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RETENTION CHARACTERISTICS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BASIC DRUGS AND PLASMA EXTRACTS ON AN ALUMINA COLUMN

MARY T. KELLY and MALCOLM R. SMYTH*
School of Chemical Sciences, National Institute for Higher Education, Dublin, Glasnevin, Dublin 9 (Ireland)
and
DARIOUSH DADGAR
IWF Research Labs Inc., 6075 Kestrel Road, Mississauga, Ontario L5T 1Y8 (Canada)
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SUMMARY

The retention characteristics of 35 drugs on an alumina column using buffered aqueous methanolic mobile phases have been investigated. It has been shown that the effect of pH on retention depends on the pK_a of the drug and the type of buffer ion used; that there is an inverse relation between ionic strength and solute retention; and that a decrease in the amount of methanol in the mobile phase causes the drugs to be held longer on the column. The chromatographic system was also coupled to an on-line column-switching assembly to facilitate extraction of the drugs from plasma, and results are presented which indicate that the alumina column lends itself well to this convenient method of plasma clean-up.

INTRODUCTION

The use of bare (unmodified) silica and alumina as stationary phases in liquid chromatography had declined in popularity with the advent of chemically modified hydrophobic silicaceous supports. In particular, the use of alumina became even less widespread, as chemical modification of its surface in a manner analogous to the reaction of silica with alkylsilanes, proved not to be a practicable proposition[1,2]. Although some workers have reacted alumina with aminosilane, cyanosilane, and pyridylsilane[3], as well as octadecylsilane[4], Laurent *et al.*[1] found that most chemical reagents which had been used successfully in the chemical modification of silica were unreactive on alumina. They were, however, able to produce a non-polar material by reacting hexamethyldisilane (HMDS) with alumina, but subsequently proved it to be strongly adsorbed rather than covalently bound, and subject to erosion by the kind of polar solvents employed in liquid chromatography.

Among the problems commonly associated with reversed-phase chromatography are poor efficiencies and peak tailing for many organic bases. These effects result from ionic interaction between the charged bases and unreacted silanol moieties

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on the silica surface. Methods which have been used to overcome these problems include ion-suppression chromatography[5], or the use of organic ammonium compounds at high pH[6,7]. More recently, attention has been focussed on the exploitation of the amine–silanol interaction, since Jane[8] first reported that basic compounds could be efficiently separated on bare silica using the kind of aqueous–organic eluents more commonly associated with reversed-phase chromatography. The mechanisms involved in these types of polar interactions are complex and multifunctional, but have been shown to consist at least in part of ion-exchange reactions[9–11], with hydrophobic interactions involving siloxane bridges contributing to some extent[5,10]. This mode of separation demands that eluents of high pH be used where a large proportion of silanol moieties are ionised, and thus available to partake in ion-exchange reactions with oppositely-charged protonated bases[9]. A limitation with the use of high pH eluents is the tendency of silica to dissolve in alkaline solutions. This problem may be partially overcome by the incorporation of a pre-column between the pump and injector in order to saturate the mobile phase with silica, and by the use of mobile phases containing a high proportion of organic component.

Recently, Laurent *et al.*[1,2] have re-appraised the application of alumina as a solid phase support in column liquid chromatography. They showed that alumina can be used in an ion-exchange mode in a manner similar to unmodified silica, with the added advantage that it is stable over a wide pH range (2–12)[1]. Furthermore, due to the amphoteric nature of alumina, it behaves (like silica) as a cation exchanger in neutral or basic solutions, and (unlike silica) as an anion exchanger in acidic solutions, thus permitting the chromatographic separation of both anions and cations.

The objective of the current study was to examine the retention characteristics of a number of basic drugs on an alumina column under varying eluent conditions, and to investigate the possibility of using a column switching assembly in order to facilitate on-line clean-up of plasma samples followed by chromatography on the alumina column.

EXPERIMENTAL

Reagents and solvents

The drugs used were received as a gift from the Institute of Clinical Pharmacology (Dublin, Ireland). Potassium dihydrogen phosphate (analytical grade) was obtained from May and Baker (Dagenham, U.K.) and potassium hydroxide (AnalaR grade) from BDH Chemicals (Poole, U.K.). Orthophosphoric acid and dipotassium hydrogenphosphate (all analytical grade) were supplied by Riedel de Haen (Seelze, Hannover, F.R.G.). HPLC-grade methanol was purchased from Labscan Analytical Sciences (Dublin, Ireland). Deionised water was obtained by passing distilled water through a Milli-Q water purification system. Dried human plasma was obtained from the Blood Transfusion Board (Dublin, Ireland) and dissolved in Milli-Q water. This plasma was then used within seven days of reconstitution.

Drug solutions

Stock solutions equivalent to 1 mg ml^{-1} of the drugs in methanol were prepared,

and working standards were made up to the required concentration, which varied between 1 and 10 μg ml^{-1} according to the detector response of the drug.

Plasma solutions

Aliquots of reconstituted drug-free plasma were spiked with drug solutions to produce the desired concentrations. These plasma solutions were then diluted (1:1) with water prior to injection into the high-performance liquid chromatographic (HPLC) system.

Instrumentation and operating conditions

The drugs were separated on a Techsphere alumina 5 μm column (150 mm × 4.6 mm I.D.), supplied by HPLC Technology (Macclesfield, U.K.). Stock solutions of phosphate buffer were prepared by mixing solutions of potassium dihydrogenphosphate and dipotassium hydrogenphosphate to produce solutions of varying pH in the range 5–8.5. Phosphoric acid was added to potassium dihydrogenphosphate to give a solution of pH 3, and potassium hydroxide was added to dipotassium hydrogenphosphate to generate solutions of pH 9 and 11. All component solutions were 1 *M*, and the mixtures were diluted with water to give the desired ionic strength. Mobile phases were made by mixing the aqueous component with methanol to produce solutions containing 30–90% organic phase. The pH of the buffer solutions and the aqueous–organic mixtures were measured at 20 ± 1°C using a standard pH glass electrode. The mobile phase was passed through a 0.45-μm filter under vacuum, and delivered by a Waters (Milford, MA, U.S.A.) Model 501 HPLC pump at a flow-rate of 1.0 ml min^{-1}. The drugs were detected by UV absorption at 254 nm using a Shimadzu SPD-6A variable-wavelength detector with a detector setting of 0.04 a.u.f.s. The resulting chromatograms were recorded with a Linseis (Selb, F.R.G.) recorder at a chart speed of 200 mm h^{-1}.

For direct injection, 20-μl aliquots of the drug solutions in mobile phase were introduced into the chromatographic system. For the purposes of column switching, a second Waters Model 501 HPLC pump and the concentration column were connected to the analytical assembly via a Rheodyne (Cotati, CA, U.S.A.) 7000 six-port switching valve. The instrument arrangement used is shown in Fig. 1.

The 10 × 1.5 mm I.D. concentration columns were dry-packed with Corasil (Waters Assoc.) RP-18 (37–50 μm) packing material. The second pump eluent was Milli-Q water filtered through a 0.45-μm filter and degassed under vacuum. When a 500-μl plasma sample is introduced via the injector port, it is swept onto the concentration column by water from pump A. The drugs are selectively retained on this column while the plasma components are eluted to waste. Meanwhile, the mobile phase eluent is being passed by pump B through the analytical column, and upon switching the valve the drugs are swept from the concentration column onto the analytical column where they are separated.

RESULTS AND DISCUSSION

Effect of pH

The retention of the 35 drugs on the alumina column was investigated as a function of pH over the range pH 3 to pH 11. The mobile phase contained

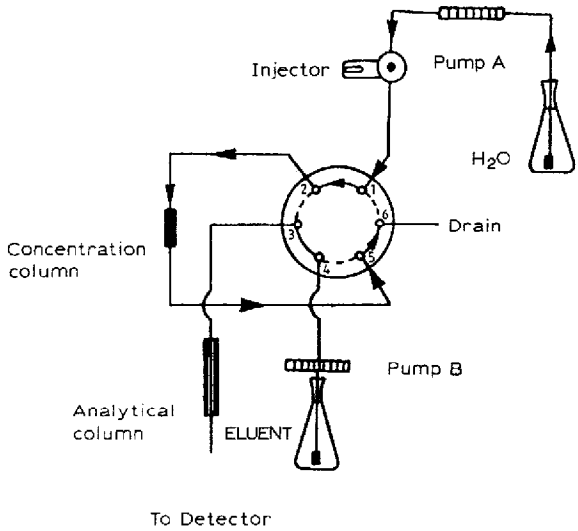


Fig. 1. Column switching assembly incorporating a six-port switching valve.

methanol–0.04 *M* potassium phosphate buffer (80:20) adjusted to the required pH. Additions were not made to compensate for variations in ionic strength from one pH to the next as it was assumed that that the changes in cation concentration would be small in relation to the changes in solute and column ionisation arising from pH variation.

The results of this study are presented in Table I. From this it can be seen that the drugs may be roughly divided into three groups; namely (A), those drugs with retention times which increase between pH 3 and pH 5, followed by a decline in retention at higher pH; (B) those drugs with exhibit decreased retention as the pH is increased; and (C), drugs whose retention times are largely unaffected by pH. These observations may be explained by considering the pK_a of the drugs, the pH of the mobile phase, and the amphoteric nature of the alumina packing. Whether the alumina surface is positively or negatively charged depends on the pH of the surrounding medium. In acidic solutions it is positively charged and behaves as an anion exchanger, whereas in basic solutions the surface is negatively charged and behaves as a cation exchanger. The pH at which the alumina surface bears no charge and is neutral is known as the zero point charge (ZPC). The ZPC is known to be related to the method of production of the alumina, and to depend heavily on the nature of ions present in the surrounding solvent. Laurent *et al.*[12] have shown that in the presence of phosphate buffer, the ZPC of alumina is reduced to 6.5 from the value of 9.2 originally quoted by Parks[13].

Group A. Although these compounds are fully ionised at low pH, interaction with the alumina packing is minimal as the latter will also bear a positive charge, thus repelling the like-charged protonated bases. As the pH is increased to 5, there is an observed increase in retention consistent with the reduced positive charge on the alumina, but as the pH is further increased to 7 and above there is a general decrease in retention. These results may be explained in terms of reduced protonation of the

TABLE I

EFFECT OF pH ON RETENTION

Mobile phase: 0.04 *M* phosphate buffer, pH 7–methanol (20:80).

Drug	pK_a	*Retention time (min)*				
		pH 3	*pH 5*	*pH 7*	*pH 9*	*pH 11*
Group A						
Amitriptyline	9.5	7.5	9.6	6.0	5.1	4.2
Atenolol	9.6	3.9	5.4	2.7	2.1	2.0
Acetopromazine	NA[a]	6.0	7.6	3.6	3.6	3.0
Chlorpheniramine	8.9	8.7	13.8	7.2	6.6	5.1
Chlorpromazine	9.3	6.2	7.5	3.6	3.0	2.7
Desipramine	10.2	5.4	7.5	7.8	6.6	5.4
Imipramine	9.6	8.1	9.6	6.0	5.1	3.9
Mefloquine	NA	7.5	10.8	5.1	4.5	3.6
Nortriptyline	9.7	5.4	8.7	7.5	6.3	5.4
Phentoloxamine	9.1	6.2	7.8	7.2	3.6	3.0
Propranolol	9.5	3.6	4.5	3.5	3.0	2.7
Pindolol	9.5	3.3	3.6	2.4	2.1	2.1
Promethazine	9.1	7.4	10.5	5.1	3.3	3.0
Protriptyline	10.1	4.8	6.6	6.9	6.3	6.0
Quinine	8.5	4.5	8.2	3.0	2.4	2.1
Tripelennamine	9.0	4.5	5.4	2.7	2.7	2.1
Tyramine	10.2	4.2	9.9	6.6	3.6	3.0
Group B						
Diltiazem	NA	4.2	3.3	1.8[b]	1.8[b]	1.8[b]
Dextromethorphan	8.3	4.8	4.5	3.0	2.7	2.4
Fluphenazine	8.1	6.1	6.0	2.4	2.4	2.4
Lidocaine	7.9	3.0	2.7	2.4	2.1	2.1
Mepivacaine	7.7	3.0	2.7	2.1	1.8[b]	1.8[b]
Perphenazine	7.8	6.9	5.1	3.3	3.0	2.7
Phentermine	10.1	5.4	4.5	3.3	3.0	2.4
Phenylpropanolamine	9.5	7.2	4.8	4.8	2.7	2.4
Trimethoprim	7.2	4.5	2.7	1.8[b]	1.8[b]	1.8[b]
Verapamil	NA	3.0	3.0	2.4	2.4	2.4
Group C						
Caffeine	14.0	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Chlorthalidone	9.4	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Chloramphenicol	NA	2.1	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Frusemide	3.6	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Nitrazepam	3.2	2.1	2.0	1.8[b]	1.8[b]	1.8[b]
Sulfamerazine	7.1	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Sulfamethoxazole	5.6	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
N-Acetylsulfamethoxazole	NA	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Theophylline	8.6	2.0	1.8[b]	1.8[b]	1.8[b]	1.8[b]

[a] NA = Not available in standard reference texts.

[b] Drug unretained under these conditions.

analytes at higher pH, and possibly a shift upwards of the ZPC of alumina, as the changing buffer composition causes a reduction in the negative charge of the column[14]. Furthermore, Laurent *et al.*[2] have demonstrated an increase in the apparent

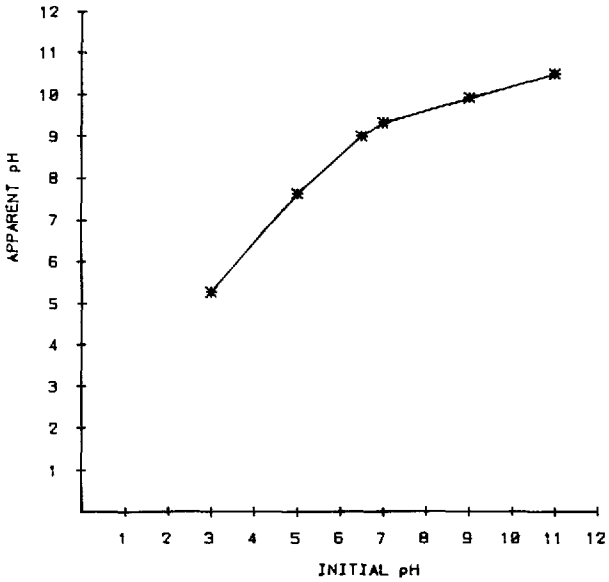


Fig. 2. Effect of the addition of 80% methanol on apparent pH. Mobile phase: 0.04 *M* potassium phosphate buffer–methanol (20:80).

pH in the presence of organic solvents, and the resulting decrease in retention is exaggerated by a concomitant decrease in solute pK_a. This is illustrated in Fig. 2, where it can be seen that there is a substantial difference between the initial and apparent pH values of solutions of initial pH 3–7. This becomes less pronounced for initial pH values of 8–10, until for a solution of initial pH of 11, the addition of methanol has the effect of producing a lower apparent pH value.

Group B. The drugs in this category exhibit reduced retention times with increasing pH. As they generally have lower pK_a values than those in group A, it is to be expected that these compounds would be less ionised than the more basic amines at any given pH, and that there would be a more pronounced reduction in their retention times under conditions of increasing eluent pH.

Group C. As expected, solutes which do not ionise, such as caffeine, are unretained by the column in this chromatographic system where the principal mechanisms of solute–column interaction are known to be ionic in nature. Likewise, compounds with low pK_a values, *e.g.* nitrazepam ($pK_a = 3.2$), are similarly unretained as they would not be sufficiently ionised to interact with the column at those pH values where alumina behaves as a cation exchanger. The acidic drug frusemide was also investigated, and was found not to be retained under any pH conditions. Frusemide has a pK_a of 3.6[15], so that at pH 3, when the apparent pH was 5.28, the drug should have been ionised. However, this pH is high enough for the column to behave more as a cation- than an anion-exchanger; hence negatively charged solutes would be repelled by the like-charged alumina surface, and therefore be unretained by the column.

Effect of ionic strength

The effect of lowering the ionic strength from 0.04 *M* to 0.02 *M* of the potassium

phosphate buffer was investigated. The results of this study are presented in Table II, where it can be seen that drug retention increases as the ionic strength is reduced. These observations agree with other workers[1,14] who also found an increase in retention on alumina as the ionic strength was reduced. These findings are consistent with the ion-exchange theory of retention which explains enhanced solute interaction in terms of decreased competition by the reduced number of competing cations for the charged sites on the column packing.

Effect of methanol content

The effect of varying the percentage methanol concentration from 90% to 30% (in a mobile phase where the aqueous component was 0.02 *M* phosphate buffer, pH 7) on the retention of 9 of the drugs is shown in Table III. From this it can be seen that as the methanol content is reduced, there is a corresponding increase in drug retention; so much so, that when the methanol content is 50% or below most of the drugs would appear to be fully retained, not having eluted after 60 min or more. These observations differ from those made by Laurent *et al.*[2], and Lingeman *et al.*[14], who found a point of maximum retention *vs.* percentage methanol concentration which is solute-dependent, but was about 40% methanol for most compounds they studied. Laurent *et al.*[2] attributed low solute retention at high methanol contents to a decrease in solute ionisation, and at low methanol contents to reduced solvation of the competing ions.

TABLE II

EFFECT OF IONIC STRENGTH ON RETENTION

Mobile phase: phosphate buffer pH 7–methanol (20:80).

Drug	*Retention time (min)*		*Drug*	*Retention time (min)*	
	0.02 M	*0.04 M*		*0.02 M*	*0.04 M*
Amitriptyline	18.3	6.0	Phentermine	6.3	3.3
Atenolol	3.6	2.7	Phenylpropanolamine	4.8	4.8
Acetopromazine	6.3	3.6	Propranolol	5.1	3.0
Caffeine	1.8[a]	1.8[a]	Pindolol	3.0	2.4
Chlorpheniramine	11.1	7.2	Promethazine	6.3	5.1
Chlorthalidone	1.8[a]	1.8[a]	Protriptyline	10.5	6.9
Chlorpromazine	4.5	3.6	Nitrazepam	1.8[a]	1.8[a]
Chloramphenicol	1.8[a]	1.8[a]	Quinine	3.0	3.0
Diltiazem	2.1	1.8[a]	Trimethoprim	1.8[a]	1.8[a]
Desipramine	11.7	7.8	Tripelennamine	3.3	2.7
Dextromethorphan	3.9	3.0	Tyramine	9.0	6.6
Fluphenazine	2.7	1.8[a]	Verapamil	2.4	2.1
Frusemide	1.8[a]	1.8[a]	Sulphamerazine	1.8[a]	1.8[a]
Imipramine	8.4	6.0	Sulphamethoxazole	1.8[a]	1.8[a]
Lidocaine	2.1	2.1	N-Acetylsulfamethoxazole	1.8[a]	1.8[a]
Mefloquine	7.4	5.1	Theophylline	1.8[a]	1.8[a]
Mepivacaine	2.4	1.8[a]			
Nortriptyline	11.4	7.5			
Perphenazine	3.3	3.0			
Phentoloxamine	4.5	7.2			

[a] Drug unretained under these conditions.

TABLE III

EFFECT OF METHANOL CONTENT ON RETENTION

Mobile phase: 0.02 *M* potassium phosphate buffer, pH 7–methanol.

Drug	*Retention time (min) at methanol content (%)*				
	30	*50*	*70*	*80*	*90*
Amitriptyline	—[a]	—	17.1	6.0	3.3
Chlorpromazine	—	—	9.3	3.6	3.0
Dextorphan	—	—	4.8	3.7	2.7
Nitrazepam	9.6	3.6	2.1	1.8	1.5
Perphenazine	—	—	6.9	3.0	2.4
Pindolol	6.9	3.9	3.0	2.4	2.4
Quinine	—	—	3.9	3.0	2.4
Tripelennamine	—	—	5.4	2.7	2.4
Sulfamethoxazole	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]

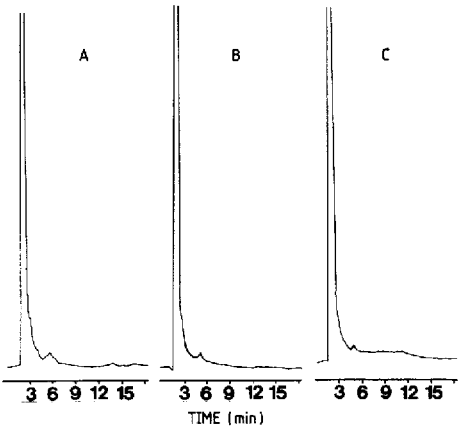
[a] Not eluted after 60 min.
[b] Drug unretained under these conditions.

At any given pH these two mechanisms will be operating in opposition to one another, as evidenced by the appearance of a retention maximum, with reduced solute ionisation predominating at high methanol contents, and reduced competing ion solvation predominating at low methanol contents. However, as Laurent *et al.*[2] point out, the enhanced solvation of large competing ions (such as tetramethylammonium hydroxide) is the opposite to what happens when the smaller lithium ion is transferred from aqueous to organic media; in this case the smaller ion is less solvated in methanol. It is quite possible that in the present study where the competing ion is the relatively small potassium ion, the degree of buffer ion solvation is reduced as the methanol content is increased, an effect which would act in concert with reduced solute ionisation to minimise the drugs interaction with the stationary phase.

Plasma experiments

Having previously carried out on-line solid–liquid extraction of drugs from plasma, followed by chromatography on a bare silica column[16], it was decided to attempt similar experiments on the alumina column. The instrument arrangement and column switching operation have been previously described in the experimental section. Plasma clean-up and drug extraction were performed on a 10 × 1.5 mm I.D. concentration column, dry-packed with Corasil C_{18} 25–40 μm packing material. In earlier work on the silica column, the C_{18} packing was compared with C_8 material in terms of drug recovery and plasma clean-up. It was found that a C_{18} concentration column retained less of the endogenous plasma components than the C_8 packing because the former is more hydrophobic and has less affinity for the relatively polar plasma constituents.

The objective of this study was to compare drug-free plasma profiles when the pH of the aqueous component was increased from pH 5 to pH 8, and when the ionic strength was increased from 0.02 *M* to 0.04 *M*. The mobile phases studied, along with the resultant chromatograms are presented in Fig. 3, where it can be seen that a slightly



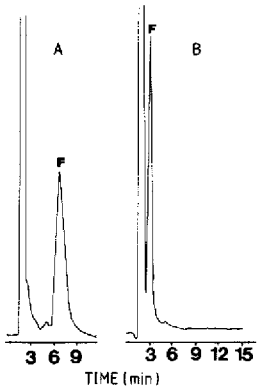


Fig. 3. Blank plasma chromatograms. Mobile phases: (A) 0.02 *M* buffer, pH 5–methanol (20:80); (B) 0.02 *M* buffer, pH 8–methanol (20:80); (C) 0.04 *M* buffer, pH 8–methanol (20:80).

Fig. 4. Spiked plasma chromatograms. Plasma spiked with fluphenazine (F) 200 ng ml^{-1}. (a) Mobile phase: 0.02 *M* phosphate buffer, pH 5–methanol (20:80); (b) mobile phase: 0.02 *M* phosphate buffer, pH 8–methanol (20:80).

better blank plasma chromatogram is obtained at the higher eluent pH with little difference observed when the ionic strength is doubled. The effect of pH on the retention of plasma constituents is not as manifest as its effect on the individual drug species. To illustrate this point, a test compound, *i.e.* fluphenazine, was extracted on-line from spiked plasma prior to chromatography. As may be seen from the chromatograms presented in Fig. 4, there is a pronounced shift in the retention time of the drug relative to that of the plasma constituents as the pH is increased from pH 5 to pH 8. These results demonstrate that it is possible to manipulate the mobile phase to produce the most appropriate retention time for the compounds of interest with little danger of introducing unacceptable interfering peaks from the plasma matrix. Hence this type of chromatography would seem to offer an advantage over conventional bonded-phase techniques, in that the plasma constituents are not sufficiently ionised to be retained by the alumina column, and hence are rapidly eluted. An important example is caffeine which can interfere with drug analysis in reversed-phase chromatography: because it does not ionise, it will not interact with alumina by the principal mechanism of retention, *i.e.* ion exchange, and as a result would elute too early to interfere with any of the peaks of interest.

Using a mobile phase containing methanol–0.04 *M* phosphate buffer, pH 5 (80:20), five replicate analyses of plasma containing 200 ng ml^{-1} fluphenazine were made. The drug eluted with a mean retention time of 7.0 min (standard deviation 0.1 min). The mean drug peak height was 65.4 mm ($\pm$ 2.3 mm) using a detector sensitivity setting of 0.04 a.u.f.s. The coefficient of variation was 3.5%, and 73.1% of the drug was recovered from plasma when compared with authentic aqueous standards injected in the same concentrations as spiked plasma. These results indicate that it should be possible to develop a fully validated bioanalytical method incorporating on-line solid-phase extraction for any drug which can be separated using this chromatographic system.

CONCLUSION

A preliminary study of the behaviour of drug compounds and plasma extracts on an alumina column has been carried out. Results would indicate that alumina presents an attractive alternative to bonded-phase separations of basic drugs which can present difficulties associated with the presence of unreacted silanol moieties on the silica surface. Alumina has an advantage over silica in that it is more stable at high pH, and thus in the separation of bases, the cationic character of the column may be maximised without fear of column degeneration. The retention of ionic compounds is strongly influenced by pH, ionic strength, and the percentage methanol in the mobile phase. Any or all of these parameters may be manipulated to produce the desired retention for the compound or compounds of interest. Chromatography on alumina would appear to be well suited to the analysis of drugs in plasma samples as it lends itself to the rapid and convenient technique of solid-phase extraction by column switching.

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