Journal of Chromatography, 196 (1980) 319–322
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,832

Note

Fast determination of free amino acids by ion-pair high-performance liquid chromatography using on-line post-column derivatization

MOHAMMAD K. RADJAI and RANDOLPH T. HATCH*

Laboratory for Biochemical Engineering and Environmental Studies, University of Maryland, College Park, MD 20742 (U.S.A.)

(First received July 2nd, 1979; revised manuscript received March 14th, 1980)

Fast and simple determination of free amino acids has been sought by many researchers. The standard procedure for separation and determination of amino acids by ion-exchange chromatography[1] using ninhydrin[2] and fluorescamine[3] is limited by the low flow-rate of the mobile phase and therefore cannot be used for quick sample analysis. The thin-layer chromatographic analysis of the highly fluorescent 1-N,N'-dimethylaminonaphthalene-5-sulfonyl (Dns) derivatives of amino acids has been reported extensively[4–7]. The strong fluorescence of this derivative has enabled detection at concentrations of at least four to five orders of magnitude lower than is possible with the ninhydrin reaction.

The use of modern high-performance liquid chromatography (HPLC) provided a turning point in the analysis of amino acids. This technique was more efficient, easier to use and offered faster analysis times (due to higher flow-rates) than the conventional ion-exchange techniques. Dns derivatives of amino acids[8,9] and phenylthiohydantoin (PTH) amino acids[10] have been successfully separated using HPLC. Post-column derivatization by *o*-phthalaldehyde–2-mercaptoethanol in conjunction with HPLC has also been investigated[11]. The advantage of this technique is that *o*-phthalaldehyde–2-mercaptoethanol reacts specifically with primary amines and hence it is very useful in analysis of amino acids in complex fermentation broths. Its disadvantage, however, lies in its long elution times. As an alternative, ion-pair chromatography can be used with significant reduction in elution times.

In the present paper, ion-pair chromatography is used to analyze some of the most common free amino acids utilizing on-line post-column derivatization by *o*-phthalaldehyde–2-mercaptoethanol. The advantage of this technique is two-fold: (1) no pretreatment of the sample is required, (2) significant improvement in elution time is realized.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph was used. The unit consisted of two Type 6000A piston pumps, a Model 396-31 Milton-Roy Minipump (an empty column was used as a pulse dampener), a Type U6K injector,

0021-9673/80/0000–0000/$02.25

a 30 cm × 3.0 mm μBondapak C_{18} column, a Model 440 UV detector and a Hewlett-Packard Model 7132A recorder.

Chemicals

Analytical grade glacial acetic acid and methanol were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Acetonitrile was purchased from Waters Assoc. The sodium salt of heptanesulfonic acid, sodium decyl sulfate, *o*-phthalaldehyde and 2-mercaptoethanol were obtained from Eastman-Kodak (Rochester, NY, U.S.A.). All chemicals were free of UV-absorbing materials, and were used without further purification.

Preparation of the solvents

Eluent A was prepared by dissolving 0.7 g sodium decyl sulfate and 5 ml of glacial acetic acid in 1 l of deionized water. Eluting solvent B was made by adding 1.2 g heptanesulfonate and 2.5 ml of glacial acetic acid to 697.5 ml of deionized water; 200 ml of acetonitrile and 100 ml of methanol were then mixed in to make up a total volume of 1 l. Both eluents were filtered through a 0.3-μm filter.

The *o*-phthalaldehyde solution was made as recommended by Roth and Hampai[11] as follows. A 25-g amount of boric acid was dissolved in 950 ml deionized water, titrated with 45% KOH solution to pH 10.4 and filtered through a 0.3-μm filter. A 800-mg amount of *o*-phthalaldehyde was dissolved in 10 ml methanol by gentle swirling, and 200 μl 2-mercaptoethanol were added. The methanol–*o*-phthalaldehyde solution was added to the boric acid mixture. It must be noted that this mixture will oxidize within 24 h if it is not kept refrigerated in an amber bottle, preferably under nitrogen. In our experiments, the boric acid mixture was prepared and stocked in the refrigerator, and the methanol–*o*-phthalaldehyde solution was freshly made and added to the boric acid mixture on the day the experiment was to be run.

It is worth mentioning here that all solvents must be degassed everyday before use. This can be done by either using an ultrasonic bath or simply by micro filtration of the solvents.

Procedure

A standard solution containing 0.1 g/l each of a combination of common amino acids was prepared. A 4-μl volume of this standard was injected into the chromatograph and eluted according to the following solvent-programming steps: isocratic condition at 0% B until 2 min after injection followed by an isocratic hold at 25% B for the next 4 min, and finally a 20-min linear program from 25 to 85% solvent B. The total flow-rate of eluents A and B was set at 1.5 ml/min. The *o*-phthalaldehyde–2-mercaptoethanol derivatizer was pumped to the column effluent at a rate of 1 ml/min via a stainless-steel three-way tee. This fluorescing mixture is subsequently passed through the UV detector where the signals are detected at 340 nm and evaluated with the recorder. All experiments were performed at room temperature. A schematic diagram of the instrument is shown in Fig. 1.

RESULTS AND DISCUSSION

Alkanesulfonic acids have been proposed as protonating compounds for the ion-pair chromatography of organic bases[12]. Heptanesulfonate and sodium decyl

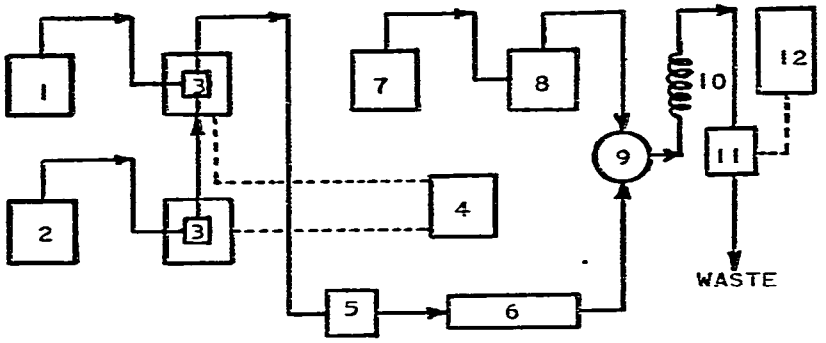

Fig. 1. Schematic diagram of the instrument: 1 and 2 = reservoirs of eluents A and B; 3 = Model 6000A pump; 4 = solvent programmer; 5 = Model U6K injector; 6 = column; 7 = post-column derivatizer reservoir; 8 = Milton-Roy minipump; 9 = three-way tee; 10 = 6 ft. Long tube: 11 = UV detector; 12 = recorder.

sulfate were used in our experiments together with acetic acid. The presence of acetic acid is primarily due to the low pH requirement for better separation of the components, and could be substituted by any other acid which does not absorb in the UV region. Small variations in the amount of acetic acid result in drastic changes in relative retentions of the amino acids. These variations are especially critical in solvent A where a pH of 2.85 was found to be optimum in separating the threonine peak from the glutamic acid and glycine pair.

Fig. 2 shows a typical chromatogram of the eighteen most common amino acids. Considering the simplicity of the method which uses direct sample injections of free amino acids without the need for time-consuming pre-derivatization steps, the short separation time of 27 min is remarkable. The order of elution under mentioned experimental conditions is as follows: aspartic acid, serine, glutamic acid and glycine (together), threonine, alanine, cystine, valine, methionine, tyrosine, isoleucine, leucine, phenylalanine, histidine, ammonia, lysine, tryptophan and arginine. From the eighteen amino acids tested, proline was not detected, because it does not react with *o*-phthalaldehyde. Glutamic acid and glycine could not be separated under these conditions. It is conceivable, however, that they could be resolved by using a higher alkanesulfate such as sodium dodecyl sulfate instead of sodium decyl sulfate used in our experiments. The concentrations of sodium decyl sulfate, 0.07%, and heptanesulfonate, 0.12%, were found to be optimum. In particular, it was observed that sodium decyl sulfate concentrations below 0.05% resulted in poor resolution of the early peaks and a total loss of the threonine peak, while concentrations above 0.08% resulted in a decrease in the relative retention time of tryptophan and its subsequent pairing with lysine. The solvent-programming profile used (refer to Fig. 2) was also found to be optimum. The isocratic condition at 0% B during the first 2 min of the run was essential for the resolution of aspartic acid, serine, glutamic acid, glycine and threonine; while the isocratic hold at 25% B over the next 4 min of the run improved the separation between the peaks for cystine, valine, and methionine. The presence of 10% methanol in eluent B improved the upward drift of the baseline, otherwise it had no other significance, and it could be replaced with more acetonitrile.

In this chromatographic system using the eluents described, 50 ng of in-

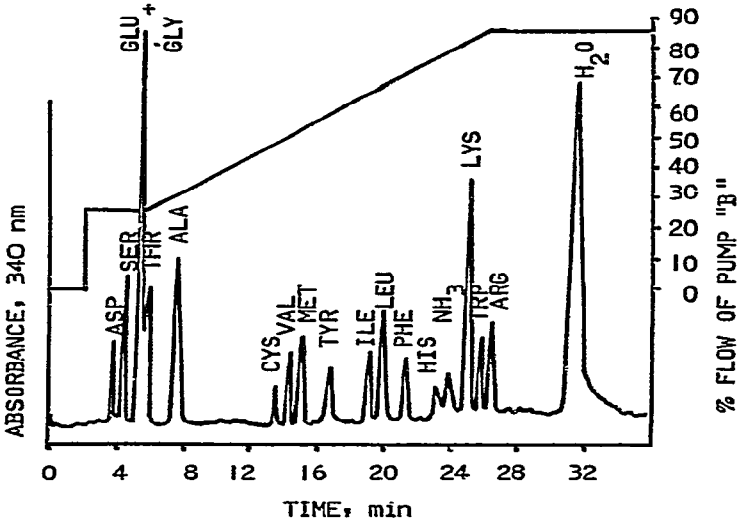

Fig. 2. Ion-pair chromatogram of free amino acid standards, 400 ng each, post-column derivatized with *o*-phthalaldehyde–2-mercaptoethanol. Conditions: solvent A, pH 2.85, 0.07% sodium decyl sulfate and 0.5% acetic acid; solvent B, 0.12% heptanesulfonate, 0.25% acetic acid, 20% acetonitrile and 10% methanol; isocratic for 2 min, then 4 min isocratic hold at 25% B, then curve No. 6 from 25 to 85% over 20 min; flow-rate of eluent 1.5 ml/min, derivatizer 1.0 ml/min; column, μBondapak C_{18} (30 cm × 3.9 mm I.D.); UV absorbance detector at 340 nm, 0.2 a.u.f.s.

dividual free amino acids can be detected at 0.1 absorbance unit full scale (a.u.f.s.). This sensitivity can be improved by two to three orders of magnitude if a fluorescence detector is used instead of the UV[13]. The concentration of the amino acid is measured by integrating the area of the chromatographic peak.

ACKNOWLEDGEMENT

The authors would like to gratefully acknowledge the National Science Foundation for support of this work under grant No. ENG 76-18480.

REFERENCES

1 D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
2 P. B. Hamilton, *Anal. Chem.*, 35 (1963) 2055.
3 S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, W. Leingruber and M. Weigele, *Science*, 178 (1972) 871.
4 Z. Deyl and J. Rosmus, *J. Chromatogr.*, 20 (1965) 514.
5 N. Seiler and M. Wiechmann, *Z. Anal. Chem.*, 220 (1966) 109.
6 J. P. Zanetta, G. Vincendon, P. Mandel and G. Gombos, *J. Chromatogr.*, 51 (1970) 441.
7 V. A. Spivak, V. V. Shcherbukhin, V. M. Orlow and J. M. Varshavsky, *Anal. Biochem.*, 39 (1971) 271.
8 H. Engelhardt, J. Asshauer, U. Neue and N. Weigand, *Anal. Chem.*, 46 (1974) 336.
9 E. Bayer, E. Grom, B. Kaltenegger and R. Uhmann, *Anal. Chem.*, 48 (1976) 1106.
10 A. Hagg and K. Langer, *Chromatographia*, 7 (1974) 659.
11 M. Roth and A. Hampai, *J. Chromatogr.*, 33 (1973) 353.
12 *Technical Note F61*, Waters Assoc., Milford, MA, 1976.
13 J. Helfrich, personal communications, Waters Assoc., Milford, MA, 1978.