Journal of Chromatography, 249 (1982) 103–110
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM 15,216

SOME PROBLEMS ASSOCIATED WITH THE ANALYSIS OF 2-AMINOETHYLPHOSPHONIC ACID USING AUTOMATED ION-EXCHANGE CHROMATOGRAPHY

J E COCKBURN* and A P WILLIAMS
Basic Ruminant Nutrition Department, National Institute for Research in Dairying Shinfield Reading RG2 9AT (Great Britain)
(Received July 19th, 1982)

SUMMARY

Amino acid analysis of 2-aminoethylphosphonic acid (2-AEP) under the conditions normally used for the analysis of acidic amino acids in protein hydrolysates resulted in the elution of 2-AEP as two incompletely resolved peaks Evidence is provided indicating that this phenomenon was due to the formation of a degradation product or an isomer on the ion-exchange column and that the resin, buffer pH and column temperature were all contributory factors.

INTRODUCTION

The aminophosphonate 2-aminoethylphosphonic acid (2-AEP) is a constituent of rumen ciliate protozoa[1–3], and has been used as a rumen protozoal marker (for the determination of the total protein in ruminant digesta which is derived from the rumen ciliate population) The concentration of 2-AEP in ruminant digesta has been determined by manual[4–6] and by automated[7–11] ion-exchange chromatography. During a study of these methods, difficulties were encountered because 2-AEP was eluted as two peaks from the ion-exchange column of an automatic amino acid analyser under the conditions normally used for the analysis of protein hydrolysates This paper describes an investigation into the possible causes of the twin peak phenomenon experienced with 2-AEP and illustrates some difficulties in attempting to quantify this compound using automated ion-exchange chromatography.

EXPERIMENTAL

A Jeol 5 AH amino acid analyser (Japan Electron Optics Laboratory, Tokyo, Japan) was used with a single column (50.0 × 0.6 cm) of LCR1 resin. The flow-rate of the eluting buffer through the column was kept constant at 0 83 ml/min throughout this investigation. Initially the column was eluted with 0.2 *N* sodium citrate buffer at pH 3 25 and a temperature of 35°C but in subsequent experiments the column temperature and buffer pH were changed. 2-AEP was obtained from Calbiochem and

0021-9673/82/0000–0000/$02 75

Sigma. Phosphonoalanine (2-amino-3-phosphonopropionic acid; PAL), 2-amino-4-phosphonobutyric acid (APB) and 1-aminoethylphosphonic acid (1-AEP) were all obtained from Calbiochem Taurine (Tau), cysteic acid (CysA), and phosphoethanolamine (PEA) were all obtained from BDH. For the infrared (IR) spectroscopy a Perkin-Elmer Model 197 spectrometer was used. For the Fourier transmission nuclear magnetic resonance (FTNMR) spectroscopy, a Jeol PFT-100P spectrometer was used.

RESULTS AND DISCUSSION

Under the conditions normally used for the elution of acidic amino acids in protein hydrolysates (0.2 *N* sodium citrate at pH 3.25, column temperature 35°C) a standard 2-AEP (Calbiochem) solution (1.0 μmole/ml) was eluted as two unresolved peaks (see Fig. 1). Other batches of 2-AEP from Calbiochem and from another source (Sigma) were analysed using the same conditions and similar chromatograms were obtained. Similar results were also obtained when the 2-AEP (Sigma) was analysed on LKB, Model 4102 (LKB, Biochrom, Cambridge, Great Britain) and Biotronik LC 2000 (Biotronik, Munich, G.F.R.) amino acid analysers It was possible that AEP from both sources contained an impurity or more than one isomeric form of AEP[12] but examination by IR spectroscopy showed that the different sources of 2-AEP, obtained from Calbiochem and Sigma, gave identical spectra which closely matched previously reported spectra from the β-form of AEP[12]. This was confirmed by FTNMR spectroscopy when ^{13}C, ^{31}P and ^{1}H spectra indicated that there were only two carbon species visible with no traces of impurities or isomers.

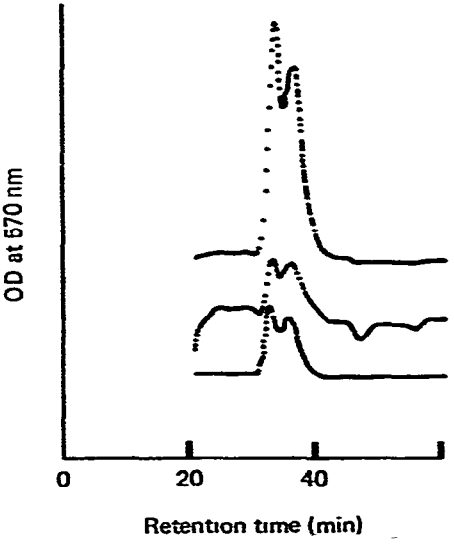

Fig. 1. Elution behaviour of 2-aminoethylphosphonic acid under the conditions normally used for the elution of acidic amino acids using automated ion-exchange chromatography; see text for analytical conditions.

The effect of column temperature on the resolution of AEP

The composition and pH of the eluting buffer was kept constant (0 2 *N* sodium citrate at pH 3 25) but the column temperature was increased in steps of 5°C over the range 30–70°C. One object of this work was to improve the resolution of the two peaks so that they could be fractionated and identified. The results obtained are given in Table I and show that 2-AEP was eluted as two incompletely resolved peaks at all column temperatures. Resolution of the two peaks (peaks 1 and 2) improved with increasing temperature but they were never completely resolved The area of peak 2 increased with increasing column temperature and the area of peak 1 decreased (see Fig 2) suggesting that peak 2 was a decomposition product formed during chromatography. The retention times of the two peaks were not markedly affected by increasing column temperature and the resolution was not improved substantially

TABLE I

THE EFFECT OF COLUMN TEMPERATURE ON THE RESOLUTION OF AEP DURING AMINO ACID ANALYSIS

See text for analytical conditions

Column temperature (°C)	*% Total peak area of peaks 1 and 2*		*Retention time (min)*	
	Peak 1	*Peak 2*	*Peak 1*	*Peak 2*
30	70	30	36	40
35	76	24	36	40
40	71	29	34	40
45	64	36	33	38
50	57	43	34	38
55	45	55	36	40
60	39	61	36	40
65	36	64	34	39
70	22	78	36	40

The effect of buffer pH

Since the best resolution of peaks 1 and 2 was obtained at the higher temperatures (see Fig. 2) in this study, column temperature was kept constant at 65°C and the pH varied over the range 2.14–3.50. 2-AEP was eluted as a single symmetrical peak in the pH range 2.14 to 2 50 but between pH 2.96 and 3 50 it was eluted as two peaks The area of peak 2 was always greater than that of peak 1 but did not appear to increase with increasing pH (see Table II) The best resolution of the two peaks, at 65°C, occurred when the pH of the eluting buffer was 3 19 or 3 50 with pH 3 19 giving the slightly better resolution and this is in agreement with the result of the study where column temperature was varied and the pH held at 3.25 (see Table I). Although at pH lower than 2.50 only one peak was observed for 2-AEP, it is possible that, under these conditions, decomposition may still occur but that the two peaks are eluted together. If both peaks have the same colour yield with ninhydrin it should still be possible to estimate 2-AEP accurately under these conditions. Using 0 2 *N* sodium citrate buffer at pH 2.10, 8 replicate analyses of 2-AEP (1.25 μmole/ml) were carried

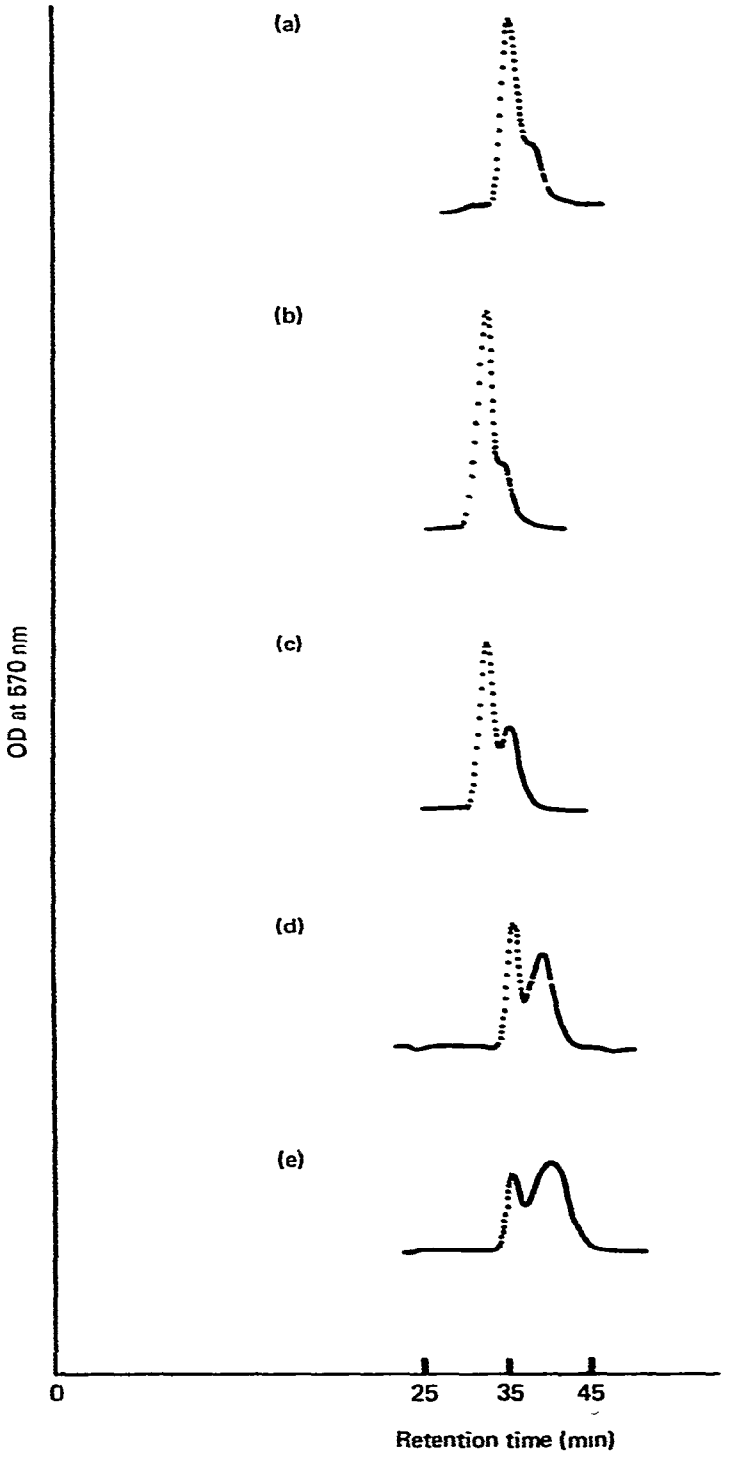

Fig. 2. The effect of column temperature on the elution of 2-aminoethylphosphonic acid from the ion-exchange column of the Jeol 5AH amino acid analyser; see text for analytical conditions (a) 30°C, (b) 40°C, (c) 50°C, (d) 60°C, (e) 70°C.

TABLE II

EFFECT OF THE pH OF THE ELUTING BUFFER ON THE RESOLUTION OF 2-AEP AT A COLUMN TEMPERATURE OF 65°C

The column (50 × 0 6 cm LCRI resin) was eluted with 0 2 *N* sodium citrate buffer at a flow-rate of 0 83 ml/min

pH of eluting buffer	*Retention time (min) of AEP*		*% of total peak area of peaks 1 and 2*	
	Peak 1	*Peak 2*	*Peak 1*	*Peak 2*
2 14		59	single symmetrical peak	
2 30		54		
2 46		52		
2 50		50		
2 96	45	46	37	63
3 09	42	44	32	68
3 19	40	42	29	71
3 26	34	39	35	65
3 39	35	39	44	56
3 50	32	37	42	58

out at a column temperature of 35°C A single symmetrical peak was obtained in each case and the mean (±S E) peak area was 0 430 ± 0 029 (see Fig 3)

Using the same conditions a standard mixture of amino acids which could possibly interfere with the resolution of 2-AEP was analysed The standard mixture contained CysA, PEA, PAL, APB and 1-AEP, all at a concentration of 0 125 μmole/ml, TAU at 0 05 μmole/ml and 2-AEP at 2 5 μmole/ml 2-AEP was well resolved from all the amino acids present but APB was not resolved from TAU and 1-AEP from PEA under these conditions (see Table III) Replacing the sodium citrate buffer with lithium citrate of similar pH and equivalent lithium ion concentration (0 2 *N*) resulted in an improvement in the resolution of these four amino acids and generally decreased the retention time of all the peaks (Table III). The precision of replicate analyses, using the lithium buffer system, is given in Table III Variation between analyses was small and a marked improvement on determinations using buffer at higher pH

IR and FTNMR spectroscopy studies

Attempts were made to characterize the decomposition products of 2-AEP chromatographed at pH 3 25 and 35°C using IR and FTNMR spectroscopy but the two peaks were never sufficiently resolved to obtain pure fractions of each component even at higher column temperature The presence of buffer citrate in the final fractions interfered with the IR spectroscopy. Attempts at desalting the fractions were also unsuccessful because the samples became contaminated with ammonia which also interfered with the spectroscopy. Since the 2-AEP used in these experiments was pure and in only one isomeric form, the two peaks appearing on the chromatogram from the amino acid analyser were probably formed as a result of decomposition or isomerisation on the ion-exchange column. To test this hypothesis samples of 2-AEP were placed in NMR tubes together with (a) the buffer (0 2 *N* sodium citrate) pH

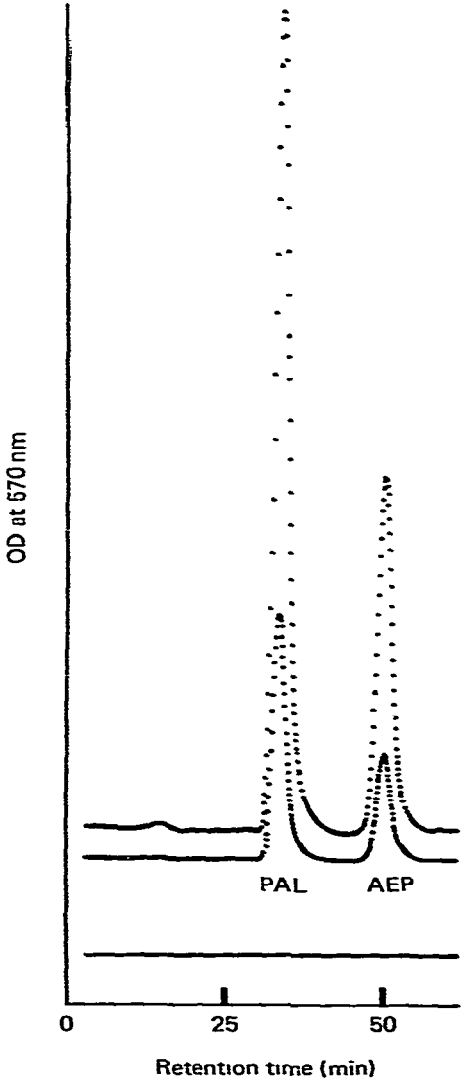

Fig. 3 Resolution of 2-aminoethylphosphonic acid (AEP) during amino acid analysis using optimum conditions for its resolution (0.20 *N* sodium citrate, pH 2.10, column temperature 35°C). The chromatogram also includes phosphonoalanine (PAL).

3.25 originally used and (b) the buffer plus the ion-exchange resin used (Jeol LCR1). Both samples were kept at 35°C, the normal column temperature, in the FTNMR spectrometer overnight and ^{31}P spectral changes were observed. With the buffer alone, no decomposition or changes occurred, but with the buffer plus resin, two peaks were observed, the minor one increasing to about 30% of the total. This again suggested that the formation of two peaks during amino acid analysis could be due to decomposition or isomerisation on the ion-exchange column.

TABLE III

RETENTION TIMES AND PRECISION OF DETERMINING SOME ACIDIC AMINO ACIDS AND AMINOPHOSPHATES INCLUDING 2-AMINOETHYLPHOSPHONIC ACID BY AMINO ACID ANALYSIS USING SODIUM AND LITHIUM CITRATE BUFFER SYSTEMS

See text for analytical details ND = not determined

Amino acid	*Retention times (min)*			*Peak areas (mean ± S.E.) Buffer system*	
	Concentration (μmole/ml)	*Buffer system*			
		Sodium	*Lithium*	*Sodium*	*Lithium**
Cysteic acid	0.125	18	15	ND	2.54 ± 0.062 (5)
2-Amino-4-phosphonobutyric acid	0.125	22	18	ND	2.40 ± 0.021 (5)
Taurine	0.05	32	23	ND	0.78 ± 0.022 (5)
Phosphonoalanine	0.125	33	25	ND	2.70 ± 0.019 (5)
1-Aminoethylphosphonic acid	0.125	30	28	ND	2.08 ± 0.108 (4)
Phosphoethanolamine	0.125	30	30	ND	1.08 ± 0.108 (4)
2-Aminoethylphosphonic acid	2.50	55	50	0.860 ± 0.058 (5)*	1.29 ± 0.012 (5)

* Value in parentheses represent the number of determinations

CONCLUSIONS

Although there have been no other reports of the appearance of two peaks on the chromatogram during automated amino acid analysis of 2-AEP it is not a unique phenomenon. Perhaps one of the best known examples is the oxidation of methionine to methionine sulphoxide and methionine sulphone when the antioxidant, thiodiglycol, is omitted from the eluting buffer[13]. There are two isomeric forms of methionine sulphoxide and these appear as two, usually incompletely resolved peaks just before aspartic acid on the chromatogram. Since methionine itself is eluted much later the conversion is clearly very rapid and very dependent on the level of thiodiglycol. N-Ethylmaleimide (NEM) has been used extensively for the modification of proteins and its condensation product with the thiol group of cysteine (cysteine-NEM) has been shown to appear as two peaks when analysed by automatic amino acid analysis (0.2 *M* sodium citrate pH 3.25, 50°C) due to the formation of diastereoisomeric forms[14,15]. The principal free amino acid of fenugreek seed, (2*S*,3*R*,4*R*)-4-hydroxyisoleucine, has been shown to become a mixture of its isomers in acid buffer (pH 2.2) with the result that three peaks appear on the chromatogram during amino acid analysis[16]. However, the formation of isomers may not be the only reason for the multiple peak phenomena. For example arginosuccinic acid (ASA), an amino acid excreted in the urine of mentally defective children is converted to two anhydrides in weakly acid aqueous solution and consequently eluted as three well separated peaks during amino acid analysis[17]. This conversion to anhydrides increased with column temperature in a manner similar to that observed for 2-AEP (Fig. 2). The peptide γ-L-glutamyl-L-aspartic acid has also been reported to elute as two peaks during amino acid analysis[18] but this was attributed to the loading of similar quantities of sample dissolved in different volumes of eluent buffer (0.2–1.4 ml) followed by 1.0 ml of 0.01 *N* HCl. The use of organic buffers such as N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES) has also been shown to result in the elution of aspartic acid (ASP) as two peaks and also to affect other amino acids in the same region[19]. Distortion of ASP associated with sample pH has also been recorded when using lithium buffer systems[20,21].

Clearly care is needed when interpreting chromatograms which contain uncommon amino acids or peptides. Previous workers analysing 2-AEP may have failed to observe the peak doubling effect although the presence of a second peak close to 2-AEP has often been reported and usually attributed to the presence of an unknown interfering amino acid in hydrolysate samples. However it is surprising that two peaks have not been observed for standard solutions of 2-AEP in those studies in which the buffer pH was close to pH 3.25 and the column temperature close to 35°C[7,22–27] normally used for the analysis of protein hydrolysates. Other workers[8–11,28] have used low pH (<3.10) to achieve better resolution of 2-AEP, conditions under which 2-AEP would probably have been eluted as a single peak so that "peak doubling" would not have been observed. The present study showed that 2-AEP was eluted as two peaks from the ion-exchange column at pH 3.25 and that the ion-exchange resin itself as well as the pH of the eluting buffer and the column temperature were all contributory factors. Whether the second peak was due to the formation of an isomer or a decomposition product was not established.

ACKNOWLEDGEMENTS

We would like to thank Dorothy Knight (NIRD, Reading) for additional amino acid analysis on the LKB 4102 analyser; Dr. D. J. Manning (NIRD, Reading) for the IR spectroscopy studies and Dr. I. K. O'Neill (Laboratory of the Government Chemist, London) for the FTNMR studies.

REFERENCES

1 M. Horiguchi and M. Kandatsu, *Nature (London)*, 184 (1959) 901.
2 M. Horiguchi and M. Kandatsu, *Bull. Agr. Chem. Soc. Jpn*, 24 (1960) 565.
3 R. M. C. Dawson and P. Kemp, *Biochem. J.*, 105 (1967) 837.
4 A. R. Abou Akkada, D. A. Messmer, L. A. Fina and E. E. Bartley, *J. Dairy Sci.*, 51 (1967) 78.
5 J. W. Czerkawski, *J. Sci. Food Agr.*, 25 (1974) 45.
6 K. El-Shazly, A. M. Nour and A. R. Abou Akkada, *Analyst (London)*, 100 (1975) 263.
7 E. A. Ibrahim, J. R. Ingalls and D. B. Bragg, *Can. J. Anim. Sci.*, 50 (1970) 397.
8 J. Hagemeister, *Kieler Milchw. Forschber.*, 27 (1975) 347.
9 J. R. Ling and P. J. Buttery, *Brit. J. Nutr.*, 39 (1978) 165.
10 C. B. Theurer, in F. N. Owen (Editor), *Protein Requirements For Cattle Symposium 1980*, Oklahoma State University, Stillwater, OK, 1982, p. 10.
11 G. S. Dufva, E. E. Bartley, M. J. Arambel, S. J. Galitzer and A. D. Dayton, *J. Anim. Sci.*, 54 (1982) 837.
12 M. Horiguchi and M. Kandatsu, *Agr. Biol. Chem.*, 28 (1964) 408.
13 S. Moore and W. H. Stein, *J. Biol. Chem.*, 192 (1951) 663.
14 D. G. Smyth, O. O. Blumenfeld and W. Konigsberg, *Biochem. J.*, 91 (1964) 589.
15 L. Bowie, J. C. Crawhall, N. Goochman, K. Johnson and J. A. Schneider, *Clin. Chim. Acta* 68 (1976) 349.
16 R. Hardman and I. M. Abu-Al Futuh, *Planta Medica*, 36 (1979) 79.
17 D. C. Cusworth and R. G. Westall, *Nature (London)*, 192 (1961) 555.
18 T. Kasai and S. Sakamura, *J. Chromatogr.*, 103 (1975) 189.
19 K. W. Joy, C. Shay and M. J. McLimont, *J. Chromatogr.*, 194 (1980) 76.
20 A. Mondino, G. Bongiovanni and S. Fumero, *J. Chromatogr.*, 71 (1972) 363.
21 Y. Houpert, P. Tarallo and G. Siest, *J. Chromatogr.*, 115 (1975) 33.
22 S. C. Chou and O. H. Sherbaum, *Exp. Cell Res.*, 45 (1966) 31.
23 T. Hori, I. Arakawa and M. Sugita, *J. Biochem. (Tokyo)*, 62 (1967) 67.
24 J. A. Alhadeff and Daves, G. D., *Biochemistry*, 9 (1970) 4866.
25 R. I. Mackie, *J. Dairy Sci.*, 56 (1973) 939.
26 E. D. Korn, D. G. Deorborn, J. M. Faies and E. A. Sokolaski, *J. Biol. Chem.*, 248 (1973) 2257.
27 D. S. Kirkpatrick and S. H. Bishop, *Biochemistry*, 12 (1973) 2829.
28 B. L. Roop and W. E. Roop, *Anal. Biochem.*, 25 (1968) 260.