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Novel solid-phase extraction strategy for the isolation of basic drugs from whole blood
Preliminary study using commercially available extraction cartridges

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Abstract

A novel strategy for the solid-phase extraction of basic drugs has been developed using commercial extraction cartridges. The procedure involves the sequential application of very different isolation mechanisms, viz. hydrophobic extraction on non-porous carbon followed by ionic extraction on a strong cation exchanger. This approach to extraction achieves both high recoveries and clean extracts when analysed by GC–MS. The potential for automation has been demonstrated using commercial sample preparation equipment.

1. Introduction

Over the last decade many new techniques have been introduced which have found wide application for the analysis of drugs and metabolites in biological materials for forensic purposes. In particular the coupling of highly effective separation methods (GC, HPLC) with sophisticated detection methods (e.g. mass spectrometry, diode-array detection) have greatly extended the limits of selectivity and sensitivity. Furthermore, with the advent of microcomputer technology, the automation of such instrumentation, to include sample introduction and report generation, has become practical and many applications have been reported [1,2].

Over a comparable time period the methods used for the preparation of samples for toxicological analysis have changed very little. Liquid–liquid extraction continues to be the method of choice for drug isolation prior to chromatographic analysis in most forensic toxicology laboratories. Such methods, though reduced in scale, would be easily recognised by a former generation of toxicologists preparing extracts for less discriminating tests. The fact that such extraction methods have remained in use for such a long time is a reflection of their practical simplicity and the flexibility of approach which can be achieved by the appropriate selection of solvent polarity, volume ratio, buffer pH, back extraction, etc. However, this flexibility has led to a multiplicity of methods in routine use throughout the world. In many cases methods

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have been optimised for the recovery of specific drugs or drug groups. The automation of liquid–liquid extraction methods has been demonstrated [3] but to date has not been widely applied in toxicological analysis.

The potential of solid-phase extraction (SPE) methods for the extraction of drugs from biological fluids has been recognised for many years [4,5]. The automation of SPE methods appears to have practical advantages over the automation of liquid–liquid methods. Extensive bibliographies of SPE methods have been published [6] and a survey of this literature reveals that the vast majority of methods use a single hydrophobic extraction column (e.g. C_{18} silica). It is also clear that most methods apply the same extraction strategy. In a typical method, the aqueous biological fluid is adjusted to an appropriate pH, where the drug has a neutral form, before being loaded onto the extraction column. Water-soluble material is then removed from the extraction column by extensive washing with water or buffer. A fractionation of the material adsorbed on the column is then attempted by subsequent washings with a specific volume of a specified aqueous organic mixture. This first fraction is discarded and then the column is washed with a further specified volume of a mixture richer in organic solvent which is collected for analysis. Thus, in this mode of operation, the extraction column is being used as an inefficient preparative reversed-phase HPLC column with the collection of the analyte along with all endogenous compounds of similar hydrophobicity. Experience has shown that such methods can give much co-extracted material with forensic samples [7]. It is our view that simple one-step hydrophobic methods do not utilise the full potential of SPE.

In the present study the authors consider the problem of a general scheme suitable for the extraction of a wide range of basic drugs from biological fluids. The method developed involves a 2-stage process using commercial SPE cartridge columns and demonstrates the general principle by which a higher degree of extraction selectivity can be achieved by the appropriate application of various extraction principles in sequence. The authors present preliminary work with the SPE method demonstrating high drug recoveries and clean extracts from whole blood, the body fluid usually submitted to forensic science laboratories. This preliminary work concentrates on four probe drugs (propranolol, nortriptyline, quinine, imipramine) and the capability of the method to be automated using the Waters Millilab system. This instrument has been discontinued but it is likely that the protocol could be used with most automated SPE systems.

2. Experimental

2.1. Materials

Propranolol hydrochloride, nortriptyline, quinine sulphate and imipramine hydrochloride were obtained from commercial suppliers and were of pharmaceutical grade. A stock solution was prepared by dissolving 10 mg of each of these drugs in 10 ml distilled ethanol. This was stored at 4°C. All solvents and reagents were of the highest quality. Methanol (MeOH) and ethyl acetate (EtOAc) were "Distol" grade (Fisons Chemicals, Loughborough, UK). The xylene was SpS grade (Romil Chemicals, Loughborough, UK). Hydrochloric acid and ammonia were both "Aristar" grade (BDH, Poole, UK) and the water used was purified in an Elgastat UHQ11 (Elga, Wycombe, UK). The SPE cartridges used were: (a) Supelclean-ENVIcarb (Supelco, Bellefonte, PA, USA, Cat. no. 57088) with a 3-ml capacity and containing 250 mg sorbent; (b) Bondelut PRS cartridges (Varian, Walton-on-Thames, UK, Cat. no. 1210–2012) with a 1-ml capacity and containing 100 mg sorbent.

2.2. Reagents

A 2% ammonia–methanol solution in ethyl acetate was prepared by adding 2.0 g ammonia and 2.0 ml methanol to a volumetric flask and making up to 100 ml with ethyl acetate. The reagent was capped and made up fresh every day.

A 1% ascorbic acid solution was prepared by

dissolving 1 g (l)-ascorbic acid (Analar grade, Fisons, Loughborough, UK) in 100 ml water and acidifying with conc. HCl to pH 2.0.

A 1% HCl–methanol solution was prepared by adding 1 ml conc. HCl to 100 ml methanol.

Xylene–methanol (8:2) was prepared by measuring 80 ml xylene and 20 ml methanol in a graduated cylinder.

A 0.15% TFA–methanol solution was prepared by adding 150 μl trifluoroacetic acid (Sigma Chemicals, Poole, UK) to 100 ml methanol. The reagent was capped and made up fresh every day.

2.3. Extraction

Blood (1 ml) was diluted 1 in 10 with water, by vortex-mixing for 5–10 s in a screw-capped culture tube, centrifuged for 10 min at 3500 *g* and the supernatant collected. This was then subjected to SPE using the Waters Millilab Sample Preparation Workstation linked to an NEC Powermate computer with Waters Millilab software.

The SPE procedure involved the sequential use of 2 extraction cartridges (carbon followed by cation exchange). A schematic representation of the procedure is outlined in Table 1. "Condition" describes the procedure by which the cartridge was prepared, prior to the sample being "loaded". A "wash" step to remove extraneous matter was followed by "elution" where the solutes of interest were removed from the cartridge.

2.4. Determination of recoveries

Whole sheep blood was spiked with the probe drugs to the required concentration using the stock solution. HPLC of the extracts was carried out using a Spectra-Physics Isochrom pump, a Philips LCUV detector set at 240 nm and a Philips chart recorder. The HPLC system was similar to Clarkes system "HA", p. 214 [8]. Extraction recoveries were measured by adding an internal standard to the final extract, computing peak-height ratios for each of the drugs under investigation and comparing these with a non-extracted mixture.

Table 1
Outline of the SPE extraction scheme described in the text

Carbon	
Condition	5 ml xylene–methanol
	5 ml methanol
	10 ml 1% ascorbic acid
	5 ml water
Load	9 ml aqueous blood supernatant
Wash	5 ml water
	5 ml 20% methanol–water
Dry	purge with dry nitrogen gas for 2 min
Elute	2 × 3 ml 0.15% TFA–methanol
	2 × 1 ml xylene–methanol
This solution is loaded onto a conditioned PRS cartridge	
PRS	
Condition	3 ml methanol
	5 ml HCl–methanol
	3 ml methanol
Load	*eluate from carbon cartridge*
Wash	2 ml methanol
	2 ml ethyl acetate
Elute	2 ml 2% ammonia–methanol–ethyl acetate

GC–MS analysis was performed using a Finnigan-MAT ITS-40 instrument (San Jose, CA, USA) equipped with a fused-silica capillary column Rtx-1, 25 m, 0.25 mm, film thickness 1 μm (Restek, from Thames Chromatography, Maidenhead, UK) in the electron ionisation mode (70 eV) scanning from 40 to 440 amu. The GC oven temp was 115°C, programmed at 25°C/min to 280°C, and held for 10 min. The injector and transfer line temperatures were 270°C. The helium carrier gas head pressure was held at 83 kPa and injection was in the splitless mode.

3. Results and discussion

The SPE procedure for basic drugs described in this paper involves the sequential use of 2 cartridges. The first step is a hydrophobic isolation on a carbon cartridge. The extract from this step is then passed through a propyl sulphonic acid cation exchanger. The entire procedure has been designed such that 3 isolation principles are applied in sequence, achieving a greater level of selectivity than would normally be achieved

using conventional approaches to SPE, while still achieving high recoveries. The first stage is a non-specific hydrophobic isolation. The second stage is a much more specific ion-exchange isolation which is then followed by the application of a further selective step based on the solubility of the analytes in organic solvents.

3.1. Stage 1: hydrophobic isolation

The adsorbent

The isolation of non-polar solutes from biological matrices has often used cartridges packed with reversed-phase bonded silica. Secondary interactions however are known to occur with this type of material causing the inefficient extraction of some drugs. Basic drugs are particularly prone to these effects and techniques used to minimise them have included pre-washing with a strong base [9] and the addition of a competing base [10]. Early work in this laboratory has shown that silica-based hydrophobic materials were not ideal for this type of extraction and a substitute was sought. Porous carbon has been used as a hydrophobic column packing material in HPLC [11,12]. In SPE, carbon in the form of graphitised carbon black (GCB) has been used to extract a range of compounds from water such as triazine herbicides [13,14] pesticides [15], oestrogen conjugates in pregnancy fluid [16] and tricyclic antidepressants from serum [17]. These carbon-based extractions were all extremely efficient. However, the published reports indicate that the surface of carbon itself is quite complex and is often contaminated, leading to a number of different interactions. The commercial extraction cartridges used in the present study contained non-porous carbon. With an adequate total surface area of 100 m^2/g these cartridges should be free of the diffusion effects which require slow, controlled flow-rates with porous GCB.

Conditioning and loading

Acetic acid conditioning has been successfully used for the isolation of pesticides on carbon [19] but our initial experiments found that with the probe drugs the surface of the carbon was affected such that the analytes were too loosely bound. Breakthrough occurred even from aqueous loading solutions if the volume applied was high enough. Quinine was particularly susceptible. Conditioning was carried out using xylene–methanol followed by methanol.

It was decided to incorporate the ascorbic acid conditioning step recommended in the pesticide extraction application [21] to prevent any possible irreversible adsorption problems with easily oxidised drugs. The loading protocol used was found to give complete isolation of the drugs.

Dilution of the blood with water and subsequent centrifugation was found to be both an efficient way of eliminating particulate matter (the cause of frit blockage in SPE cartridges) and allowed complete drug isolation onto preconditioned carbon cartridges.

Elution

Initial experiments showed that elution from carbon was difficult. Xylene–methanol (8:2) was found to be the best simple organic mixture for the drugs although methylene chloride–methanol has been used by several workers to elute drugs from carbon.

Eluotropic data for porous carbon HPLC packing material [22] supports this finding. Methanol (the eluting solvent normally associated with bonded silica) was found to be a very poor elution solvent for carbon; this again agreed with the same eluotropic data. In fact, as much as 50% methanol in water could be used as a wash solution with confidence that no breakthrough would occur. However, washing with 20% methanol was found to be more than adequate to elute coloured material originating from the blood, so cleaning the cartridge.

It has been shown [20] that the presence of TFA in methanolic mobile phases dramatically improves peak shape in carbon-based HPLC. Adding TFA to the methanol used to elute the drugs from carbon SPE was found to produce consistently high recoveries.

Total elution for all drugs was only complete whenever the methanolic eluent was followed by the xylene-based eluent. While TFA–methanol was successful in eluting almost all of the drugs,

the xylene–methanol aliquot was necessary to ensure complete elution of some drugs.

The presence of TFA in the carbon elution solvent at a concentration of 0.15% was chosen to be the optimum concentration, sufficient to be effective as an elution modifier for the carbon SPE cartridge and yet below that concentration found to cause some breakthrough when loading the cation-exchange cartridge.

The final carbon extraction protocol was tested for recovery efficiency with a number of drugs (including the probe drugs) at a concentration of 50 μg/ml in blood. The high drug levels allowed direct injection of the extract onto HPLC for quantification. These results (Table 2) showed that extraction and recovery were complete and reproducible with good within-run precision (n = 9). A number of other drugs were also quantified along with the 4 probe drugs. Data for these drugs demonstrated similar high recovery efficiencies.

3.2. Stage 2: cation-exchange isolation

Cation exchange has been used to isolate basic drugs and other compounds from a variety of matrices. Basic drugs were extracted from the urine of racing greyhounds [23] using a strong cation exchanger. Initial experiments in this laboratory with forensic blood samples however showed that ion exchange as an initial isolation mechanism was unreliable, probably due to the presence of high salt concentrations in these samples—sodium fluoride and potassium oxalate (preservative and anticoagulant) are often added to the sample vials. However, ion exchange as a second-stage isolation technique is very useful. It is highly selective and can be successfully used when the ionic strength of the solutions involved can be controlled.

Table 2
Mean total absolute recoveries (±S.D.) of drugs from carbon (n = 9)

Drug	Total recovery (%)	C.V. (%)
Propranalol	101.0 ± 4.8	4.7
Nortriptyline	96.7 ± 4.4	4.5
Quinine	92.6 ± 3.0	3.2
Imipramine	100.0 ± 4.0	4.0
Haloperidol	108.9 ± 4.1	3.7
Dothiepin	102.6 ± 2.9	2.8
Nitrazepam	99.8 ± 1.7	1.7
Chlorpromazine	88.1 ± 4.7	5.3

Blood (1 ml) extracted using procedure; blood drug concentration 50 μg/ml; recovery determination was made by adding the internal standard after extraction.

A propyl sulphonic acid cation exchanger was chosen for this work. As a strong acid it will remain charged under a wide range of pH conditions. Furthermore it is less hydrophobic than the other strong cation-exchange SPE cartridges (SCX) which are based on bonded benzene sulphonate chemistry. It was considered desirable that the second extraction phase should involve ionic rather than hydrophobic interaction.

Conditioning of the PRS cartridge involved washing with methanolic HCl to convert the exchanger into its protonated form (it is supplied from the manufacturer as a sodium salt). Our experience has shown that basic drugs in non-aqueous solution extract on the conditioned cation exchanger easily and reliably.

Elution from the cation exchanger was more complex. Initially we used ammoniacal methanol to elute the basic drugs, the ammonia acting to both displace the drugs from the anionic sites on the cartridge and to raise the pH so that the basic drugs were in their neutral forms. Although this approach was successful it was found that even cleaner extracts could be achieved by the application of a more selective elution solvent (see stage 3 below).

3.3. Stage 3: organic solubility

It was found that ammoniacal ethyl acetate provided the required extra selectivity. The distinct narrow, brown-coloured band visible at the top of the PRS cartridge (after loading the first stage blood–carbon extract) remained in place after elution. This coloured band was washed off with the drugs when using ammoniac-

al methanol. Initial problems with the reproducibility of this elution step were solved by adding a small quantity of methanol to the ammoniacal ethyl acetate. Another advantage in using ammonia-based eluents is that evaporation of the final extract prior to chromatographic analysis did not cause problems due to the presence of non-volatile salts.

The final dual-stage procedure was tested with the probe drugs, and the results are presented in Table 3. It can be seen that the high recoveries associated with the first stage have been maintained throughout the whole procedure.

Results from the previously published work using porous carbon strongly indicate that extraction efficiencies at very low levels should not deteriorate. Preliminary work shows that recovery does not deteriorate at low levels. Imipramine recovery was found to be 105.8% (±0.6) at 1 μg/ml, and 107.9% (±2.7) at 0.1 μg/ml ($n = 3$). The procedure has been used successfully to process forensic casework blood samples and further work on the validation of the technique for low levels with detection by GC–MS is underway at present.

Fig. 1 shows a GC–MS chromatogram of an extract prepared using the described method from a whole blood sample spiked with a mixture of drugs at a concentration of approximately 0.2 μg/ml.

The extraction of drugs from plasma and urine using a mixed-mode sorbent (Bondelut Certify)

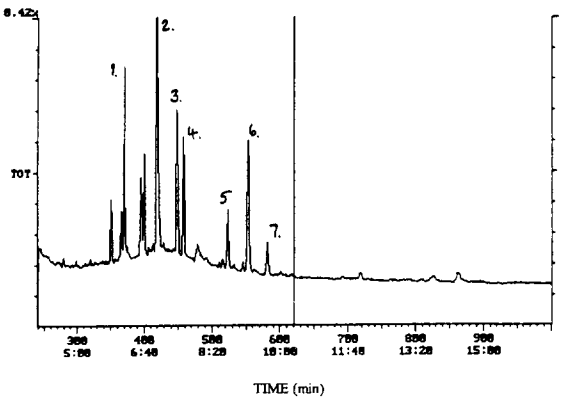

Fig. 1. Chromatogram of an extract of whole blood spiked with a mixture of drugs. Extraction method as discussed in text. Peaks: 1 = orphenadrine; 2 = diphenylpyraline (internal standard); 3 = amitriptyline; 4 = imipramine; 5 = dothiepin; 6 = diazepam; 7 = chlorpromazine. Blood concentrations 0.2 μg/ml.

has been described [24,25]. The mechanism of action seems to be mixed hydrophobic–ion exchange, similar in concept to the present study but involving only one extraction cartridge. These cartridges have been used mainly for urine samples but recovery data from whole blood down to 5 μg/ml have been reported [26]. Because the ion-exchange process is involved from the beginning of the procedure it may not be suitable for forensic samples where the salt concentrations may well be as high as 20% in those cases where only a small sample of blood or urine has been added to the prepared sample container.

Extraction using mixed-mode sorbents, such as Certify, has the advantage of simplicity (single cartridge) but the combination of hydrophobic and ion-exchange principles in a single step does not allow the separate stages to be optimised individually. Our procedure, therefore, provides a better hope of higher recoveries and cleaner extracts. Furthermore the advent of automated extraction equipment means that procedures with fewer steps are no longer as advantageous as they may have been if manual SPE were the only option.

Table 3
Mean total absolute recoveries (±S.D.) of drugs through entire SPE procedure ($n = 5$)

Drug	Total recovery (%)	C.V. (%)
Propranolol	92.9 ± 3.2	3.4
Nortriptyline	90.8 ± 3.0	3.3
Quinine	89.9 ± 2.2	2.4
Imipramine	93.7 ± 1.7	1.8

Blood (1 ml) extracted using procedure; blood drug concentration 50 μg/ml; recovery determination was made by adding the internal standard after extraction.

4. Conclusions

The method described in this paper introduces a highly selective means of solid-phase extraction using multiple-isolation mechanisms achieving high recoveries. Each extraction step has been designed to isolate the drugs completely, such that increased selectivity is not compromised by poor recovery. Clean extracts have been achieved by the sequential application of these extraction steps employing very different isolation mechanisms. The use of non-porous carbon as a hydrophobic sorbent with subsequent isolation and elution from a cation exchanger has been shown to be successful for the range of drugs studied. Furthermore the automated extraction of these drugs from whole blood using this protocol has been demonstrated. Thus the SPE strategy outlined in this paper provides a prospect of automated sample preparation for blood samples with high recoveries and clean extracts.

References

[1] B. Hardinger, LC–GC Int., 3 (1990) 32.
[2] J.C. Pearce, R.D. McDowell, A. El Sayed and B. Pichon, Int. Lab., 20 (1990) 34.
[3] A.S. Curry, Advances in Forensic and Clinical Toxicology, CRC Press, Cleveland, OH, 1972, p. 83.
[4] E. Doyle, J.C. Pearce, U.S. Picot and R.M. Lee, J. Chromatogr., 411 (1987) 325.
[5] K.C. Van Horne (Editor), Sorbent Extraction Technology, Analytichem International, Harbour City, CA, 1985.
[6] Waters Applications Bibliography, Waters Chromatography Division, Harrow, 1991.
[7] R.J. Hughes and M.D. Osselton, J. Anal. Toxicol., 13 (1989) 77.
[8] A.C. Moffat (Editor), Clarkes Isolation and Identification of Drugs, The Pharmaceutical Press, London, 1986.
[9] G.M. Roberts and C.S. Hann, Biomed. Chromatogr., 1 (1986) 49.
[10] V. Marko, L. Soltes and I. Novak, J. Pharm. Biomed. Anal., 8 (1990) 297.
[11] J.H. Knox, K.K. Unger and H. Mueller, J. Liq. Chromatogr., 6 (1983) 1.
[12] K.K. Unger, Anal. Chem., 55 (1983) 361.
[13] M. Battista, A. Di Corcia and M. Marchetti, Anal. Chem., 61 (1989) 935.
[14] A. Di Corcia, M. Marchetti and R. Samperi, J. Chromatogr., 405 (1987) 357.
[15] F. Mangani, G. Crescentini and F. Bruner, Anal. Chem., 53 (1981) 1627.
[16] F. Andreolini, C. Borra, F. Caccamo, A. Di Corcia and R. Semperini, Anal. Chem., 59 (1987) 1720.
[17] G. Carfagnini, A. Di Corcia, M. Marchetti and R. Samperi, J. Chromatogr., 530 (1990) 359.
[18] A. Di Corcia, R. Samperi, E. Sebastiani and C. Severini, Anal. Chem., 52 (1980) 1345.
[19] The extraction of non-volatile pesticides using graphitised carbon, The Supelco Reporter, 1992.
[20] C.K. Lim and G. Guanghua, Separation of anionic and cationic compounds of biomedical interest on porous graphitic carbon, Proc. CIS'89, Tokyo, 1989.
[21] A. Di Corcia and M. Marchetti, Anal. Chem., 63 (1991) 580.
[22] J.H. Knox, K.K. Unger and H. Mueller, J. Liq. Chromatogr., 6 (1983) 1.
[23] C.M. Moore, J. Forensic Sci. Soc., 30 (1990) 121.
[24] X.H. Chen, J. Wijsbeek, J.V. Veen, J.P. Franke and R.A. de Zeeuw, J. Chromatogr., 529 (1990) 161.
[25] X.H. Chen, J. Wijsbeek, J.P. Franke and R.A. de Zeeuw, J. Forensic Sci., 37 (1992) 61.
[26] X.H. Chen, J. Franke, K. Ensing, J. Wijsbeek and R.A. de Zeeuw, J. Anal. Toxicol., 17 (1993) 421.