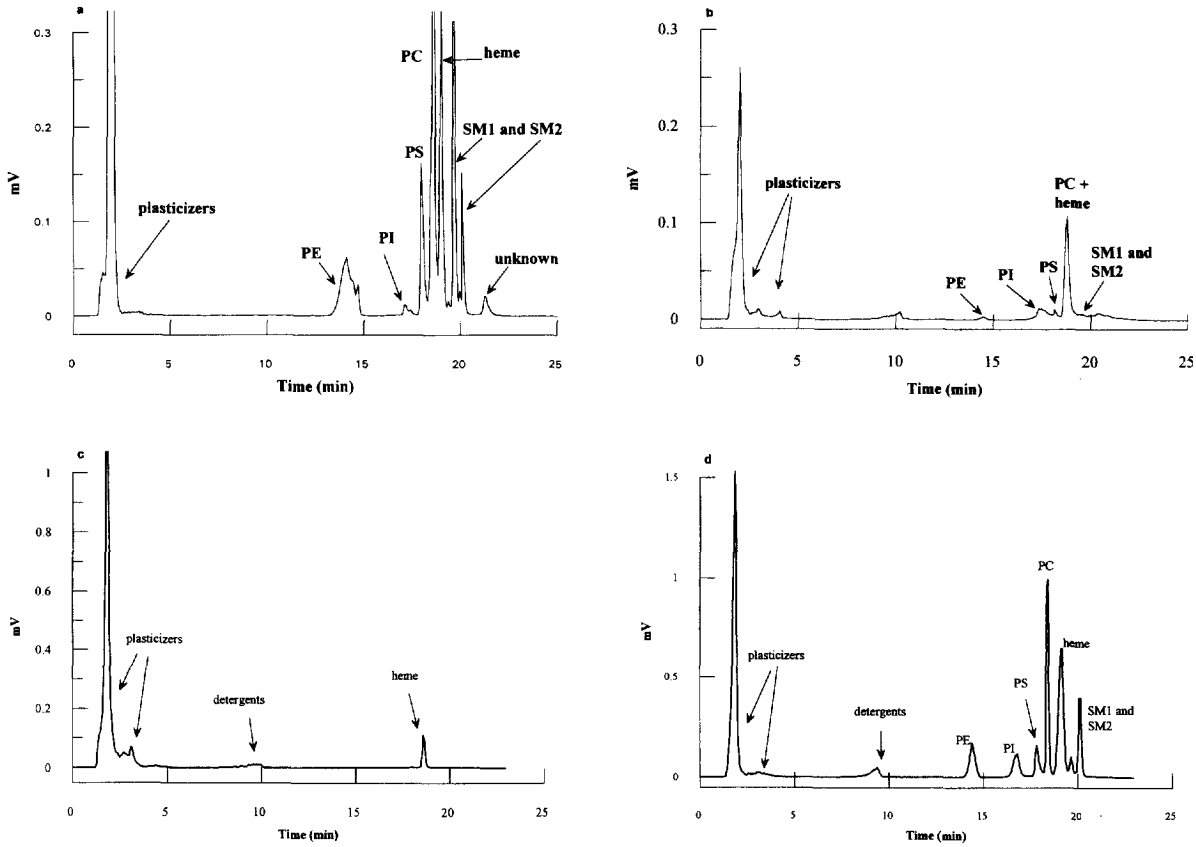

Fig. 2. (a) Chromatogram of SDB extract of RBCs (1 ml, total hemoglobin is 10.8 g dl^{-1}). (b) Chromatogram of SDB extract of stroma free Hb (90 ml of 1.35 g dl^{-1} Hb). Most of the phospholipids have been removed. (c) Chromatogram of SDB extract of purified stroma free Hb (30 ml of 7.8 g dl^{-1} Hb). No phospholipids were detected. (d) Chromatogram of SDB extract of hemoglobin which was spiked with 26 μg of the five phospholipids.

approximately 10 min. The extraction efficiency was identical to that without the pre-filter.

The LLE and SDB methods were compared to published values for the 5 phospholipids in RBCs (and therefore other lipids such as cholesterol were not included). For the comparative study the five lipids were summed up and the percent of each lipid was calculated from the total. The same calculation was made for published values for phospholipids in RBCs and the comparison is shown in Table 1. The results indicate excellent correlation of the two methods with published values [20–22].

The decrease in the phospholipid levels was measured after RBCs lysis and microfiltration. The SDB analysis of a representative post lysis/microfiltration sample is shown in Fig. 2b. There was a decrease in the phospholipid levels from 3185 μg g^{-1} Hb in the RBCs sample to 24 μg g^{-1} Hb in the stroma free Hb sample. The analysis of the RBCs and stroma free Hb solutions demonstrate the flexibility of the method since sample size was varied from 1 ml for the RBCs to 90 ml for the stroma free Hb sample with no change in equipment or procedure. Samples as large as 1 l have been tested with this method (data not shown).

The stroma free Hb solution was further purified by pasteurization and chromatographic steps. The phospholipid levels in the final purified hemoglobin were routinely tested by the SDB disk method and found to be below 2 μg g^{-1} Hb (or 0.2 nmol ml^{-1}, Fig. 2c). Any Hb lot containing phospholipids above this level would be rejected.

Table 1
Comparison of the LLE method with the SDB method for extraction of red blood cells

Lipid	Phospholipid (%)		
	Method		Published values[a]
	LLE	SDB	
PE	27.7	24.8	29.2
PI	2.0	3.4	1.2
PS	29.3	31.1	30.2
SM	27.4	27.0	25.0

Experimental conditions: Four 1-ml samples of red blood cells (total Hb = 10.8 g dl^{-1}) were extracted by each method. The concentration of phospholipids present were calculated versus a standard curve. The total concentration of phospholipids was calculated for each sample and the percent of the individual lipids were calculated from the total (g%). The standard deviations were less than 8% for both methods.

[a] Averaged from several sources (Refs. [20–22]).

Table 2
Recoveries of spiked purified hemoglobin, extracted by SDB method

Lipid	26-μg spike		5 μg-spike	
	Recovery (%)	SD	Recovery (%)	SD
PE	90.5	6.4	91.2	10.3
PI	87.7	8.5	109.6	19.4
PS	77.7	6.0	98.3	13.8
PC	77.7	6.0	98.3	13.8
SM	98.0	4.1	115.2	11.9

Experimental conditions: 30-ml samples of 8 d dl^{-1} hemoglobin were spiked with 5 or 26 μg of phospholipid. The hemoglobin was extracted by the SDB method and the concentrations were calculated from a standard curve. The recovery was calculated versus the concentration of the spike. Note that PC and PS were not completely resolved in this set of data and the recoveries were calculated together. $n = 6$ for both experiments.

In a parenteral product it is necessary to identify the source of all the components from a regulatory and safety viewpoint. For these reasons, we examined the identity of all the components in the extract of purified Hb (example Fig. 2c) and of purified Hb spiked with the 5 phospholipids (Fig. 2d). A tentative identification of the peaks by mass spectroscopy (MS) are labelled on the figures. The MS analysis confirmed that the SDB disk extracted phospholipids and confirmed the purity of the Hb solutions. Detection with MS analysis was very sensitive: with this method the phospholipids in a 190-ml sample of purified hemoglobin (8 g dl^{-1}) were found to be less than 3 pg ml^{-1}.

The other components shown in chromatogram Fig. 2c and d were identified as detergents and phthalates (plasticizers) and were shown to be related to sample preparation. The interference at 18.6 min in both the spiked and non-spiked sample had a M_r of 616 u, and was tentatively identified as heme. The presence of the heme was further indicated by the reddish-brown colour of the fraction and its spectrum determined by a diode array scan from 220–600 nm. The heme can be separated from the other peaks or it can be identified by a UV–Vis detector placed in-line before the evaporative light scattering detector.

The efficiency with which the SDB disks extract phospholipids from purified hemoglobin solutions was determined by MOSA. The recovery ranged from 77–98% for a 26 μg spike and from 91–115% for a 5-μg spike (Table 2). These results were reproduced in an inter-day precision study over a three week period with 14 samples, yielding comparable results. The >100% recoveries are present only with the lower spiking concentration and could arise from imprecision in the chromatography or errors in the extraction. The error in the chromatographic step on a 1-μg injection of phospholipid ranges from 2–15%, whereas the error on a 30-μg injection is less than 3%, indicating the error in the chromatography at the lower concentrations is significant. The error attributed to sample handling has not been quantified but can be minimized by the use of an internal standard. Overall, recoveries are essentially complete and are very acceptable for an extraction method.

The effect of Hb on the extraction efficiency was investigated and the results are summarized in Fig. 3a–e, where the recovery was plotted against the concentration of each lipid. The recoveries of the five phospholipids in the presence of Hb averaged 15% lower than in its absence, indicating a masking effect. To account for any masking effects, the target concentration of 0.2 nmol ml^{-1} was multiplied by 1.15. By applying this correction there is still less than 0.23 nmol ml^{-1} of phospholipid in the final purified hemoglobin which is well below possible toxic levels.

Fig. 3 also indicates the capacity of the disk. If the recovery of phospholipid starts to decrease with

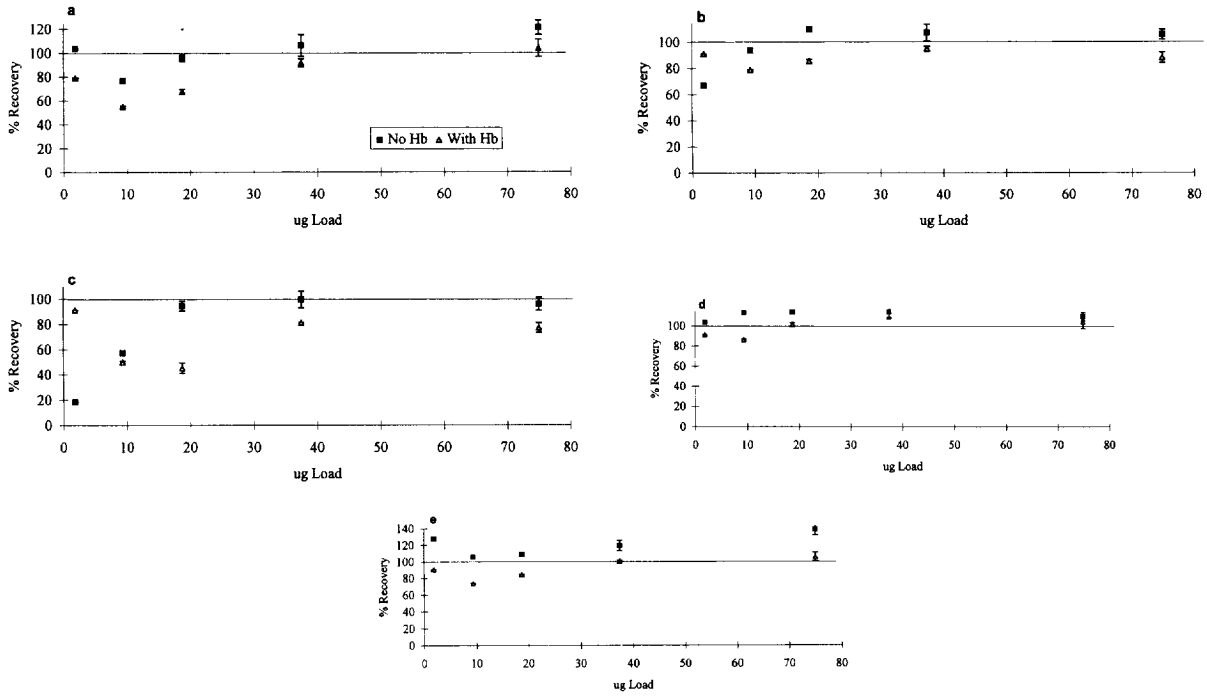

Fig. 3. Percentage recovery of phospholipids at lipid loads from 1.8–80 μg. (a) Phosphatidylethanolamine, (b) phosphatidylinositol, (c) phosphatidylserine, (d) phosphatidylcholine and (e) sphingomyelin.

increasing concentrations, this may indicate the disk is saturated. As is evident from Fig. 3a to e, disk capacity was not reached when 75 μg of each lipid was spiked and extracted. The capacity was determined to be at least 75 μg of each phospholipid or 375 μg total, which is within the range reported by the manufacturer [23].

The accuracy of the SDB method to extract phospholipids from purified Hb spiked with 26 μg of the 5 phospholipids was determined by comparison with the classical LLE method. The results are shown in Table 3 and indicate that the recovery of PE, PC and SM is very similar for both methods, but that of PI and PS is much lower for the LLE method than for the SDB method. In addition, there was interference in the chromatograms of the LLE extracts near the PE peak, which may have caused the excess recovery. Overall the recovery was superior with the SDB method.

This study aimed to develop a simple method using minimal solvent which could be used routinely to extract phospholipids from hemoglobin or RBCs. The SDB filtration method is flexible since sample size is easily varied from 1 ml up to 1 l with no change in the operating procedure. The method is rapid; for example, a 30-ml sample of purified Hb is filtered in less than 2 min and the elution step is complete in approximately 30 min. This is a 2/3 reduction in time versus the classical LLE method.

Table 3
LLE method versus SDB disk method for spiked hemoglobin

Lipid	26-μg spike		5-μg spike	
	Recovery (%)	SD	Recovery (%)	SD
PE	81.1	4.1	111.2[a]	7.5
PI	93.1	1.9	53.7	20.4
PS	65.5	5.4	38.2	15.8
PC	100.0	3.3	91.3	4.0
SM	95.7	1.7	101.9	5.9

Experimental conditions: 30-ml samples (for SDB method) or 5-ml samples (for LLE method) of hemoglobin were spiked with 26 μg of each of the 5 phospholipids. Four samples were extracted for both methods.

[a] There was interference near the PE peak in the chromatogram of the liquid extract, whose identity has not yet been determined.

The solvent use is 16 ml per sample regardless of sample size, versus 300 ml for a 30-ml sample for LLE, giving a 95% reduction in solvent use. The SDB method is both accurate and reproducible and has been shown to be useful for RBCs, stroma free Hb solutions and purified Hb samples.

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