Journal of Chromatography, 303 (1984) 185–189
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,062

Note

Separation of nucleobases on polar amino cyano high-performance liquid chromatography columns

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(First received November 11th, 1982; revised manuscript received July 2nd, 1984)

The chromatography of nucleic acid constituents has been reported in numerous articles and has been the subject of various reviews[1–3]. This reflects the importance of these biological compounds in the clinical study of normal and defective metabolisms. Some recent examples of nucleic acid constituent separations by reversed-phase high-performance liquid chromatography (HPLC) include those of R. J. Simmonds and R. A. Harkness[4], M. Ryba[5] and M. W. Dong and J. R. Gant[6]. A systematic study of the separation of nucleobases, nucleosides and nucleotides by ion-exchange chromatography was reported by P. J. M. van Haastert[7] while M. Ryba and T. Beranek[8] reported on normal-phase separation of purines, pyrimidines and their nucleosides on silica gel columns.

In the examination of fermentation broth extracts for guanine and hypoxanthine, we found the use of isocratic reversed-phase HPLC unsatisfactory due to broth components which could not always be resolved from the compounds of interest and due to the presence of late-eluting components; these considerable amounts of lipophilic entities also precluded the use of aqueous ion exchange. Furthermore, the general nature of our samples also counterindicated the use of normal phase chromatography on silica gel due to the presence of highly polar components.

These considerations led us to examine the applicability of the polar amino cyano (PAC) bonded-phase column packing*. We found that isocratic elution of the PAC column with buffered eluents consisting of 8% (v/v) water and 92% (v/v) acetonitrile gave excellent resolution of the seven common nucleobases. We also found that we could use eluent "pH" changes to affect the selectivity and capacity factors for the nucleobases. These effects were especially pronounced for xanthine.

EXPERIMENTAL

Experiments were performed using a LDC Constametric IIIG pump, a Micromeritics Model 725 automatic injector equipped with an AURA Industries MVI interface and a Valco electrically actuated 6-port valve with a 15-μl sample loop. A

* A normal-phase separation mechanism may be operative with this packing since we found that increasing the water content of the eluent decreases the retention times for the compounds examined in this study.

0021-9673/84/$03.00

Kratos Spectroflow 773 absorbance detector was used at 254 nm and 0.04 a.u.f.s. A Spectra-Physics SP 4100 recording integrator plotted chromatograms and determined retention times. Data points were obtained in triplicate and were found to be highly reproducible. A Whatman polar amino cyano column (Partisil PXS 5/25 PAC, 25 cm × 4.6 mm, 5-μm particle size) was used equipped with an Upchurch C-130 precolumn packed with Whatman Co-Pell PAC 30–38 μm particles. The temperature of the column and precolumn was maintained at 40°C with a constant temperature water jacket. The acetonitrile was HPLC grade (Fisher Scientific) while the water was purified prior to use by Millipore Milli RO-4 and Milli Q water purification systems.

The buffer components were as follows: cyanoacetic acid, Matheson Coleman and Bell; trifluoroacetic acid (TFA), J. T. Baker; acetic acid, Fisher Scientific; tris-(hydroxymethyl)aminomethane (Tris), Calbiochem; morpholinopropanesulfonic acid (MOPS) Calbiochem-Behring; triethylamine (TEA), Eastman Kodak.

The buffered eluents were prepared as illustrated for the cyanoacetic acid–TEA eluent. To 100 ml of 0.5 *N* cyanoacetic acid in acetonitrile–water (1:1) was added 50 ml 0.5 *N* TEA in acetonitrile–water (1:1). The mixture was brought up to 160 ml volume with acetonitrile–water (1:1) and added to 840 ml acetonitrile. After mixing, the eluent was degassed by vacuum filtration through a 1–2-μm pore size polytetrafluoroethylene filter membrane.

The seven nucleobases were purchased from Sigma and will be abbreviated as follows: adenine, Ade; cytosine, Cyt; guanine, Gua; hypoxanthine, Hyp; thymine, Thy; uracil, Ura; xanthine, Xan.

The injection standard solution of the bases was prepared as follows: Thy, Ura, Ade, Cyt, Hyp, Xan and Gua, 5.0, 6.0, 20, 24, 22, 100 and 100 mg each, respectively, were added to 500 ml water–acetonitrile (1:2) and sonicated for 1 h. The resultant solution was filtered and stored at room temperature. For each buffered eluent, a series of 3-ml aliquots of the above solution was transferred to test tubes, solvent removed and replaced with the same amount of buffered eluent. The test tubes were sonicated and aliquots transferred to sample vials of the Micromeritics injector. The preparation of fermentation broth extracts was as follows: 40 ml methanol was added to a 40 ml (nominal) fermentation broth and thoroughly mixed and

TABLE I

THE pK_a VALUES AND THE pH OBSERVED IN ACETONITRILE–WATER (1:1) FOR THE BUFFERS

Buffer	*pK_a in water*	*Observed pH****
Cyanoacetic acid (+ TEA)	2.45*	3.32
Acetic acid (+ TEA)	4.75**	5.73
MOPS (+ TEA)	7.20**	7.12
Tris (+ TFA)	8.30**	8.12

* From ref. 9.

** From ref. 10.

*** Beckman combination electrode Model 39505; Leeds and Northrup Model 7411 pH meter; using a mixed solution of 100 ml of 0.5 *N* buffer and 50 ml of 0.5 *N* TEA (or TFA), both in acetonitrile–water (1:1).

centrifuged. To one part of the supernatant was added one part of buffered eluent; the hypoxanthine spiked sample was prepared by mixing one part of supernatant and one part of buffered eluent containing 5.4 μg/ml Hyp.

RESULTS

The pK_a and pH values in water-acetonitrile (1:1) of the buffers for the eluents used to chromatograph the nucleobases are listed in Table I. Table II lists the capacity

TABLE II

CAPACITY FACTORS (k') FOR THE SEVEN NUCLEOBASES IN THE FOUR BUFFERED ELUENTS

Nucleobase	*Buffer*			
	Cyanoacetic acid + TEA	*Acetic acid + TEA*	*MOPS + TEA*	*Tris + TFA*
THY	0.60	0.56	0.70	0.73
URA	0.72	0.70	0.95	0.98
ADE	2.25	2.01	2.44	2.80
CYT	3.56	3.58	5.93	5.32
GUA	5.40	5.17	8.70	8.43
HYP	2.48	2.42	3.21	4.93
XAN	1.65	2.27	3.21	13.74

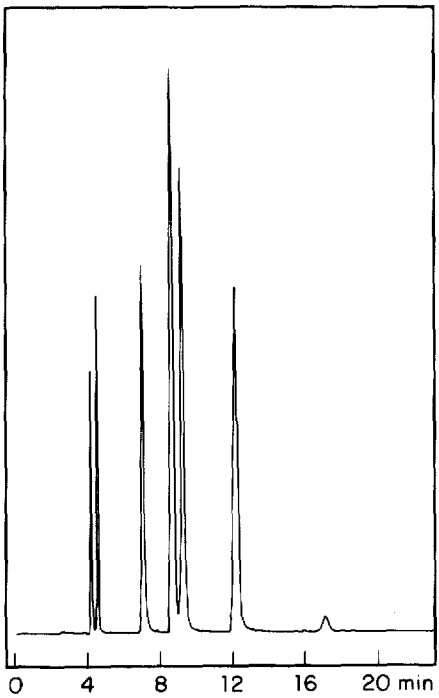

Fig. 1. Chromatogram of the nucleobases using the cyanoacetate-buffered eluent at 1 ml/min. In order of elution THY, URA, XAN, ADE, HYP, CYT, GUA.

factors (k') observed for the nucleobases in each of the four eluents. Fig. 1 and Fig. 2 are chromatograms of the nucleobases in the cyanoacetate-buffered eluent and the Tris-buffered eluent respectively. Fig. 3A and B are chromatograms of a fermentation broth extract, respectively as is and spiked with hypoxanthine.

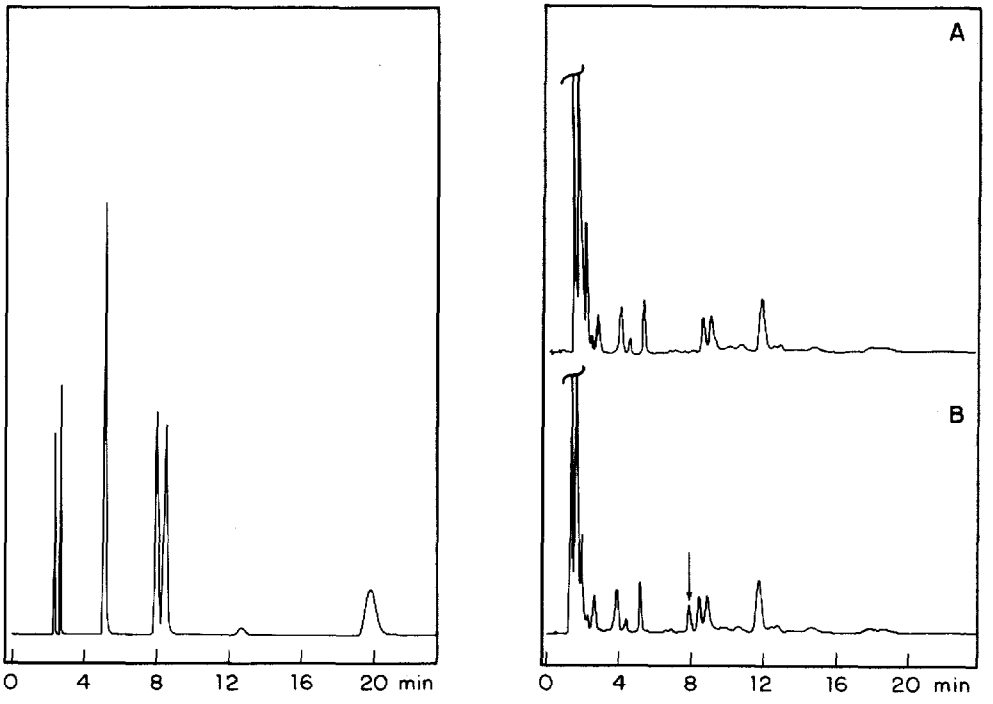

Fig. 2. Chromatogram of the nucleobases using the Tris-buffered eluent at 2 ml/min. In order of elution THY, URA, ADE, HYP, CYT, GUA, XAN.

Fig. 3. Chromatogram of a fermentation broth extract using the Tris-buffered eluent at 2 ml/min. (A) Fermentation broth; (B) fermentation broth spiked with HYP.

DISCUSSION

The use of a polar amino cyano HPLC column in conjunction with buffered water–acetonitrile (8:92) eluents affords a viable alternative to the standard reversed-phase, silica gel and ion-exchange methods for the chromatographic separation of nucleobases. The PAC method provides baseline separations and the ability to change k' and α values by the appropriate choice of buffers. The high organic content allows for the facile removal of solvent for preparative isolation work and the rapid elution of lipophilic components. The excellent separations coupled with low k' values and freedom from late eluting components obviates the need for gradient elution with its ancillary expense, complications and need for reequilibration.

The capability of dramatically changing the k' value of xanthine by varying the buffer components of the eluents may make this method especially attractive in the analysis of caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxan-

thine) and related compounds important in the food industry. Xanthine is the only nucleobase of the seven used in this study sufficiently acidic (pK_a 7.72) to be in its anionic form within part of the pH range covered by the buffers in the four elements. Experiments are now in progress to confirm whether the high susceptibility of xanthine's k' and α values to buffer changes are related to its pK_a.

ACKNOWLEDGEMENT

We thank Dr. William VandenHeuvel of these laboratories for his encouragement and helpful discussions.

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