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# AMMONIUM ACETATE: A GENERAL PURPOSE BUFFER FOR CLINICAL APPLICATIONS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

The chromatographic properties of ammonium acetate have been studied, and its use as a general purpose buffer for reversed-phase chromatography has been investigated. Simple ammonium acetate systems can often replace complicated and expensive buffers, with or without ion-pairing agents, with improved column selectivity and efficiency. A wide range of clinically important compounds have been successfully separated by ammonium acetate buffer systems. These include creatinine, bilirubin, verapamil and its metabolites, etoposide and teniposide, vitamin A, biogenic amines and porphyrins.

## INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) with buffered mobile phases is a powerful technique for the separation of both neutral and ionic compounds. It is the preferred method for the clinical laboratory, since most medically important compounds are water-soluble. The nature of the buffer can significantly alter the selectivity and efficiency of a column and, therefore, the resolution. This question has been investigated, and acidic amine phosphate buffers have been shown to be highly effective[1].

HPLC has great potential in clinical analysis, but the existence of many and often complicated and expensive mobile phases required for chromatographing different groups of compounds is one reason why this technique has not been more widely accepted by routine clinical laboratories. An inexpensive, simple, and easily accessible buffer with good chromatographic properties is needed.

Ammonium acetate has many properties which are particularly attractive as a general purpose buffer. It is: (1) chemically stable, non-toxic, inexpensive, and readily available; (2) fully ionized and almost neutral in water; (3) a good buffering medium in the pH range most useful for RP-HPLC; (4) highly soluble in methanol and acetonitrile; (5) an excellent masking agent for residual silanol groups on chromatographic media leading to greatly improved separations; (6) compatible with all commonly used HPLC detectors (UV–Vis, fluorescence, electrochemical); (7) able to

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accelerate rates of proton equilibrium, important for chromatography of ionic compounds; and (8) relatively volatile and easily removed after preparative separation or when used in conjunction with a mass spectrometer. The analysis of a wide range of clinically important compounds is acheived with simple ammonium acetate buffer systems, and the superior chromatographic properties and versatility of ammonium acetate are demonstrated by selected examples of applications.

## EXPERIMENTAL

### *Materials and reagents*

Creatinine, bilirubin, retinoids and catecholamines were from Sigma (Poole, U.K.). Porphyrins were isolated from urine and faeces of porphyric patients, as previously described[2]. Verapamil and its metabolites were from Abbott Labs., (Queenborough, U.K.). Etoposide (VP16-213), Teniposide (VM-26) and α-peltatin were gifts from Dr. D. C. Ayres (Queen Mary College, London, U.K.). Their picro isomers were prepared by treating 1 mg of VP16-213 and VM-26, respectively, with 1 drop of triethylamine. Acetic acid, ammonium acetate and EDTA were AnalaR grade from BDH (Poole, U.K.). Acetonitrile and methanol were HPLC grade from Rathburn Chemicaals (Walterburn, U.K.).

### *High-performance liquid chromatography*

A Varian (Walnut Creek, CA, U.S.A.) Model 5000 liquid chromatograph was used with either a Varian UV-100 variable-wavelength detector, a Perkin-Elmer (Beaconsfield, U.K.) LS-3 fluorescence spectrometer, or a model LCA-15 electrochemical detector (EDT Research, London, U.K.). A Rheodyne 7125 injection valve was used for sample injection.

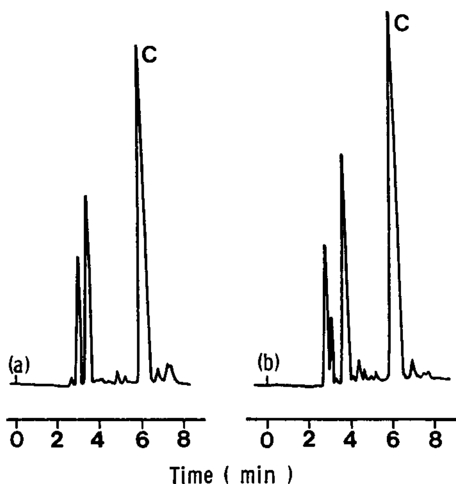
All column packing materials, ODS-Hypersil ($C_{18}$), MOS-Hypersil ($C_8$) and SAS-Hypersil ($C_1$), were from Shandon Southern Products (Runcorn, U.K.).

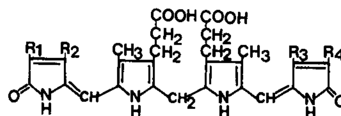
## RESULTS AND DISCUSSION

### *Creatinine*

Creatinine in serum and in urine is commonly measured in clinical laboratories as an index of renal function. Chromatograms of creatinine in serum and in urine are shown in Fig. 1. The system, with 0.1 *M* ammonium acetate as eluent, has many advantages over other HPLC methods. As ion-pairing agents and/or organic solvents [3–5] are avoided, it is cheaper. A steady capacity ratio value (k′) is obtained with ammonium acetate above 0.05 *M*. It is therefore unlikely to suffer from retention time fluctuations and is thus more reproducible.

Replacing ammonium acetate with water did not alter the retention time of creatinine, but did result in severe tailing. The greatly improved peak shape is due to the excellent masking of residual silanol groups by ammonium acetate and by its ability to accelerate proton equilibration in the chromatographic process. Acidic amine phosphate buffers have also been shown to possess properties leading to elimination of peak broadening[1].



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| IIIα | $CH{=}CH_2$ | $CH_3$ | $CH_3$ | $CH{=}CH_2$ |
| IXα | $CH_3$ | $CH{=}CH_2$ | $CH_3$ | $CH{=}CH_2$ |
| XIIIα | $CH_3$ | $CH{=}CH_2$ | $CH{=}CH_2$ | $CH_3$ |

Fig. 1. Separation of creatinine in (a) serum and (b) urine. Column, 25 cm × 5 mm ODS-Hypersil; eluent, 0.1 *M* ammonium acetate; flow-rate, 1 ml/min; detector UV 235 nm. C = creatinine.

Fig. 2. Structures of bilirubin isomers.

*Bilirubin*

The masking of accessible silanol groups and the acceleration of proton equilibrium by ammonium acetate can be further illustrated by considering the separation of bilirubin isomers (Fig. 2). The IIIα, IXα and XIIIα isomers were completely resolved when 50% acetonitrile in 0.1 *M* ammonium acetate was the mobile phase (Fig. 3a); with 50% acetonitrile in water as eluent, excessive peak broadening with the consequent loss of isomer separation was observed (Fig. 3b).

The system compares favourably to other HPLC methods for separating bilirubin isomers[6–8] and is also applicable to the determination of conjugated and unconjugated bilirubin in plasma following transesterification[9].

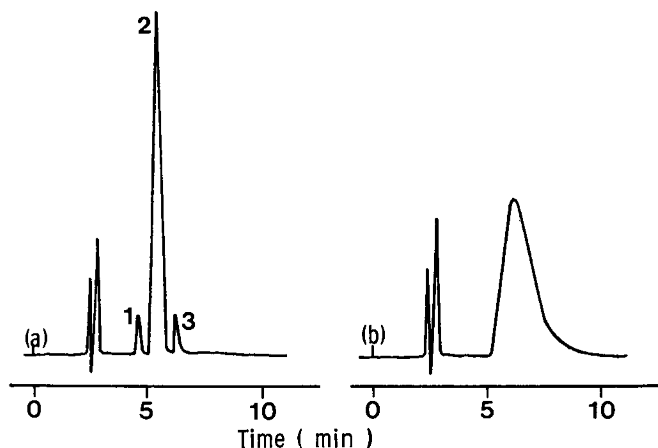



Fig. 3. Separation of bilirubin isomers. Column, 25 cm × 5 mm ODS-Hypersil; eluents: (a) 50% acetonitrile in 0.1 *M* ammonium acetate and (b) 50% acetonitrile in water; flow-rate, 1 ml/min; detector, UV-vis 450 nm. PEAKS: 1 = XIIIα; 2 = IXα; 3 = IIIα.

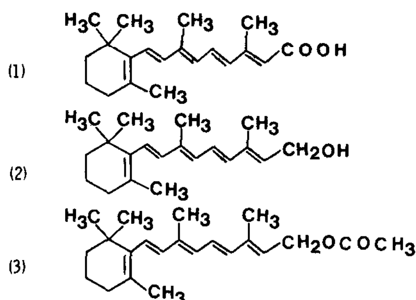



Fig. 4. Structures of retinoic acid (1), retinol (2) and retinyl acetate (3).

*Vitamin A*

The separation of retinoic acid, retinol and retinyl acetate (Fig. 4) demonstrates the ability of ammonium acetate buffers to separate simultaneously ionogenic, neutral, and strongly hydrophobic compounds. These retinoids were effectively resolved, with excellent peak symmetry, by elution with 70% acetonitrile in 0.1 *M* ammonium acetate (Fig. 5a). The system is also suitable for the estimation of vitamin A (retinol) in plasma (Fig. 5b).

*Verapamil and metabolites*

This relatively new anti-anginal, anti-arrhythmic, and antihypertensive drug (Fig. 6) was easily separable from its major metabolites norverapamil, D617 and D620 with 55% methanol in 0.1 *M* ammonium acetate as eluent (Fig. 7a). The buffer selectivity effect is evident, for, under otherwise identical conditions, 0.1 *M* sodium acetate was unable to separate verapamil from norverapamil and D617 from D620 (Fig. 7b). The most likely explanation for the altered column selectivity is effective masking of accessible silanol groups by the $NH_4^+$ of ammonium acetate.

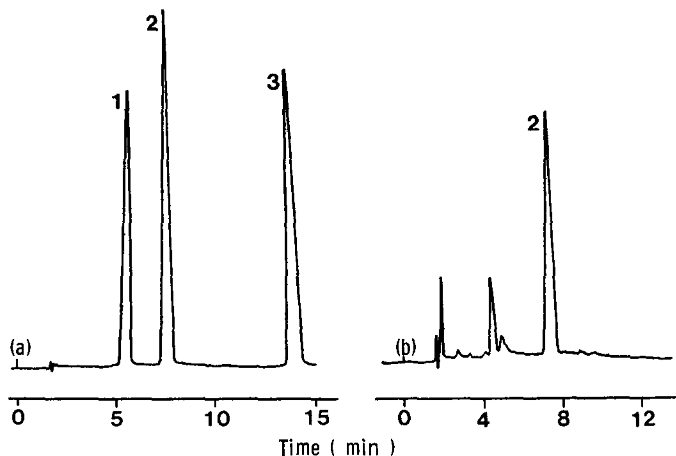



Fig. 5. Separation of retinoic acid, retinol and retinyl acetate in (a) standard solution and (b) plasma. Column, 25 cm × 5 mm MOS-Hypersil; eluent, 70% acetonitrile in 0.1 *M* ammonium acetate; flow-rate, 1 ml/min; detector, UV 330 nm. Peaks: 1 = retinoic acid; 2 = retinol; 3 = retinyl acetate.

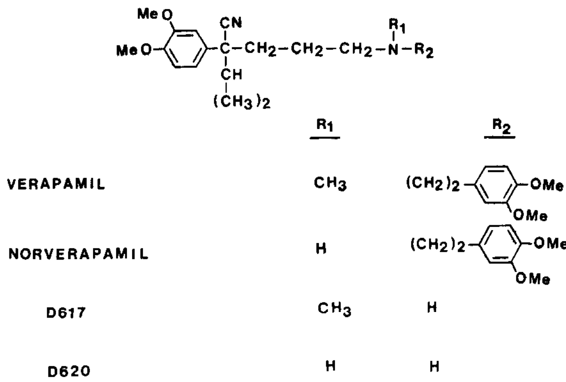



Fig. 6. Structures of verapamil and metabolites.

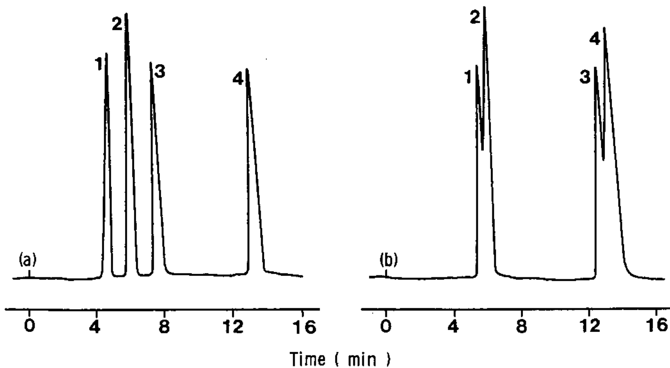



Fig. 7. Separation of verapamil and metabolites. Column, 10 cm × 5 mm MOS-Hypersil; eluents: (a) 55% methanol in 0.1 *M* ammonium acetate and (b) 55% methanol in 0.1 *M* sodium acetate; flow-rate, 1 ml/min; detector, fluorescence, ex. 278 nm and em. 320 nm. Peaks: 1 = D620; 2 = D616; 3 = norverapamil; 4 = verapamil.

*Etoside (VP16-213) and Teniposide (VM-26)*

These two anti-cancer agents (Fig. 8) are easily isomerized, in the presence of a weak base, to the pharmacologically inactive C-2 epimers (picro VP16-213 and picro VM-26[9].

It has been reported that reversed-phase chromatography on $C_{18}$-bonded columns failed to separate the picro isomers from the parent compounds, and a $\mu$Bon-

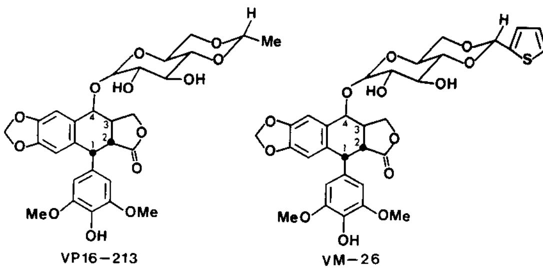



Fig. 8. Structures of Etoposide (VP16-213) and Teniposide (VM-26). ● denotes $\beta$-H configuration. The picro isomers have 2$\alpha$-H configurations.

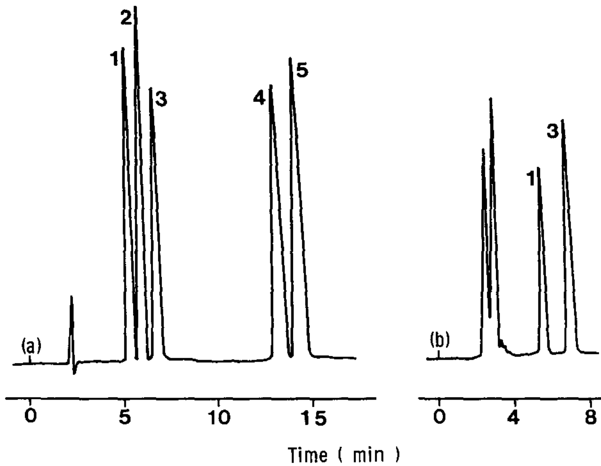



Fig. 9. Separation of VP16-213, VM-26 and their isomers in (a) standard solution and (b) serum of a patient undergoing VP16-213 chemotherapy. Column, 25 cm × 5 mm ODS-Hypersil; eluent, 37.5% acetonitrile in 0.5 *M* ammonium acetate; flow-rate, 1 ml/min; detector, electrochemical, +0.9 V. Peaks: 1 = VP16-213; 2 = picro VP16-213; 3 = α-peltatin (internal standard); 4 = VM-26; 5 = picro VM-26.

dapak phenyl column had to be used for the separation[10]. However, the inclusion of ammonium acetate in the mobile phase led to greatly improved column efficiency and resolution of the isomers was achieved (Fig. 9).

A relatively high buffer concentration (0.5 *M*) was used, as it was observed that peak symmetry improved with increasing buffer concentration. This may simply reflect the requirement for excess masking agent to compete for adsorption sites with the larger numbers of polar groups present in these molecules.

*Biogenic amines*

The catecholamines and indoleamines (Fig. 10) are usually analysed by reversed-phase ion-pair chromatography[11–14]. These rather complicated mobile phases can be replaced by a simple ammonium acetate buffer (pH 5.15). The system effectively resolves the catecholamines and their major metabolites (Fig. 11a) and is applicable to the determination of catecholamines in plasma, urine (Fig- 11b), and cerebrospinal fluid. The retention times of these compounds can be controlled by adjustments of pH and buffer concentrations.

Solvent degassing is important in the electrochemical detection of the biogenic

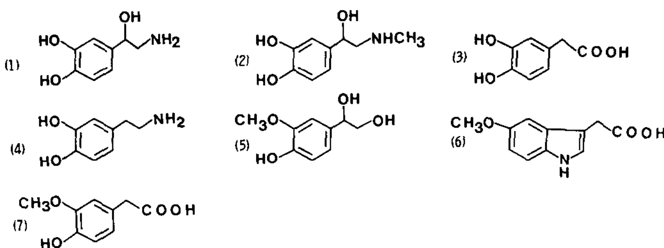



Fig. 10. Structures of biogenic amines. 1, Noradrenaline; 2, adrenaline; 3, 3,4-dihydroxyphenylacetic acid; 4, dopamine; 5, 3-methoxy-4-hydroxyphenylglycol; 6, 5-hydroxyindoleacetic acid; 7, homovanillic acid.

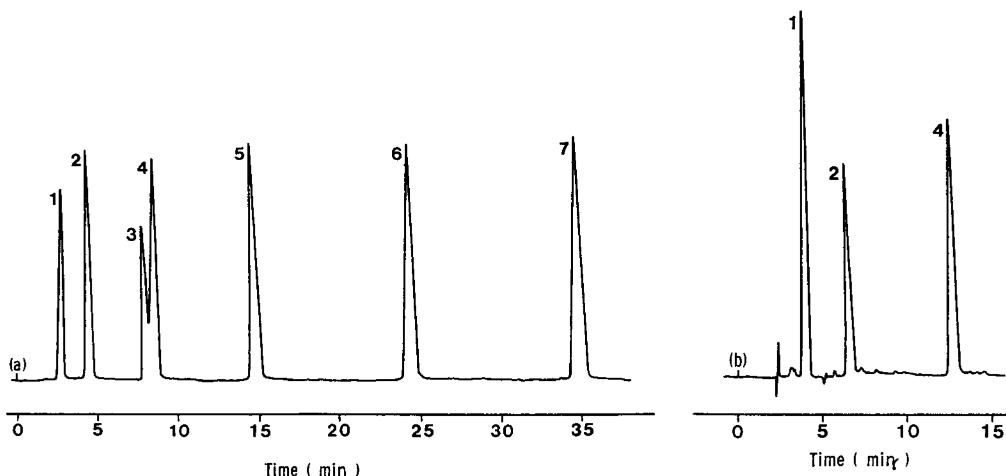



Fig. 11. Separation of biogenic amines in (a) standard solution and (b) urine of a patient with pheochromocytoma. Column, 25 cm × 5 mm ODS-Hypersil; eluent, 0.1 *M* ammonium acetate pH 5.15; flow-rate, (a) 1.5 ml/min and (b) 1.0 ml/min; detector, electrochemical, +0.65V. Peak numbers correspond to compound numbers in Fig. 10.

amines. A system free of organic solvents is therefore more reproducible, since it is not likely to suffer from gradual composition changes due to on-line solvent degassing.

*Porphyrin profiles*

The porphyrins (Fig. 12, Table I) have been separated by reversed-phase ion-pair chromatography[15–17]. These systems provide adequate separation of porphyrins into groups but are unable to resolve the isomers (Table I) important for the biochemical diagnosis of diseases.

A complete porphyrin profile (Fig. 13) is at present only possible with the acetonitrile-methanol-ammonium acetate system recently described by us[18]. A high molar concentration of ammonium acetate (1 *M*) is essential for the separation. This is obviously difficult or impossible to achieve with buffers less soluble in acetonitrile and methanol. The high buffer concentration is also advantageous as it allows acidic solutions of porphyrins to be injected directly into the column without damaging the stationary phase. This property of ammonium acetate is especially useful for the analysis of compounds which are only soluble in strong acids or alkali.

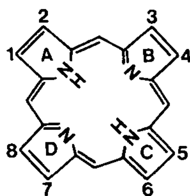



Fig. 12. Porphyrin skeleton. For structures see Table I.

TABLE I

STRUCTURES OF PORPHYRINS

Side-chain abbreviations: Me = Methyl; Et = Ethyl; V = Vinyl; PrH = $CH_2CH_2COOH$; AcH = $CH_2COOH$.

| *HPLC Peak No.* | *Porphyrins* | *Side-chain substitution pattern* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* |
| 1 | Uroporphyrin-I | AcH | PrH | AcH | PrH | AcH | PrH | AcH | PrH |
| 2 | Uroporphyrin-III | AcH | PrH | AcH | PrH | AcH | PrH | PrH | AcH |
| 3 | Heptacarboxylic porphyrin-I | AcH | PrH | AcH | PrH | AcH | PrH | Me | PrH |
| 4 | Heptacarboxylic porphyrin-III* | AcH | PrH | AcH | PrH | AcH | PrH | PrH | Me |
| 5 | Hexacarboxylic porphyrin-I* | Me | PrH | AcH | PrH | AcH | PrH | Me | PrH |
| 6,7 | Hexacarboxylic porphyrin-III* | Me | PrH | AcH | PrH | AcH | PrH | PrH | Me |
| 8 | Pentacarboxylic porphyrin-I | Me | PrH | Me | PrH | AcH | PrH | Me | PrH |
| 9–11 | Pentacarboxylic porphyrin-III* | Me | PrH | Me | PrH | AcH | PrH | PrH | Me |
| 12 | Coproporphyrin-I | Me | PrH | Me | PrH | Me | PrH | Me | PrH |
| 13 | Coproporphyrin-III | Me | PrH | Me | PrH | Me | PrH | PrH | Me |
| 14 | Deethylisocoproporphyrin | Me | H | Me | PrH | AcH | PrH | PrH | Me |
| 15 | Isocoproporphyrin | Me | Et | Me | PrH | AcH | PrH | PrH | Me |
| 16 | Mesoporphyrin | Me | Et | Me | Et | Me | PrH | PrH | Me |
| 17 | Protoporphyrin | Me | V | Me | V | Me | PrH | PrH | Me |

* Only one isomeric form is shown.

*Retention mechanisms with ammonium acetate buffers*

The most important retention mechanism with ammonium acetate buffer on reversed-phase columns is undoubtedly hydrophobic interaction between the solutes and the non-polar stationary phase surface. The retention behaviour of the retinoids,

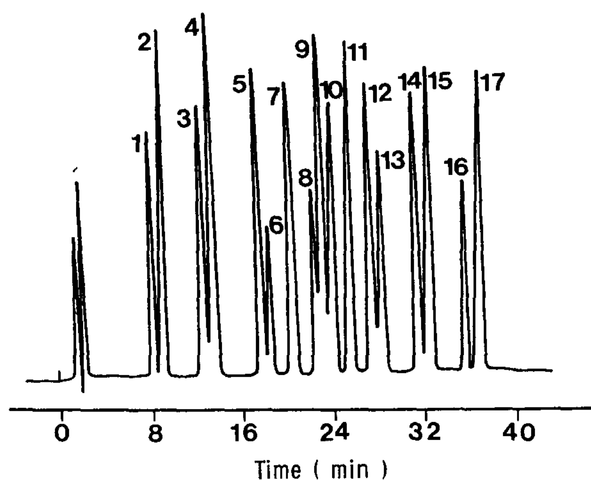



Fig. 13. Separation of porphyrins and porphyrin isomers. Column, 15 cm × 5 mm SAS-Hypersil; eluents, (A) 10% acetonitrile in 1 *M* amonium acetate pH 5.15 and (B) 10% acetonitrile in methanol; elution, 30 min linear gradient from 100% (A), 0% (B), to 65% (B) followed by isocratic elution at 65% (B) for 10 min; flow-rate, 1 ml/min; detector, UV–vis 404 nm. For peak identification, see Table I.

catecholamines, and porphyrins clearly showed that retention increased with increasing hydrophobicity of the solutes.

The hydrophobic interaction is, however, not the only basis for separation. It is known that highly polar or strongly basic molecules can interact with the free silanol functions at the surface of the stationary phase through hydrogen bonding and/or ion-pairing mechanisms[1,19]. Furthermore, basic ions extracted by the stationary phase may act as ion-exchangers. The need for high molar concentrations of ammonium acetate (0.5–1.0 *M*) for the satisfactory elution of VP16-213, VM-26 and the porphyrins demonstrates the presence of hydrogen bonding and/or ion-pairing of solutes on the stationary phase, since higher concentrations of ammonium acetate should result in more effective masking of silanol groups, thus preventing the excessive interaction with the solutes.

Ammonium acetate is also able to form ion-pairs with solutes, while the association of the $NH_4^+$ ions with the stationary phase may lead to ion-exchange properties. The retention of porphyrins, for example, decreases with increasing ammonium acetate concentrations. This observation is directly opposite from that expected in hydrophobic chromatography[20] and is consistent with an ion-exchange mechanism.

*Disadvantages of ammonium acetate*

One of the most obvious disadvantages of ammonium acetate, as of all carboxylic acid buffers, is its relatively high UV cut-off value (220 nm). However, since the great majority of clinically important compounds have absorptions above 220 nm, this disadvantage is minimal. Another limitation of ammonium acetate is that it reacts with derivatization reagents for primary amines, such as in the phenylthiohydantoin reaction for amino acids. It is thus incompatible with on-line post-column reactors when these reagents are used.

## CONCLUSIONS

(1) Ammonium acetate is an ideal general purpose buffer for RP-HPLC in clinical applications.

(2) Ammonium acetate is an excellent masking agent for accessible silanol groups of reversed-phase packings and significantly improves the chromatography of both neutral and ionogenic compounds.

(3) The ability of ammonium acetate to accelerate proton equilibrium in the chromatographic process is particularly useful for the chromatography of ionogenic compounds.

(4) Reversed-phase ion-pair eluents can usually be replaced by simple ammonium acetate systems with improved column selectivity, efficiency, and resolution.

(5) Retention of ionogenic compounds can be precisely controlled by adjusting the ammonium acetate concentration, pH, organic solvent concentration, and temperature of the mobile phase.

(6) Very high molarities of ammonium acetate can be used to achieve separations otherwise difficult or impossible.

(7) Hydrophobic mechanism dominates, but ion-pairing and ion-exchange behaviour are also observed in systems containing ammonium acetate.

(8) It is anticipated that ammonium acetate will provide excellent chromatography for a very wide range of compounds.

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REFERENCES

1 W. R. Melander, J. Stoveken and Cs. Horváth, *J. Chromatogr.*, 185 (1979) 111.
2 C. K. Lim and J. M. Rideout, *J. Liq. Chromatogr.*, 6 (1983) 1969.
3 S. J. Soldin and J. G. Hill, *Clin. Chem.*, 24 (1978) 747.
4 D. N. Buchanan, D. N. Tait and E. F. Domino, *J. Chromatogr.*, 163 (1979) 212.
5 A. Von Hausen, D. Fuchs and H. Wachter, *J. Clin. Chem. Clin. Biochem.*, 19 (1981) 375.
6 T. A. Wooldridge and D. A. Lightner, *J. Liq. Chromatogr.*, 1 (1978) 653.
7 S. Onishi, S. Itoh, N. Kawade, K. Isobe and S. Sugiyama, *Biochem. Biophys. Res. Commun.*, 90 (1979) 890.
8 C. K. Lim, R. V. A. Bull and J. M. Rideout, *J. Chromatogr.*, 204 (1981) 219.
9 N. Blanckaert, P. M. Kabra, F. A. Farina, B. E. Stafford, L. J. Morton and R. Schmid, *J. Lab. Clin. Med.*, 96 (1980) 198.
10 R. J. Strife, I. Jardine and M. Colvin, *J. Chromatogr.*, 182 (1980) 211.
11 F. Smedes, H. Poppe and J. C. Kraak, *J. Chromatogr.*, 231 (1982) 25.
12 K. Mori, *Adv. Biosci.*, 36 (1982) 275.
13 A. M. Krstulovic, *J. Chromatogr.*, 229 (1982) 1.
14 A. Allenmark, *J. Liq. Chromatogr.*, 5, Suppl. 1 (1982) 1.
15 R. Bonnett, A. A. Charalambides, K. Jones, I. A. Magnus and R. J. Ridge, *Biochem. J.*, 173 (1978) 693.
16 H. D. Meyer, K. Jacob, W. Vogt and M. Knedel, *J. Chromatogr.*, 199 (1980) 339.
17 M. Chiba and S. Sassa, *Anal. Biochem.*, 124 (1982) 279.
18 C. K. Lim and T. J. Peters, *Clin. Chim. Acta*, 139 (1984) 55.
19 M. Zakaria and P. R. Brown, *J. Chromatogr.*, 255 (1983) 151.
20 Cs. Horváth, W. Melander and I. Molnar, *Anal. Chem.*, 49 (1977) 142.