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Fundamental studies in reversed-phase liquid–solid extraction of basic drugs; III: sample matrix effects

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Abstract: Using a set of 13 basic solutes (including a quarternary ammonium compound) with controlled pK_a, log P and plasma protein binding, the factors which influence the extraction of these compounds from plasma by reversed-phase liquid–solid extraction have been studied. Direct analysis of plasma, avoiding deproteination, etc., is possible providing the sample is applied to the cartridge under the appropriate conditions. These conditions are dictated by the degree of plasma protein binding and are controlled and predicted from the lipophilicity of the compound. Plasma samples containing lipophilic compounds (log D(pH 7.4)>2.03) require acidification and samples containing more polar compounds (log D(pH 7.4)<0.9) should be applied untreated. Failure to use the appropriate conditions can result in significant losses from the cartridge on application or washing. In the intermediate lipophilicity range the optimal application conditions cannot be predicted. The effect of various aqueous–organic wash solvents have been investigated, and it was found that the cationic but not the proteinaceous components of plasma also affect the extraction process. This phenomenon which is probably due to an attenuation of the secondary cation exchange mechanism, results in more facile elution than when compounds are applied in water.

Keywords: *Solid-phase extraction; liquid–solid extraction; basic drugs; plasma; protein binding; physico-chemical parameters; wash solvents.*

Introduction

Previous work in this series [1, 2] has shown how ionic and hydrogen bonding interactions play an important role in the extraction of basic compounds using nominally reversed-phase (C2 and C18) extraction cartridges. For the sake of simplicity and to avoid confounding effects of the matrix, etc. this work was carried out with the test solute being applied in water or weak methanol solution. To further extend the relevance of our work, the present report examines the extraction of plasma, which is commonplace in pharmaceutical and other areas involving drug testing such as forensic analysis.

In carrying out this work we have borne in mind problems experienced by other workers in the analysis of plasma [3, 4]. These problems which may be related to protein binding, can lead to the need for additional sample pretreatment stages. These have included deproteinization and dilution of the sample, as well as evaporation and redissolution. However these extra steps are in themselves problematical, occasionally leading to unexpected drug losses [4], and they are also frequently time-consuming. Consequently, they detract from the view that many workers possess of solid-phase extraction, that it is an efficient, fast and easily automated procedure.

The object of the present study is to extend our understanding of the use of reversed-phase cartridges to the extraction of basic drugs from plasma. In particular, attention has been given to the control and effective manipulation of the application and wash stages.

Experimental

Materials and equipment

With the exception of ^{14}C-labelled paraquat (1,1′-dimethyl-4,4′-bipyridinium, specific activity 362 μCi mg^{-1}, radio-chemical purity >98%) obtained from ICI Central Toxicology Laboratory (Macclesfield, UK) all the radiolabelled compounds were as previously described [1]. Reagent chemicals of the purest grades available were obtained from a variety of sources as were the unlabelled drug compounds. Bond Elut cartridges (C2 and C18, 100 mg size) and scintillation counting materials were as described previously [1].

High-performance liquid chromatography (HPLC) was carried out using a 100 × 4.6 mm i.d. column packed with Spherisorb S5W silica. The eluent was methanol–aqueous ammonium

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acetate buffer (9:1, v/v) (pH 9.1) as previously described [5]. Detection was by means of a Perkin–Elmer LC135 UV detector set at the appropriate wavelength (typically the absorption maximum) for the compound of interest.

Log *P* and pK_a data were either measured in-house using standard techniques or obtained from the literature. Protein binding data were obtained from the literature where possible, generated previously in-house using equilibrium dialysis, or were determined here using an ultra-centrifugation technique employing the Amicon Centrifree™ system. Pressure dialysis was carried out using an Amicon ultrafiltration stirred cell fitted with a YM5 membrane (molecular weight cut off 5000 Daltons).

Human plasma was obtained from normal volunteers. Blood was taken into sodium oxalate (1.5 mg ml^{-1}) centrifuged to give plasma which was stored at 4°C with sodium azide (1%).

Phosphate buffered saline (PBS) was prepared from KH_2PO_4 (0.268 g), Na_2HPO_4 (1.140 g) and NaCl (9.0 g) per litre of water.

Methods

Solid-phase extraction. A typical experiment involved conditioning of an extraction cartridge with methanol (1 ml), followed by de-ionized water (1 ml). The radiolabelled compound, in either plasma (with or without pretreatment), or water was then drawn through the cartridge, and the cartridge washed with water (2 × 1 ml), this was followed by successive washes of methanol–water. The application volume along with the various washes were collected for scintillation counting.

Protein binding. Plasma was spiked with the compound of interest at a concentration of approximately 1–2 μg ml^{-1}. Each determination was carried out in duplicate using the Centrifree™ system according to the manufacturer's instructions. Non-specific binding to the membrane was determined for each compound and shown to be negligible.

HPLC analysis. Plasma was spiked with each compound of interest at a concentration of approximately 1 μg ml^{-1}. An aliquot of each sample with or without the addition of an equal volume of acetic acid (1.0 M), was then applied to a C2 cartridge and the cartridge washed with water (2 × 1 ml). The compound was eluted with ammonium acetate (3.33×10^{-2} M) in 90% aqueous methanol (0.5 ml). The resultant extract was analysed directly by HPLC using the conditions described above.

Results and Discussion

Application stage

Atenolol and propranolol were applied to C2 and C18 cartridges in both water and plasma (1 ml of each). Following application, the cartridges were washed with water (2 × 1 ml). The results of this experiment (Table 1) showed a very significant loss of propranolol in the application and water wash volumes when the compound was applied in plasma. In contrast, the loss for the other combinations studied was minimal, typically <2.6%. Reducing the volume of plasma to 0.1 ml gave a small reduction in the amount of compound lost, as did dilution of the sample with water prior to application. However, neither of these procedures reduced the loss for propranolol to that seen with atenolol or when propranolol itself was applied in water.

The protein binding of propranolol is relatively high (90%), compared to atenolol where the protein binding is <5% [6]. It was postulated therefore, that the loss observed with propranolol was due to protein binding which prevented effective interaction of the compound with the stationary phase. Dilution of the sample, which was shown above to be partially successful in reducing the propranolol loss, is a common procedure for reducing protein binding.

Using propranolol as a test probe various sample pretreatment procedures were examined with a view to minimizing or eliminating the protein binding and hence improving the recovery of this and hopefully other highly protein bound compounds.

The pretreatment procedures and their resultant effects are shown in Table 2. These procedures were selected on the basis of their known effect on protein binding but also for their simplicity of operation. With one exception, all the procedures gave a small improvement in retention on the cartridges. The use of methanol or acetonitrile as employed by Massart and coworkers [3, 4], although effective, involved considerable pre-preparation prior to the solid-phase extraction

Table 1
Proportion of atenolol and propranolol lost on application and washing of C2 and C18 reversed-phase cartridges when applied in various media

	Percentage lost			
	Atenolol		Propranolol	
Sample and volume	C2	C18	C2	C18
Water (1 ml)	0.1	0.1	1.0	0.8
Plasma (1 ml)	1.6	2.6	9.3	14.3
Plasma (1 ml) + water (9 ml)	3.6	2.0	4.6	7.4
Plasma (0.1 ml)	0.4	1.2	4.0	5.2
Plasma (0.1 ml) + water (0.9 ml)	0.9	0.6	4.7	6.8

Table 2
The effect of various plasma pretreatment procedures on the loss of propranolol when applied to C2 and C18 cartridges

	Propranolol lost on application and 2 × 1 ml water washes (%)	
Conditions	C2	C18
0.1 ml plasma	4.0	5.2
0.1 ml plasma + 0.9 ml acetic acid*	2.3	3.6
0.1 ml plasma + 0.9 ml trifluoroacetic acid*	2.2	2.0
0.1 ml plasma + 0.9 ml HCl*	2.2	2.8
0.1 ml plasma + 0.1 ml urea (10 M)	3.1	3.1
0.1 ml plasma + 0.2 ml methanol	41.9	37.8
0.1 ml plasma + 0.1 ml methanol + 0.9 ml H_2O†	3.1	3.1
0.1 ml plasma + 0.1 ml acetonitrile + 0.9 ml H_2O†	2.7	2.2

* All acids 0.5 M.
† Sample mixed with organic solvent, filtered then diluted with water prior to application to the cartridge.

stage *per se* and was considered less favourable. There was some variability in the data for the acids investigated although there proved to be little significant difference between them. Acetic acid was chosen for further work on the basis that it involved the same anion as used for elution [1] and also in subsequent HPLC analysis [5].

Using the full range of radiolabelled compounds, which showed protein binding covering the maximal range (0–100%), recovery from plasma was investigated with and without acidification of the sample. This work was carried out using the C2 cartridge which has been shown previously to be optimal when dealing with a series of compounds having widely different lipophilicities [1]. Surprisingly, it was found that acidification could not be used as a general treatment for plasma, its use being dependent on the physico-chemical characteristics of the compound in question. The data in Table 3 show that compounds with high protein binding require acidification of the sample prior to application if losses are to be reduced to a minimum. Conversely, compounds with low protein binding require no pretreatment, and acidification of the sample actually causes a very significant loss.

The effect of acidification on the extraction of lipophilic compounds can be rationalized through a reduction in the plasma protein binding leading to a more favourable interaction between the stationary-phase and the drug molecule. The deleterious effect of acidification with weakly protein bound compounds however is somewhat inexplicable. It is possible that the hydrophobic effect is reduced due to increased ionization, although it is also expected that the electrostatic effect would be increased in opposition to this.

The above phenomenon, as well as being dependent on protein binding, also appears to be related to the solute distribution coefficient (log D, Table 3). The distribution coefficient is the octanol–water partition coefficient (log P) which has been corrected for the state of ionization. For a basic compound log P and log D are related in the following way:

$$\log D = \log P - \log\left[1 + \text{antilog}\,(\mathrm{p}K_a - \mathrm{pH})\right],$$

Table 3
The effect of pre-acidification of plasma prior to application to a C2 cartridge on the loss of various drugs

Compound	Drug lost on application and 2 × 1 ml water washes (%)		Protein binding* (%)	log D (7.4)
	Plasma	Acidified plasma		
Paraquat	1.1	86.4	0 (0)†	−3.06
Atenolol	0.4	68.1	4.5 (<5)‡	−1.87
ICI 138061	1.0	48.1	24.7	0.34
ICI 95527	11.4	9.2	42.9	0.43
Propranolol	10.3	2.3	89.7 (90)‡	1.53
ICI 42464	12.8	0.8	98.1	2.93
ICI 169369	25.4	0.8	99.9 (99.2%)†	3.61
Tamoxifen	83.5	7.9	100.0 (99%)†	5.43

* Figures in parenthesis were reported previously.
† ICI unpublished data.
‡ From ref. 6.

where pK_a is the dissociation constant of the base in question and pH refers to the medium. The lipophilicity of a compound as measured by its log P or log D is probably one of the dominant factors controlling drug–protein binding [7]. As log D is easily calculated, more clearly defined and less variable than protein binding, it was used in preference to protein binding in all subsequent work.

The data in Table 3 although showing the necessary conditions (i.e. acidified or un-acidified) to use at the extremes of lipophilicity, do not give a clear indication of the appropriate conditions for a compound of intermediate lipophilicity. A clear definition of the cross-over point from acidification to non-acidification necessitated additional data. This was generated by the analysis of several further compounds using, in this instance, HPLC analysis. The compounds which are listed in Table 4 had known protein binding and lipophilicity which was complementary to that of the compounds already studied. These samples were analysed with and without acidification, and the loss on application and following two water washes was determined by comparison with unextracted standards. Typical chromatograms for acebutolol are shown in Fig. 1. Although acidification of plasma gave more early eluting material, in practice there was little difference in chromatographic performance between either approach.

Using the data for all compounds, various methods of transformation were investigated to give a clearer indication of when acidification was required. The most useful involved a plot of [% drug lost on direct application −% drug lost on acidified application] against the solute log D calculated at pH 7.4. The experimental data, plotted in this form are shown in Fig. 2. The best fit straight line has a correlation coefficient of 0.9415, and the cross-

Table 4
Additional compounds studied using HPLC analysis, and their reported protein binding and physico-chemical data

Compound	Protein binding (%)	Log P	pK_a (25°C)
Nortriptyline	90–95%*	4.62‡	9.7*
Penbutolol	>95†	4.15†	9.5‡
Acebutolol	20*	1.87†	9.55‡
Amiloride	NS	−0.13‡	8.7*
Metoprolol	12*	2.15†	9.6‡

NS = not significant, taken to be zero.
* From ref. 6.
† From ref. 7.
‡ ICI unpublished data.

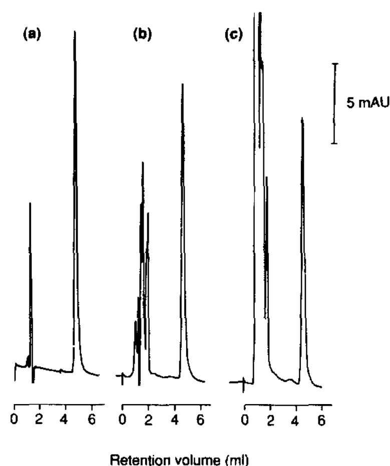

Figure 1
Typical chromatograms for acebutolol: (a) unextracted; (b) extracted from plasma; and (c) extracted from acidified plasma.

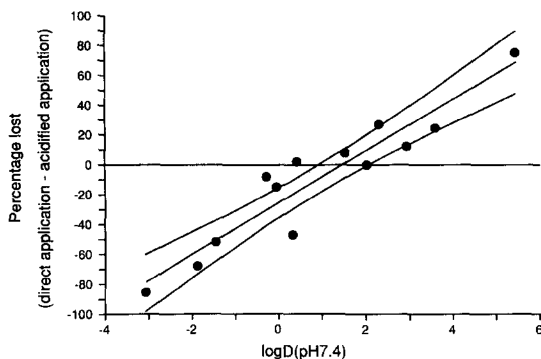

Figure 2
Correlation of [% lost on direct application − % lost on acidified application] against solute log D (7.4) for 13 basic solutes. The best fit straight line is shown along with the 95% confidence interval.

over point occurs at a solute log D (7.4) of 1.47. Although the correlation is significant, the scatter in the data suggests that this is less than ideal in terms of defining a decision point as to whether plasma should be acidified or not. Also shown in Fig. 2 are the 95% confidence intervals around the best fit line, which can probably be used more successfully to formulate general rules for extraction. Compounds with a log D (7.4) > 2.03 require acidification of the plasma whereas compounds with log D (7.4) < 0.95 do not require acidification. In the intermediate range $0.95 >$ log D (7.4) < 2.03 the optimal conditions cannot be predicted; these must be determined by experiment. However, even if the wrong conditions are employed, the extraction efficiency should still be greater than 80%.

Through use of the appropriate conditions excellent recoveries for all compounds studied, typically >85% could be achieved (Table 3 and Fig. 2). In combination with the use of previously defined elution procedures [1], a simple but highly selective and versatile extraction technique for basic drugs is obtained.

Wash stage

It is common practice in solid-phase extraction of plasma to wash the cartridge with water following application of the sample. This water wash serves to remove salts and proteins which may be loosely bound to, or trapped in the interstices of the packing material. The cartridge can then be further washed with either organic or hydro-organic solvents to remove potential interferents and improve the specificity of the procedure. We have shown previously that when the compound is applied in water, it is possible to wash a C2 or a C18 cartridge with relatively large volumes of methanol and not obtain elution of basic compounds. These types of compound are effectively held by secondary ionic interactions. In the case where the compound is applied in plasma however, a different situation exists, and a significant proportion of the drug is eluted when the cartridge is washed using a stepwise gradient of methanol–water (Fig. 3). These data suggest that some component in the plasma is adversely affecting the interactions on the column leading to more facile elution than when the compounds are applied in water.

Two possible factors could be responsible for this difference. Firstly the ionic components in the plasma could be interacting with the ionized silanols and hence reducing the secondary interaction. Secondly it could be possible that the proteins in the plasma are actually coating the surface of the stationary

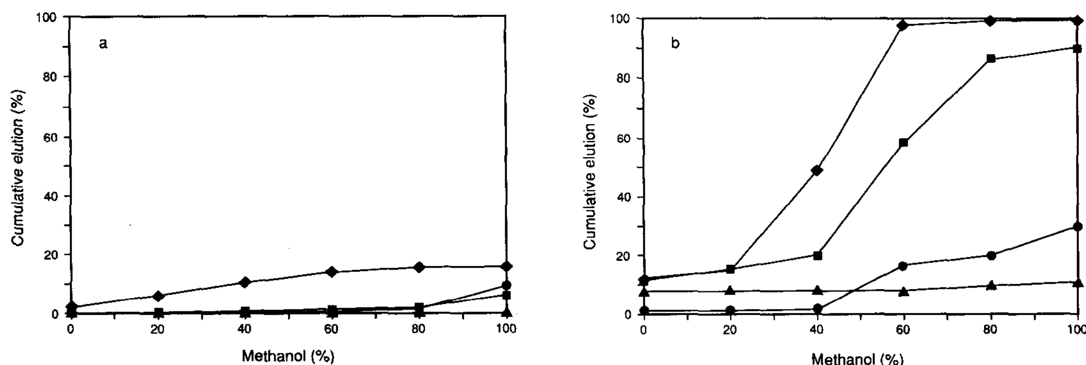

Figure 3
Cumulative elution profiles for a range of basic drugs eluted from a C2 cartridge with a stepwise methanol–water gradient following application in water (a) or plasma (b), 1 ml of each. Atenolol (■), ICI 95527 (◆), ICI 42464 (●), and tamoxifen (▲).

phase and thus preventing the drugs from interacting with both the primary reversed-phase and secondary ionic sites.

To resolve this matter a series of experiments were carried out which involved the application of drugs in plasma, separated plasma constituents, or pseudo plasma containing only the ionic or major protein components.

A 20 ml aliquot of plasma was dialysed and the plasma filtrate obtained. The residue was further washed with water and repeatedly dialysed to give plasma proteins free of ionic and other small molecular weight components. These residual plasma proteins were then redissolved in water or phosphate buffered saline. Phosphate buffered saline alone served to represent the ionic constituents of plasma, and the proteinaceous components were represented by a solution of human serum albumin (44.5 mg ml^{-1}) and human gamma globulins (12.0 mg ml^{-1}) in water. A 'synthetic' plasma was also prepared by dissolving the above proteins in phosphate buffered saline.

Aliquots (1 ml) of each of these sample matrices were then tested and compared with plasma for their effect on loss of two compounds (ICI95527 and atenolol) during washing with 1 ml aliquots of a stepwise gradient of methanol in water, from 0 to 100%, in 20% increments. The results, which are shown in Table 5, are presented as methanol ED_{50} values. These are the concentrations of methanol required to elute half the compound. These data clearly show that it is the ionic components of plasma which result in more facile elution, i.e. giving lower ED_{50} values, rather than the protein constituents. Although there were some problems in dissolving the dialysis residue in water, the results in terms of a total absence of elution are very significant. The small amount of elution observed with protein dissolved in water, we believe to be due to contamination of the proteins with salts. A simple silver nitrate test on the aqueous protein solution gave a white precipitate indicating the presence of chloride presumably as the potassium or sodium salt, in either or both of the proteins.

Table 5
The effect of sample application in different matrices on the elution of two compounds from a C2 cartridge with a stepwise gradient of methanol–water

Matrix	Methanol ED_{50} (%)	
	Atenolol	ICI 95527
Plasma	56	41
Plasma filtrate	53	36
Phosphate-buffered saline (PBS)	74	50
Plasma residue in water	>100	>100
Protein in water*	92	66
Protein in PBS	73	49

* Proteins contain traces of alkali halides.

Allowing for hydrogen bonding effects, which have been previously shown to alter the elution order from reversed-phase cartridges [2], the more lipophilic compounds could be more heavily washed without loss from the cartridge being observed. For example, tamoxifen (log *P* 6.63) when applied in acidified plasma could be washed with the full methanol–water gradient and the cumulative elution was still only 11%. As observed previously [1] and reported by other workers [3, 8] the strength of acetonitrile as a wash or elution solvent appeared to be less than that of methanol. If the function of the wash solvent is merely to remove non-basic, non-polar materials then acetonitrile would appear to be preferred over methanol.

The ionic and hydrogen bonding interactions which have been demonstrated previously, when compounds were applied in water [1, 2], were all shown to operate in this present work where the sample was plasma.

Conclusions

This work shows that the success of solid-phase extraction procedures for basic drugs is very dependent on the protein binding characteristics of the drug in question. Direct analysis of plasma is possible, although it appears that no one set of conditions can be used to extract all drug types. Under the conditions described here lipophilic compounds require the sample to be acidified prior to application to the cartridge, whereas more polar compounds do not. The conditions necessary for the successful extraction of a highly protein bound lipophilic base are actually disadvantageous when applied to a weakly protein bound drug and *vice versa*. The conditions necessary for the optimal extraction of a basic drug can be readily predicted from the distribution coefficient (log *D* pH 7.4) of the drug. Failure to use the correct conditions can result in signifi-

cant losses on application of the sample or washing of the cartridge with water.

The conditions necessary for the selective washing and elution of basic compounds using reversed-phase cartridges are similar to those determined previously [1, 2] where the compounds were applied in water. Small differences do exist however, particularly, in relation to washing of the cartridge with aqueous organic solvents. These differences are related to the ionic, as opposed to the proteinaceous components of the plasma and are probably caused by attenuation of the ion-exchange interactions of the charged base and silanols by the cationic constituents of the plasma.

Not surprisingly, the more lipophilic compounds are more resistant to elution with aqueous–organic mixtures. Hence cleaner extracts should be possible with this type of compound, compared to more polar drugs. Earlier findings [1, 3, 8], which suggest that acetonitrile is preferable to methanol as a wash solvent, have also been confirmed.

This work, taken with previously published data [1, 2], helps define the optimal conditions for the extraction of basic drugs from plasma. The methods described, which are in general use in this laboratory can be used to extract basic drugs in good yield (>90%), with excellent selectivity. These papers also serve to show the importance of physico-chemical parameters e.g. log P and pK_a in designing optimal analytical methods.

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