SEPARATION OF BASIC DRUGS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A SILICA COLUMN USING A METHANOL-ETHYLENEDIAMINE BUFFER

ROGER M. SMITH* and JOHN O. RABUOR[a]

Department of Chemistry, Loughborough University of Technology, Loughborough, Leics LE11 3TU (U.K.)
(Received August 30th, 1988)

SUMMARY

The application of methanol–aqueous ethylenediamine–ammonium nitrate eluents has been investigated for the high-performance liquid chromatographic separation of basic drugs on silica stationary phases. These eluents were shown to be more reproducible than previously studied systems based on methanol–aqueous ammonia–ammonium nitrate eluents. The effects of different eluent pH and buffer concentrations have been examined.

INTRODUCTION

Basic drugs frequently cause problems when analysed by reversed-phase high-performance liquid chromatography (HPLC) because of their interaction with acidic silanol groups on the surface of the stationary phase. A number of alternative methods have therefore been proposed for their separation and one of the most successful approaches has been the use of silica columns with high pH buffered eluents containing a high proportion of methanol. This method was originally proposed by Jane[1], who used an ammonium nitrate buffer, and has subsequently been studied by Law and co-workers[2,3]. In a recent study Schmid and Wolf[4] have examined a similar high-methanolic system and looked at the effect of sodium acetate buffer and ammonia concentration by using the tricyclic drugs. In all these systems the separation is effectively based on the ion-exchange properties of the silica stationary phase.

There has been some concern that although HPLC is a very widely used technique for analytical separations, intra- and interlaboratory reproducibility of separations can be very poor. As a consequence, we have carried out a series of studies of separations of forensic interest to investigate the sources of these variations and to examine methods to standardise the separations and the recording of the results. As part of this work, the separation of basic drugs on silica using a methanol–aqueous ammonia–ammonium nitrate eluent has been examined in detail.

---

[a] Present address: National Agricultural Laboratories, P.O. Box 14733, Nairobi, Kenya.

In our studies the effects of changes in the composition of the mobile phase and in the operating conditions have been examined[5] and differences in the separation on different batches and brands of silica have been reported[6]. Similar differences between brands have also been noted previously[2,4]. There were significant differences between separations even on a single column when the results were expressed as capacity factors but considerably more reproducible results could be obtained if the results were recorded as relative retentions compared to protriptyline as an internal standard. To test the conclusions of these studies the separation has been examined in national[7] and international collaborative studies[8]. In both these studies the variance of the results was higher than in intralaboratory studies. It was suggested that differences in the operating temperature of the laboratories could have had an effect as few of the laboratories used a thermostated column. However, it appeared that a major factor which could not be controlled was the concentration of the ammonia stock solutions used to prepare the aqueous buffer. This would affect the pH and ionic strength of the mobile phase.

In setting up a standard eluent system to determine the reproducibility of batches of column materials[6], it was demonstrated that consistent results could be obtained over a three-month period using a single source of ammonia, even though its concentration changed due to evaporation. However, deliberate larger changes in the ammonia concentration to 200, 180, 160, 90, 80 or 60% of the original value caused major effects on the relative and absolute retentions. Some of the analyte drugs were more susceptible to the changes than others. These problems with the reproducibility and stability of the concentrated ammonia solution have led to an interest in alternative eluent systems based on less volatile buffer components. If possible the buffer should be prepared by weight or defined volume of a single liquid compound rather than as a volume of a dilute aqueous solution of a volatile base. It is also important to avoid methods which would require pH adjustment as this could cause differences in the ionic strength of the eluent, which were also shown to affect the separations.

The present study reports a system based on the use of a relatively involatile amine, ethylenediamine, as the base, whose proportion in the mobile phase can be precisely defined as the volume of a neat liquid. The effects of differences in the pH and buffer concentrations and of different batches of the stationary phase have been studied.

EXPERIMENTAL

Chemicals and standards

Ammonium nitrate, analytical-reagent grade, and methanol, HPLC grade, were from FSA Laboratory Supplies, Loughborough, U.K. Ethylenediamine was reagent grade from Aldrich, Poole, U.K. Concentrated ammonia solution (sp. gr. 0.880) was laboratory grade from BDH Chemicals, Poole, U.K. Samples of basic drugs were from from the reference collection of the Central Research Establishment, Home Office Forensic Science Service.

HPLC equipment

HPLC separations were carried out using a Pye Unicam LC-XPS pump and an

Altex 153 fixed-wavelength detector at 254 nm. The samples (5 μl) were injected using a 7125 Rheodyne valve fitted with a 20-μl loop onto a Shandon column (250 $\times$ 5 mm I.D.) packed with Spherisorb S5W 5 μm (Batch 2752 or 5493, Phase Separations Queensferry, U.K.). The methanol–buffer eluent was pumped at 2 ml min^{-1} and was passed through a pre-column packed with silica, installed between the pump and the injection valve. The pre-column and the analytical column were maintained at 30°C in a circulating-water bath. Peaks were recorded using a chart recorder.

Ammonia buffer solutions

Ammonia–ammonium nitrate buffer was prepared by mixing ammonia (sp. gr. 0.880) (90 ml), ammonium nitrate (27 g) and water (900 ml).

Ethylenediamine–ammonium nitrate buffers

The standard buffer (pH 10.2) was prepared by a ten-fold dilution of a mixture of ammonium nitrate (10.5 g), ethylenediamine (15.0 ml) and water (200 ml).

A buffer of pH 9.47 was prepared by a ten-fold dilution of a mixture of ethylenediamine (5 ml) and ammonium nitrate (10.0 g) in water (200 ml).

A buffer of pH 10.56 was prepared by a ten-fold dilution of a mixture of ethylenediamine (15 ml) and ammonium nitrate (5.02 g) in water (200 ml).

Sample solutions of basic drugs

Solution of mixtures of the basic drugs were made up as described for the collaborative study[7], each including protriptyline hydrochloride as an internal standard, in ethanol–water (90:10, v/v) with concentrations (0.02–8 mg ml^{-1}) chosen to give a similar detector response for each drug.

For much of the work a simplified set of test solutions containing characteristic drugs was used[5]. The detailed composition of these solutions are given below [concentrations in mg ml^{-1} in ethanol–water (90:1, v/v)].

(A) Dipipanone hydrochloride, 0.40; prolintane hydrochloride, 1.24; protriptyline hydrochloride, 0.15; strychnine, 0.07.

(B) Promazine, 0.006; phenylephrine bitartrate, 1.44; protriptyline hydrochloride, 0.15.

(C) Codeine phosphate, 1.07; ephedrine, 2.25; protriptyline hydrochloride, 0.15.

(D) Sodium nitrate, 30 mg ml^{-1} in methanol–water (90:10, v/v) as a column void volume marker.

Calculations

The separations were carried out in triplicate and the mean retention times were used to calculate the capacity factors as $k' = (t_R - t_0)/t_0$.

Relative capacity factors were calculated as k'/k'_p where k'_p is the capacity factor for the protriptyline present as an internal standard in each test solution.

RESULTS AND DISCUSSION

In order to design an eluent that can be prepared reproducibly in different laboratories it must be possible to specify precisely all the constituents, as even small differences may significantly affect the relative retentions of the analytes. Rather than

preparing buffers by adjusting them to a specified pH it is also preferable to use fixed weights or volumes to give a predictable pH. This was demonstrated in earlier studies of the separation of barbiturates in which preparing the buffer by weight from solid salts gave highly reproducible results[9]. In initially investigating the separation of drugs on a silica column, it appeared that the ammonia–ammonium nitrate buffer was robust as the pH was unaffected even by significant changes in the amount of ammonia or ammonium nitrate used in the preparation of the buffer[5]. However, the retentions did appear to be sensitive to the ionic strength of the mobile phase[5]. In collaborative studies[7,8] the relatively poor reproducibility suggested that one area which could not be controlled was the strength of the ammonia solutions used in the preparation of the eluent.

In the present study a limited group of basic drugs was examined. This included dipipanone, pipazethate, phenylephrine and strychnine, compounds which have been found to be particularily sensitive to changes in the experimental conditions[5,6]. The study started by examining the use of other bases as possible alternatives to concentrated ammonia solution for the preparation of buffers with pH of about 10. Piperidine was too insoluble in water to give a pH for the buffer greater than 8. Diethylamine was more satisfactory but it is relatively volatile (b.p. 55°C) and could be lost from the mobile phase during the separation in a similar manner to ammonia. The less volatile amine, ethylenediamine (b.p. 118°C), was also apparently suitable. When it was used in place of ammonia at a similar strength, it gave an aqueous buffer solution with a pH of 10.2. However, when a methanol–buffer (90:10, v/v) eluent was used to separate the limited test set of basic drugs, the drugs were barely retained on the column compared to the corresponding ammonia–ammonium nitrate system. The internal standard, protriptyline, was eluted with a capacity factor of less than 0.5 compared to 2.5 and the retention times of the other analytes were even shorter. These small retentions are insufficient for resolution and identification of the basic drugs.

As the rate of elution is governed by the ionic strength of the mobile phase, this initial buffer mixture was diluted ten-fold with water to give a buffer with a pH of 10.22 and the separation was re-examined. This eluent gave comparable retentions to those achieved earlier (Table I) but the relative retentions for some of the drugs differed from those obtained with the ammonia buffer eluent.

Mobile phases were then prepared using buffer solutions of pH 10.55 and 9.47 by altering the rato of the ethylenediamine and ammonium nitrate (see Experimental) and the separations were repeated. At pH 9.47 the resolution of the test mixtures was very poor. With the pH 10.55 eluent (Table I) a better separation was obtained but the order of elution changed. Strychnine was now eluted much more rapidly than protriptyline. This could cause problems as all the components would be eluted within too short a time span. It was therefore decided to use the pH 10.2 solution as the standard buffer in future studies. Using this buffer, repeated separations on successive days showed that the repeatability of these eluents was satisfactory.

The work up to this point had been carried using a Spherisorb S5W (Batch 2752) column as the stationary phase. However, it has been observed that retention on different batches of Spherisorb S5W gave different selectivities with the ammonium nitrate system[6]. As the initial batch of silica had been exhausted, a second batch of stationary phase (Spherisorb S5W Batch 5493) was examined. The same mixtures of 28 drugs that had been used in the collaborative study[7] were separated using the ethylenediamine eluent and gave good peak samples (Fig. 1).

TABLE I

EFFECT OF DIFFERENT AMINES AND BUFFER CONCENTRATIONS ON THE RETENTION OF BASIC DRUGS ON SPHERISORB S5W

Based on columns prepared from Spherisorb S5W batch 2752. Temperature, 30°C. Eluent, methanol–buffer (90:10, v/v). Values using the ammonia buffer from ref. 6.

Compounds	Capacity factors			Relative capacity factors (× 100)		
	Buffer			Buffer		
	Ammonia	Ethylenediamine		Ammonia	Ethylenediamine	
		pH 10.22	pH 10.55		pH 10.22	pH 10.55
Dipipanone	0.37	0.58	0.62	20.3	27.2	22.1
Promazine	0.71	0.78	0.76	38.8	36.6	27.1
Codeine	0.88	0.92	0.87	48.2	43.2	31.1
Prolintane	0.88	1.11	1.12	48.2	52.1	40.0
Phenylephrine	1.17	1.26	1.43	64.0	59.1	51.1
Ephedrine	1.28	1.50	1.78	70.0	70.4	63.6
Protriptyline	1.83	2.13	2.80	–	–	–
Strychnine	2.65	2.77	2.52	144.4	130.0	90.0

The separation was repeated three times on successive days, using a fresh eluent each day, to determine the repeatability of the separation. The capacity factors and relative capacity factors compared to protriptyline were determined and the standard deviations for the repeated studies were calculated (Table II). The repeatability of the capacity factors and the relative capacity factors were good and in most case better than those obtained for the ammonia-based system[5,6]. The retentions of some analytes could not be distinguished from the solvent front and codeine and dipipanone now
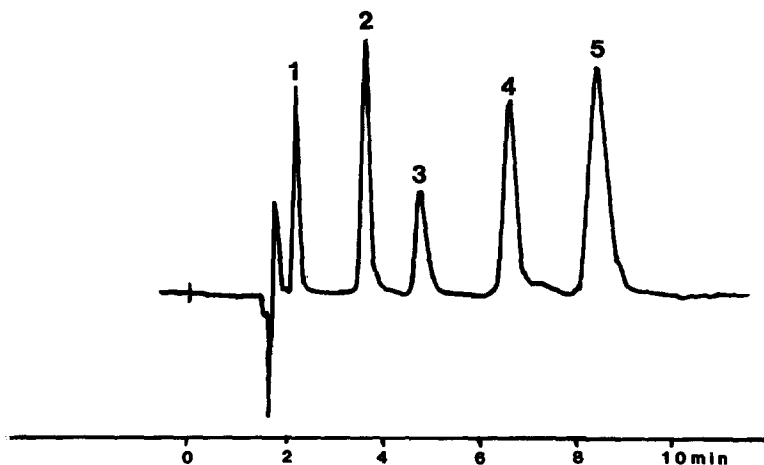

Fig. 1. Separation of drug test mixture on Spherisorb S5W Batch 5493. Eluent: methanol–(ethylenediamine–ammonium nitrate buffer pH 10.2) (90:10, v/v). Basic drugs: (1) procaine; (2) promazine; (3) ethoheptazine; (4) protriptyline (internal standard); (5) strychnine.

TABLE II

REPRODUCIBILITY OF CAPACITY FACTORS AND RELATIVE CAPACITY FACTORS OF BASIC DRUGS DETERMINED USING ETHYLENEDIAMINE BUFFER ELUENT

Based on three separations on successive days on column prepared from Spherisorb S5W Batch 5493. Eluent, methanol–(aqueous ethylenediamine–ammonium nitrate buffer pH 10.2) (90:10, v/v); temperature, 30°C.

Compounds	Capacity factors			Relative capacity factors (× 100)			Ammonia[c]
	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)	
Diazepam	[a]	–	–	–	–	–	
Nitrazepam	[a]	–	–	–	–	–	
Papaverine	[a]	–	–	–	–	–	
Caffeine	[a]	–	–	–	–	–	
Dextropropoxyphene	0.09	0.01	11.1	3.5	0.5	14.3	
Cocaine	0.17	0.02	11.8	6.5	0.8	12.3	
Procaine	0.24	0.02	8.3	9.3	0.9	9.7	
Amitriptyline	0.54	0.03	5.5	20.3	0.6	3.0	
Chlorpromazine	0.60	0.04	6.7	22.4	1.0	4.5	
Propranolol	0.68	0.02	2.9	26.1	0.4	1.5	
Imipramine	0.84	0.03	3.6	31.7	0.8	2.5	
Phentermine	0.95	0.03	3.2	35.7	0.5	1.4	
Amphetamine	0.99	0.03	3.0	37.7	0.5	1.3	
Promazine	1.01	0.03	3.0	38.5	0.8	2.1	36.4
Dipipanone[b] ⎫	1.06	0.03	2.8	40.1	0.8	2.0	33.5
Codeine[b] ⎭							46.0
Morphine	1.22	0.04	3.3	45.9	0.4	0.9	
Pholcodine	1.35	0.05	3.7	51.3	0.8	1.6	
Phenylephrine	1.50	0.01	0.7	56.5	0.1	0.2	60.7
Prolintane	1.62	0.04	2.5	60.9	0.8	1.3	62.8
Ethoheptazine	1.63	0.03	1.8	62.0	0.5	0.8	
Nortriptyline	1.68	0.03	1.8	64.0	0.4	0.6	
Ephedrine	1.85	0.06	3.2	69.6	0.4	0.6	69.9
Methdilazine	1.86	0.05	2.7	70.3	0.7	0.9	
Pipazethate	1.89	0.08	4.2	71.0	1.5	2.1	
Methylamphetamine	2.09	0.08	3.8	79.0	0.7	0.8	
Protriptyline	2.63	0.03	1.1	100.0	–	–	
Strychnine	3.62	0.06	1.7	137.4	0.9	0.7	159.8

[a] Unresolved from solvent front.
[b] Unresolved (in same test mixture).
[c] From ref. 6.

coeluted, whereas previously they had been well separated. The retentions and order of elution of the basic drugs differed markedy from a separation on this batch using the ammonia based eluents[6]. There were also significant differences from the retentions of the smaller group of drugs with the ethylenediamine eluent measured on the older batch of Spherisorb S5W (Table I).

Further studies of this approach to the separation of basic drugs on silica are in progress with the aim of achieving a buffer solution prepared by weight from solid components that will give a highly reproducible mobile phase. This can then be used to examine in detail the differences between batches and columns of the stationary phase.

CONCLUSION

The use of a non-volatile liquid amine to prepare the buffer solution improves the reproducibility of the separation of basic drugs on a silica column. The discrimination of the separation is similar to that obtained with the original ammonia–ammonium nitrate eluent. The selectivity of the separation is susceptible to differences in the silica column material.

ACKNOWLEDGEMENTS

REFERENCES

1 I. Jane, *J. Chromatogr.*, 111 (1975) 227.
2 B. Law, R. Gill and A. C. Moffat, *J. Chromatogr.*, 301 (1984) 165.
3 B Law, *J. Chromatogr.*, 407 (1987) 1.
4 R. W. Schmid and Ch. Wolf, *Chromatographia.*, 24 (1987) 713.
5 R. M. Smith, T. G. Hurdley, R. Gill and M. D. Osselton, *J. Chromatogr.*, 398 (1987) 73.
6 R. M. Smith, T. G. Hurdley, J. P. Westlake, R. Gill and M. D. Osselton, *J. Chromatogr.*, 455 (1988) 77.
7 R. Gill, M. D. Osselton, R. M. Smith and T. G. Hurdley, *J. Chromatogr.*, 386 (1987) 65.
8 R. Gill, M. D. Osselton, R. M. Smith and T. G. Hurdley, *J. Pharm. Biomed. Anal.*, in press.
9 R. M. Smith, T. G. Hurdley, R. Gill and A. C. Moffat, *Chromatographia.*, 19 (1984) 401.