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Aqueous two-phase partitioning sample preparation prior to liquid chromatography of hydrophilic drugs in blood

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Abstract

The technique of aqueous two-phase partitioning is investigated as a sample preparation procedure prior to LC determination of drugs in blood. In the extraction 500 μl of whole blood is added to 2.00 g of PEG 300 and 18.0 g of phosphate buffer. The phases are mixed by stirring and allowed to separate before the clear lower salt phase is drained out using a specially designed glass adapter. After filtration 20 μl is injected into the LC system. When validated for the X-ray contrast medium iohexol R.S.D. was 2.0% and 1.2% at 10 μg/ml and 100 μg/ml of iohexol in blood, respectively.

Keywords: Hydrophilic drugs; Aqueous two-phase systems; Partitioning

1. Introduction

In liquid–liquid extraction one aqueous phase is usually combined with one water-immiscible organic phase. It is also possible to prepare systems with two immiscible phases, which are entirely aqueous. When a polymer such as polyethylene glycol (PEG) is mixed with either a second polymer (*e.g.* dextran) or a salt (*e.g.* potassium phosphate) in certain concentrations in water, the solution separates into two phases. In the case of PEG and potassium phosphate, the system consists of one PEG-rich and one salt-rich phase. Such systems are used for partitioning/separation/fractionation of biological materials such as cells, viruses, nucleic acids and proteins, without any loss of biological activity [1–5]. The technique has also been applied to inorganic ions [1].

The partitioning behaviour of a solute between the two aqueous phases is governed by solute properties and by the type(s), molecular weight(s) and concentration(s) of polymer(s), as well as type(s) and concentration(s) of salt(s) present, pH, and temperature. The range of polymer ratios or polymer/salt ratios that is required to obtain phase separation can be seen from the phase diagram for the relevant system [2]. The closer the composition of the system is to the critical point, the lower is the interfacial tension. There is a tendency for adsorption of particles at the interface, which increases the larger the particles and the larger the interfacial tension [2]. Compared to a conventional aqueous–organic two-phase system, differences in properties between two aqueous phases are small. This corresponds to a

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smaller range of partitioning coefficients; *K* usually varies over a range of 0.1 to 10 [1].

Prior to liquid chromatographic determination of drugs in blood, cells and proteins have to be removed. Sample preparation techniques that minimize consumption of organic solvents harmful to the environment as compared to conventional liquid–liquid extraction are highly desirable.

The author wished to evaluate the technique of aqueous two-phase partitioning as a sample preparation technique, and in this paper this principle is used prior to liquid chromatography of the drugs paracetamol and theophylline and the X-ray contrast medium iohexol (Omnipaque) in blood. These compounds were chosen because of their hydrophilicity and high blood concentration levels. The extraction is carried out in phosphate buffer pH=7.4/PEG 300 by magnetic stirring using some recently innovated [6] and simple glass equipment that will be described.

2. Experimental

2.1. Chemicals and materials

Omnipaque 180 mg I/ml (Nycomed Amersham), theophylline ethylenediamine and polyethylene glycol 300 (macrogolum 300) were obtained from Norsk Medisinaldepot (Oslo, Norway), whereas paracetamol was obtained from Sigma (St. Louis, MO, USA). Potassium dihydrogen phosphate, dipotassium phosphate (trihydrate), sodium chloride (all of analytical grade) and Coomassie Brilliant Blue G250 (for electrophoresis) were obtained from Merck (Darmstadt, Germany), orthophosphoric acid (85%, analytical reagent grade) from May and Baker (Dagenham, UK), sodium hydroxide (analytical grade) from Eka Nobel (Bohus, Sweden), acetonitrile (HPLC grade) from Rathburn (Walkerburn, UK), ethanol from Arcus (Oslo, Norway) and bovine albumine (crystallized, min. 98% purity, for electrophoresis) from ICN (Costa Mesa, CA, USA). Water was obtained from a Milli-RO PLUS system (Millipore, Bedford, MA, USA). Citrated blood from cattle was obtained frozen from Nord–Norges salgslag (Tromsø, Norway). Human blood from a healthy donor was collected in citrate-containing tubes.

2.2. HPLC

The HPLC system was composed of a Model 616 pump/Model 600 S controller unit (Waters, Milford, MA, USA), a Model 996 photodiode array detector (Waters) and a Model 717plus autosampler (Waters). Data were acquired and processed by Millennium software (Waters).

The chromatographic separation was carried out at 25°C on an ABZ+Plus analytical column (5 μm particle size, 150×4.6 mm I.D.) protected by a 20× 4.6 mm I.D. ABZ+Plus Supelguard column (Supelco, Bellefonte, PA, USA). The mobile phase flow-rate was 0.7 ml/min.

2.3. Sample extraction

To 18.0 g of extraction buffer and 2.00 g of PEG 300 in a 50 ml Erlenmeyer flask (Quickfit 19/26 joint) 500 μl of blood and a 3 cm teflon-coated magnetic bar were added. The mixture was stirred on a Variomag Model HP 6 six-point magnetic stirrer (H+P Labortechnik GmbH, München, Germany) at 400 rpm for 3 min. Using a specially designed glass adapter (Fig. 1) that fits into the opening of the flask and features a stopcock and a tube for equalizing flask air pressure with atmospheric pressure, the flask was turned upside-down after phase separation (*ca*. 5 min). After 30–60 s an aliquot of the lower salt phase was drained into a vessel, and 0.6–0.7 ml was filtered through a 0.2 μm Puradisc 25AS syringe filter (Whatman, Clifton, NJ, USA) into a 1 ml glass autosampler vial from which 20 μl was injected into the HPLC system. Autosampler temperature was kept at 8°C.

2.4. Estimation of protein content

Protein content in the extract was estimated using the Bradford method [7], which is a spectrophotometric quantification of protein utilizing the principle of protein-dye binding. The «standard» method was used. Standards containing 250, 500, 750 and 1000 μg/ml of albumine in 0.15 *M* NaCl were made from a stock solution made by diluting 100.0 mg of albumine to 5.00 ml with 0.15 *M* NaCl. To 100 μl of protein standard or extract 5.00 ml of protein reagent was added. The reagent blank for the standards was

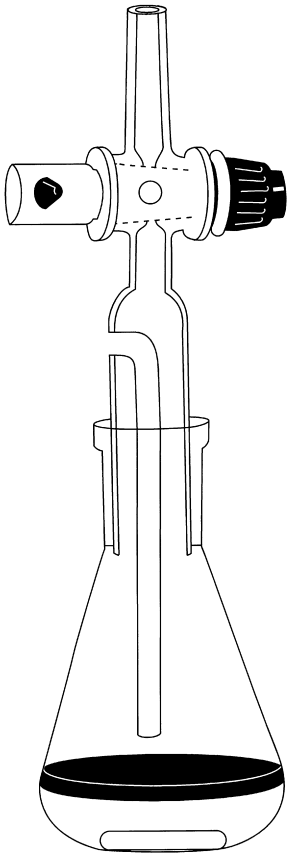

Fig. 1. Erlenmeyer flask containing sample solution after phase separation with the glass adapter inserted.

prepared from 0.15 *M* NaCl and protein reagent. For the sample extract the reagent blank was prepared from blank salt-rich phase and protein reagent. Absorbance at 595 nm was measured in a Hitachi (Hitachi Instruments, San Jose, CA, USA) Model U-2001 spectrophotometer in 1 cm plastic cells (Ratiolab, Dreieich, Germany).

2.5. Preparation of solutions

Working standards of iohexol, paracetamol and theophylline ethylenediamine as well as stock solutions of the latter two were prepared in water. Extraction buffer was prepared by mixing 253.7 g of dipotassium phosphate trihydrate, 106.4 g of potassium dihydrogen phosphate and 719.9 g of water. Mobile phase components were degassed with helium and mixed in the HPLC system.

3. Results and discussion

3.1. Extraction

Using this phosphate/PEG system for extraction of hemolysed blood, after stirring and phase separation the red colour was in the upper PEG-rich phase, while the lower salt-rich phase was essentially clear and colourless. The red colour comes from hemoglobin, which constitutes *ca.* 10% of whole blood and is the most abundant protein in blood. It was then desirable to have the drugs in sufficient amounts in the lower clear phase. This phase is also the more polar one [2]. In this paper hemolysed blood from cattle was always used unless otherwise stated. When using fresh human blood the red colour also distributed into the upper phase and the lower phase was clear. Sufficient analyte for detection at relevant concentrations was extracted by using a system composition with a relatively large salt-rich phase, and near the lower concentration limit for phase separation. Recovery under conditions optimised for iohexol is shown in Table 1. For theophylline, which is approximately 60% bound to plasma proteins [8], extraction from a solution of

Table 1
Extraction recovery [d] ($n=2$)

Compound	Concentration added	From hemolysed blood	From water
Iohexol	100 μg/ml	35% [a]	
Paracetamol	10 μg/ml	25% [b]	
Theophylline ethylenediamine	10 μg/ml	47% [c]	50%

[a] Mobile phase: 4% acetonitrile in water. Detection: 244 nm.
[b] Mobile phase: 7% acetonitrile in water. Detection: 245 nm.
[c] Mobile phase: 8% acetonitrile in 0.05M phosphate buffer pH=3.7. Detection: 270 nm.
[d] Percentage of what would have been an even distribution in the system.

theophylline in water was also carried out, to see how much the blood constituents lowered extraction recovery. As can be seen from Table 1 the extraction recoveries from hemolysed blood were in the range of 25–47%, and the recovery for theophylline in water somewhat higher than for the same drug in hemolysed blood. In recent papers on method validation the extent of recovery is not considered to be important provided sufficient accuracy, precision and detection limit is obtained [9,10].

The protein content of the extract was estimated to be 35 μg/ml after extraction of 500 μl of blood with 16.2 ml of extrant (sum of buffer and PEG). The blood contained 17.4 g of protein per 100 g. If the protein had been evenly distributed in the system, the protein concentration would have been *ca*. 5.2 mg/ml. Thus the extract contained less than one per cent of what would have been an even distribution of protein in the system.

After stirring and phase separation an aliquot of the lower phase is drained out using a glass adapter. The adapter is described above and shown when mounted in an Erlenmeyer flask in Fig. 1 and can easily be made by a glassblower. Extraction using this equipment has been previously described [6] as an alternative to conventional separatory funnels prior to titrimetric determination of a drug substance (amphetamine sulphate).

In the present extraction procedure 500 μl of blood is extracted with 18.0 g of buffer and 2.00 g of PEG 300. This involves quite a large dilution of the sample. An experiment was carried out to see how the amount of iohexol extracted varied at different concentrations of phosphate buffer and different volumes of sample added (50 μg/ml of iohexol in hemolysed blood). The amount of PEG was kept constant at 2.00 g, whereas buffer composition was varied within 18.0 g keeping the ratio of the phosphate salts constant. The system composition described under «experimental» contains 25% w/w of salt, and system salt content was varied from 24 to 42% in the experiment. The higher the salt content, the larger the volume of sample that could be added without red colour occurring in the salt phase. The largest amount of iohexol in a clear lower phase was obtained when adding 2.5 ml of sample to a system containing 30% of salt. Under these conditions the area of the iohexol main peak was 2.3 times larger than the area obtained for the system containing 25% of salt (that was added 500 μl of sample). The recovery is, however, larger for the latter system. Peak shape distortions were not observed. In routine application specifying volumes of added solutions would be preferable, but in the present case weight of solutions was considered to be convenient, since system compositions in the literature are given by weight.

When protein precipitation is used for sample preparation the most efficient reagents are acids [11], and their use is dependent of analyte stability in acid. Otherwise organic solvents are used. When comparing the deproteinizing efficiencies of various precipitating agents (for plasma samples) [11] with the present deproteinizing of blood samples, these efficiencies are in the same range. Another alternative technique for clean-up of blood samples is dialysis, where proteins and particles are removed by a semipermeable membrane. Dialysis can be carried out either in an off-line mode or on-line [12–14] with liquid chromatography. Used alone, dialysis only gives removal of particles and large molecular-weight compounds.

3.2. Liquid chromatography

Chromatograms are shown in Fig. 2. When comparing the blanks of hemolysed blood and fresh human blood with the blank salt phase (prepared by «extraction» of 500 μl of water) it can be seen that the biological matrix does not contribute much to the front/background in the chromatogram. Iohexol gives rise to two peaks in the chromatogram (because of isomerism [14,15]), at *ca.* 6.1 min and at *ca*. 7.3 min, respectively. The largest of these, the peak at 7.3 min, was used for quantification. The blanks did not contain substances that would interfere with the detection of this iohexol peak. Co-extraction of hydrophobic substances does not seem to be a problem in this sample preparation technique.

3.3. Validation

The standard curve prepared from biological matrix spiked at three different concentrations of iohexol in the range 5–500 μg/ml was linear ($r=0.9999$). Accuracy and precision were determined by spiking

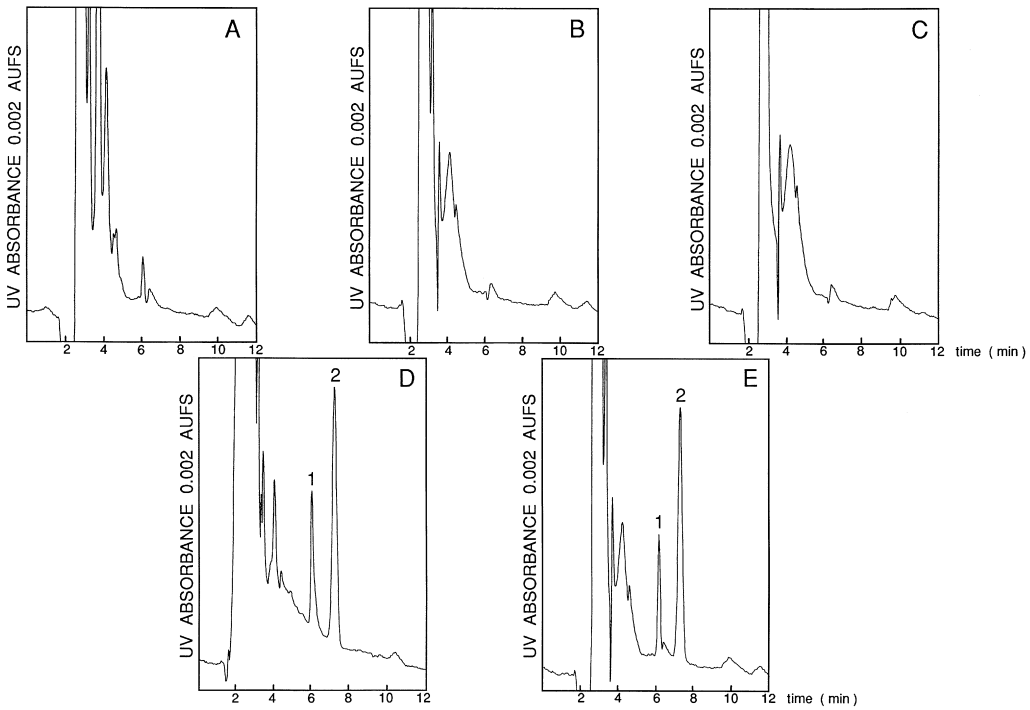

Fig. 2. Chromatograms of (A) blank of hemolysed blood, (B) blank of fresh human blood, (C) blank salt phase, (D) sample of hemolysed blood spiked with 50 μg/ml of iohexol and (E) sample of fresh human blood spiked with 50 μg/ml of iohexol. The iohexol peaks are numbered 1 and 2, respectively. For more information about conditions see Table 1.

hemolysed blood at 10 and 100 μg/ml and analysing the respective extracts. Each extract was injected once, and peak area was used for quantisation. The results are shown in Table 2. The results show good accuracy and precision for the extraction and analysis of iohexol.

4. Conclusion

The technique of aqueous two-phase partitioning has been shown for some hydrophilic drugs in blood to be suitable for sample preparation prior to liquid chromatography. No organic solvent is consumed, and the procedure is gentle to the drugs. However, the analyte should be present at a relatively large concentration, or sensitive detection would be necessary.

Table 2
Accuracy and precision for the extraction of iohexol from hemolysed blood

Concentration added	Concentration found (mean)	R.S.D.
10 μg/ml (n=5)	10.5 μg/ml	2.0%
100 μg/ml (n=6)	96.4 μg/ml	1.2%

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