Amino Acids (1992) 3: 303–309

Amino
Acids

Analysis of amino acids as DABS-derivatives with a sensitivity to the femtomole level using RP-HPLC narrow-bore columns

V. Stocchi, F. Palma, G. Piccoli, B. Biagiarelli, M. Magnani, L. Masat, and **L. Cucchiarini**

Istituto di Chimica Biologica "Giorgio Fornaini", Università degli Studi di Urbino, Urbino, Italy

Accepted December 16, 1991

Summary. In this paper we report the complete separation of amino acids as DABS-derivatives using a 3 μm Supelcosil LC-18 (25 cm × 2.1 mm I.D.) narrow-bore column. The system described makes it possible to perform the analysis of DABS-amino acids with a sensitivity to the femtomole level. We have also studied the conditions necessary for using the narrow-bore columns for routine analysis, paying particular attention to the problem of providing adequate protection for the analytical column. We have found it very suitable to use a (2 cm × 2.1 mm I.D.) guard column filled with a 40 μm Pelliguard LC-18, pellicular packing resin, without affecting the complete resolution of the DABS-amino acids. Comparing the results obtained using conventional HPLC columns (3–5 μm Supelcosil LC-18) of different lengths (15 and 25 cm × 4.6 mm I.D.) with those obtainable with the narrow-bore columns used in this work, it is possible to achieve a much greater sensitivity using the narrow-bore columns. In short, using the appropriate guard column and the "standard" HPLC apparatus used, the narrow-bore columns are very useful for routine analyses of DABS-amino acids with a sensitivity at the femtomole level.

Keywords: Amino acids – Reversed-phase high performance liquid chromatography – DABS-Amino acids – Narrow-bore columns – Amino and imino acid analysis

Introduction

The analysis of amino acids can be considered one of the most important applications which has strongly influenced the different biological fields in the last thirty years. The early separations based on ion-exchange chromatography and post-column detection with ninhydrin (Spackman, 1981) with a sensitivity at nanomole level have been used to acquire the majority of information we have

at present concerning the chemistry and biochemistry of proteins as well as the distribution of free amino acids in physiological fluids. More recently, the precolumn derivatization of amino acids followed by reversed-phase high-performance liquid chromatography (Engelhart, 1974; Bayer, 1976; Schmidt, 1979; Wilkinson, 1979; Tapuhi, 1981; Kaneda, 1982; De Jong, 1982; Negro, 1987; Benson, 1975; Cronin, 1977; Bohlen, 1982; Cooper, 1984; Allison, 1884; Chang, 1981a and b; Chang, 1982; Chang, 1983; Winkler, 1984; Chang 1984; Stocchi, 1985; Knecht, 1986; Vendrell, 1986; Hughes, 1987; Stocchi, 1989) has become the most widely used method for the analysis of amino acids, reducing the analysis time and increasing the sensitivity to the picomole level. Among the different reagents proposed for precolumn derivatization of amino acids the DABS-Cl allows the detection of the DABS-derivatives in the visible region (Chang, 1981a and b); Chang, 1982; Chang, 1983; Winkler, 1984; Chang, 1984; Stocchi, 1985; Knecht, 1986; Vendrell, 1986; Hughes, 1987; Stocchi, 1989), reacting with primary and secondary amino acids, and giving derivatives stable at room temperature (Knecht, 1986). Furthermore RP-HPLC methods have already been reported for their complete separation using standard HPLC columns at room temperature (Stocchi, 1985; Stocchi, 1989). These methods are simple, reproducible and reliable. In this paper we describe the experimental conditions for using a Supelcosil LC-18 narrow-bore column for routine analysis to achieve the complete separation of 35 DABS-amino acids and by-products including the separation of tryptophan, cysteic acid, carboxymethylcysteine, S-sulfocysteine, hydroxy-proline, methionine sulfone, methionine sulfoxide, taurine, cystine, and δ-hydroxy-lysine. The use of narrow-bore columns, under the experimental conditions described, makes it possible to significantly increase the sensitivity of amino acid analysis at the femtomole level.

Experimental

Chemicals

4-Dimethylaminoazobenzene-4′-sulphonyl chloride (DABS-Cl) was purchased from Fluka (Buchs, Switzerland). Free amino acids for the preparation of DABS-amino acids were obtained from Sigma (Lot. 96F-9000) (Sigma Chemical Company, St. Louis, Missouri, U.S.A.). Amino acid standard mixture calibrations (Pierce No. 20077 and Sigma No. A 2908) of the highest grade were obtained from Pierce and Sigma (Pierce Chemical Company, Rockford, IL, U.S.A.). Acetonitrile, methanol, and acetone of HPLC grade were obtained from Fluka. Analytical reagent-grade potassium dihydrogen-phosphate and other reagents were purchased from Merck (Darmstadt, F.R.G.). Hydrochloric acid (6N) constant boiling, sequanal grade, was obtained from Pierce. Millipore filters (0.22 μm) were obtained from Millipore (Bedford, MA, U.S.A.). Bovine serum albumin and lysozyme were purchased from Boeringer-Manneheim (West Germany). Bradykinin, β-lipotropin, insulin chain B, substance P and other peptides were obtained from Sigma. The Supelcosil LC-18 column (25 cm $\times$ 2.1 mm I.D.), 3 μm particles, and the Pelliguard LC-18, pellicular packing resin, 40 μm particles, were obtained from Supelco (Supelco, Bellefonte, Pennsylvania, U.S.A.). Triple distilled water was prepared in the laboratory and used for the preparation of buffers. The buffers used for HPLC analysis were filtered through a 0.22 μm Millipore filter.

Preparation of DABS-derivatives

DABS-Cl was recrystallized as previously described by Chang [Chang (1981), Chang (1983)]. Briefly one gram of DABS-Cl was dissolved in 100 ml of boiling acetone (HPLC

grade) and the insoluble materials were removed by passing the solution through a sintered-glass filter funnel. The solution was left to cool at room temperature and then maintained at −20°C overnight to obtain a needle-shaped crystal precipitation. The supernatant was removed by suction and the DABS-Cl crystals were dried under vacuum, using a Savant concentrator (Savant, Hicksville, NY, U.S.A.). A stock solution of DABS-Cl (1.30 mg/ml) in acetone (HPLC grade) was prepared. Aliquots (100, 200, and 500 μl) of the stock solution were pipetted into Eppendorf tubes, dried under vacuum and stored at −20°C over a period of months without appreciable degradation. Fresh DABS-Cl solution (4 nmol/μl acetonitrile) was prepared by redissolving dried DABS-Cl stored at −20°C before use. Standards of mono and bis-DABS-amino acids were prepared according to Chang et al (Chang, 1981, Chang, 1983) with slight modifications as previously described (Stocchi, 1985). Standard calibration mixtures of DABS-amino acids were prepared using Pierce or Sigma standard mixtures of free amino acids. An aliquot of these standard mixtures was diluted to obtain 5 nmol/ml of each amino acid (except cystine). This solution (0.01 ml) was pipetted into an Eppendorf tube, dried under vacuum, using a Savant concentrator, and then resuspended in 10 μl of 0.05 M sodium bicarbonate, pH 9.0 and treated with 20 μl DABS-Cl solution (4 nmol/μl acetonitrile). The mixture was heated at 70°C for 10 minutes, dried under vacuum and the residue redissolved in 500 μl of 1 : 1 (v/v) 70% (v/v) ethanol and 25 mM potassium diydrogen phosphate buffer, pH 6.8. The final concentration of each DABS-amino acid of this standard solution was 2 picomoles/5 μl, except for cystine which was 1 picomole/5 μl.

HPLC Analysis

A liquid chromatographic system from Beckman, the Gold system, (Beckman, Berkely, CA, U.S.A.) was used throughout this work. The HPLC apparatus consisted of two model 126 pumps, a PC-8300 solvent programmer, a Model 210 sample injection valve, a 5 μl injection loop, and a Model 166 variable-wavelength UV-visible range detector, equipped with a 12 μl flow cell. Integration of peak areas was obtained by means of a Shimadzu C-R6A Chromatopac electronic integrator (Shimadzu Corporation, Kyoto, Japan). The separation of DABS-amino acids was performed using a 3 μm Supelcosil LC-18 column (25 cm × 2.1 mm I.D.) protected with a guard column (2 cm × 2.1 mm I.D.), 40 μm Pelliguard LC-18 pellicular packing resin. The solvent A was 25 mM potassium dihydrogen phosphate buffer, pH 6.8 and the solvent B was acetonitrile-methanol (70 : 30). The separation of the DABS-amino acid derivatives was obtained using the gradient shown in Fig. 1. The gradient was then immediately returned to 27% of solvent B and the initial conditions restored in 12–15 minutes. The flow rate was 0.3 ml/min and the detection was performed at 436 nm.

Results and discussion

The aim of our work was to check if it was possible to use the narrow-bore columns for routine analysis of amino acids as DABS-derivatives using standard HPLC equipment with a loop of 5 μl and a flow-cell of 12 μl. It could be of some interest to have the possibility of significantly increasing the sensitivity only changing the analytical column. In fact, using the narrow-bore column under the conditions reported in this paper it is possible, firstly, to have the complete separation of over 35 DABS-amino acids and by- products working at room temperature and, secondly, to increase the sensitivity at femtomole level. Furthermore the main problem in using HPLC narrow-bore columns for routine analysis is providing adequate protection for them in order to maintain the best analytical conditions for a long time. Fig. 1 shows the complete separation of about 35 DABS-amino acids and by-products at room temperature. The flow rate was 0.3 ml/min and the gradient program as reported under "Experimental".

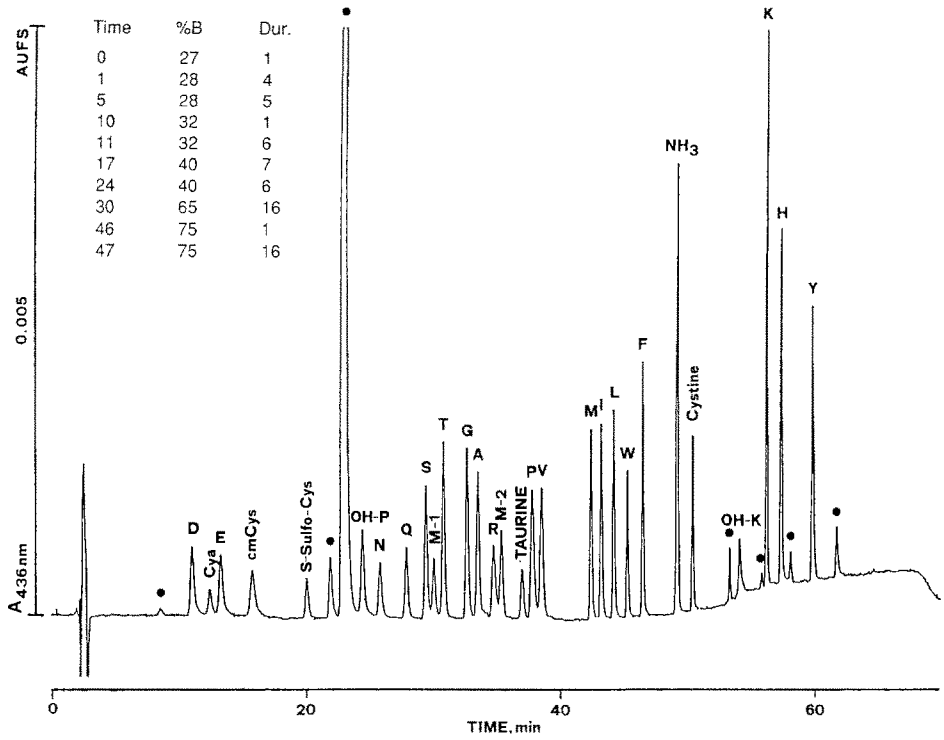

Fig. 1. Complete RP-HPLC separation of about 35 DABS-amino acids and by-products using a Supelcosil LC-18 column (25 cm × 4.6 mm I.D.), 3 μm particles. Solvent A, 25 mM potassium dihydrogen phosphate buffer (pH 6.8); solvent B, acetonitrile-methanol (70 : 30). The separation was obtained at room temperature using the conditions reported under "Experimental". The amount of each standard DABS-amino acid present in the mixture ranged from 0.7 to 2 picomoles. *Cya* cysteic acid; *cm-Cys* carboxymethylcysteine; *OH-P* hydroxyproline; *M-1* methionine sulfoxide; *M-2* methionine sulfone; *OH-K* δ-hydrolysine. • by-products originating from the excess reagent. The chromatogram was obtained using a variable wavelength detector, Model 166, from Beckman equipped with a 12 μl flow- cell. The volume injected was 5 μl. Flow-rate, 0.3 ml/min

The main problem that we observed using narrow-bore columns is their protection with a suitable guard column. For the separation of the DABS-amino acids this becomes a critical feature. We observed over-pressure problems after only 3–4 runs, injecting 5 μl of DABS-amino acid mixture corresponding to about 1–2 picomoles. The over pressure problems observed for the separation of DABS-amino acids are more serious than those observed with the separation of other compounds such as the DABTH-amino acids and nucleotides (data not shown), making the use of narrow-bore columns without adequate protection impossible. This problem was not previously observed working with columns of (15 cm × 4.6 mm I.D.), 3 μm particles protected with a guard column (2 cm × 4.6 mm I.D), 5 μm particles (Stocchi, 1989). This is probably due to the absorption or precipitation of aspecific material on the top of the column that in the case of narrow-bore columns of 2.1 mm I.D., reducing the surface area by about 5 times compared with columns of 4.6 mm I.D., makes the over pressure problem evident. Furthermore, we have tried to solve this problem by centri-

fuging the solution before injection at 12,000 g for 10–15 min without success. These problems were observed at the beginning, using a (2 cm × 2.1 mm I.D.) guard column, filled with a Supelcosil LC-18 resin, 5 μm particles. We were able to solve the protection of the analytical column using a (2 cm × 2.1 mm I.D.) guard column, filled with Pelliguard LC-18 pellicular packing materials, 40 μm particles. In this way it is possible to use the narrow-bore columns for routine analysis, without observing any drawbacks, only changing the guard column every week. If we consider that the amount of resin present in a guard column (2 cm × 2.1 mm I.D.) is almost 5 times less than that contained in a guard column (2 cm × 4.6 mm I.D.), to change the guard column every week becomes a reasonable procedure, making it possible to maintain the best analytical conditions of the column for a long time(over months). The separation reported in Fig. 1 is even a little better than that previously described by us (Stocchi, 1989) using a (15 cm × 4.6 mm I.D.) column, 3 μm particles. The use of a guard column filled with a pellicular packing material of 40 μm does not affect the complete resolution of the amino acids under investigation. It should be pointed out that a (25 cm × 2.1 mm I.D.) column, 3 μm particles, usually shows about 28,000–29,000 theoretical plates, a significantly higher efficiency compared with standard HPLC columns of (25 cm × 4.6 mm I.D.), 5 μm particles or (15 cm × 4.6 mm I.D.), 3 μm particles. Therefore, these columns could also be suggested for the resolution of complex mixtures. From our experience, we have observed that it is possible to use the narrow-bore column with standard HPLC equipment obtaining a true increase in sensitivity as shown in Table 1 which compares the results obtained using different columns of varied lengths, filled with different particles and injecting the same amount of sample in the same volume that was 5 μl. If we consider equal to 1 the sensitivity obtained using a Supelcosil LC-18 column (25 cm × 4.6 mm I.D.), 5 μm particles, using the narrow-bore columns (25 cm × 2.1 mm I.D.), 3 μm particles it is possible to increase the sensitivity to at least 10 times. This result represents a significant improvement which makes it possible to perform the amino acid analysis at femtomole level. Fig. 2 shows an original chromatogram obtained setting the sensitivity at 0.00125 AUFS and injecting 250 femtomoles of each amino acid as DABS-derivatives. The detection limit, calculated from this chromatogram, is about 70–80 femtomoles. This represents a very high level of sensitivity which makes it possible to cope with many different biological problems, espe-

Table 1. Comparison in sensitivity using different HPLC columns[1]

Column	Dimension	Particles μm	Sensitivity[2]
LC-18	(25 cm × 4.6 mm I.D.)	5	1
LC-18	(15 cm × 4.6 mm I.D.)	3	1.4–1.5
LC-18	(25 cm × 2.1 mm I.D.)	3	10

[1] The volume injected was 5 μl.
[2] The sensitivity was established equal to 1 considering the peak height obtained injecting the same amount of sample. The flow-cell volume was 12 μl.

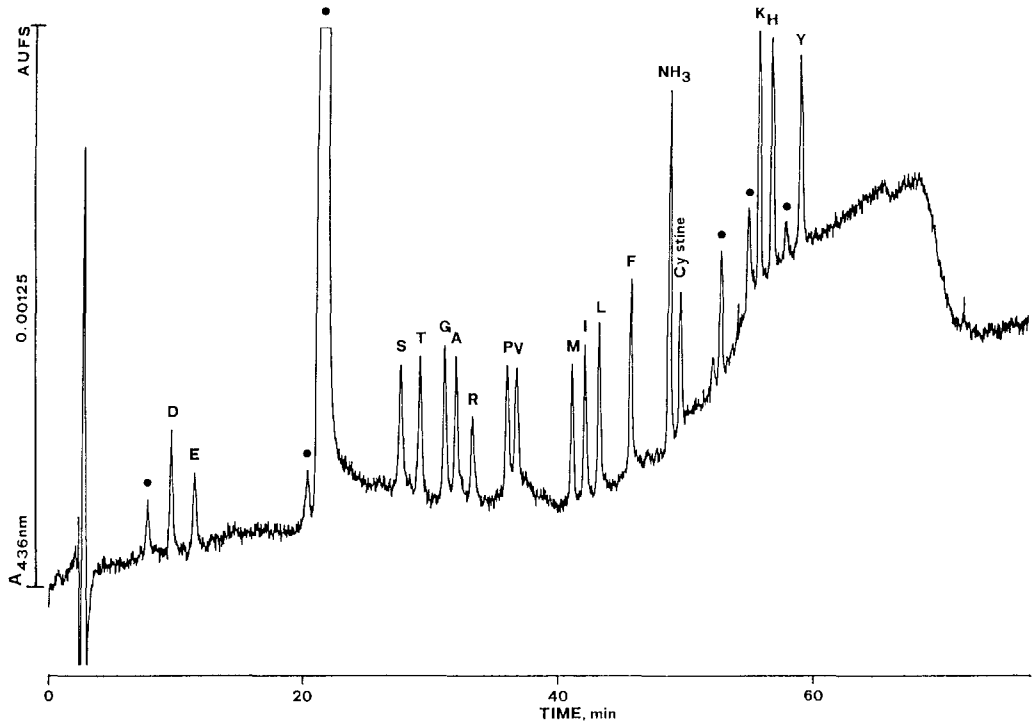

Fig. 2. Separation of a standard DABS-amino acids mixture. The amount of each standard amino acid was 250 femtomole. The experimental conditions used were the same as Fig. 1.
• by-products originating from the excess of reagent

cially when small amounts of peptide or protein are available. However, using the narrow-bore columns it is possible to perform routine analyses of amino acid using only 0.5–1 picomole of each amino acid, with a stable baseline (Fig. 1), and without problems in their correct evaluation. Performing the HPLC analysis at this very high sensitivity the main problem is the "background problem". We found it very useful to use HCl gas-phase hydrolysis and to treat the pyrex tubes used for hydrolysis overnight at 600°C. This was tested by hydrolyzing the several standard peptides and proteins reported under "Experimental" observing that the "background problem" could be reduced substantially making it possible to work at very high sensitivity (data not shown). Other useful applications requiring high sensitivity (tryptophan estimation, amino acid analysis of small amounts of protein electroeluted from SDS-polyacrylamide gel electrophoresis, etc) have already been described elsewhere (Stocchi, 1989).

Conclusions

The method described here, using a reversed-phase narrow-bore column, permits the complete separation of about 35 DABS-amino acids and by-products at room temperature. The procedure proposed is simple, reproducible and reliable. Furthermore the experimental conditions reported make it possible to use the narrow-bore columns with standard HPLC equipment for routine

analysis significantly increasing the sensitivity, when compared to standard HPLC columns, permitting amino acid analysis at femtomole level. The strategy described in this paper could also be applied for the separation of other compounds when more efficiency and sensitivity are necessary.

Acknowledgment

This work was supported by P. F. Chimica Fine II and P. F. Ingegneria Genetica, CNR.

References

Benson JR, Hare PE (1975) Proc Natl Acad Sci USA 72: 619–622
Bohlen P, Schroeder R (1982) Anal Biochem 126: 144–156
Bayer E, Grom E, Kaltenegger B, Uhman R (1976) Anal Chem 48: 1106–1109
Cronin JR, Hare PE (1977) Anal Biochem 81: 151–156
Cooper HDJ, Ogden G, McIntosh J, Turnell CD (1984) Anal Biochem 142: 98–102
Chang J-Y, Knecht R, Braun G (1981) Biochem J 199: 547–556
Chang J-Y, Martini P, Bernasconi R, Braun GD (1981) FEBS Lett 132: 117–120
Chang J-Y, Knecht R, Braun DG (1982) Biochem J 203: 803–806
Chang J-Y, Knecht R, Braun DG (1983) Methods in enzymology, vol 91. Academic Press, New York, p 41
Chang J-Y (1984) J Chromatogr 295: 193–120
De Jong C, Hughes JG, Van Vieringen E, Wilson KJ (1982) J Chromatogr 241: 345–359
Engelhart H, Asshauer J, Neve U, Weigand N (1974) Anal Chem 46: 336–340
Hughes JG, Frutiger S, Fonk C (1987) J Chromatogr 389: 327–333
Knecht R, Chang J-Y (1986) Anal Chem 58: 2375–2379
Kaneda N, Stato M, Yagi K (1982) Anal Biochem 127: 49–54
Negro A, Garbisa S, Gotte L, Spina M (1987) Anal Biochem 160: 39–46
Spackman HD, Moore S, Stein HW (1958) Anal Chem 30: 1190–1198
Stocchi V, Cucchiarini L, Piccoli G, Magnani M (1985) J Chromatogr 349: 77–82
Stocchi V, Piccoli G, Magnani M, Palma F, Biagiarelli B, Cucchiarini L (1989) Anal Biochem 178: 107–117
Schmidt GJ, Olson DG, Slavin W (1979) J Liq Chromatogr 2: 1031–1045
Tapuhi Y, Schmidt DE, Linder W, Karger BL (1981) Anal Biochem 115: 123–129
Vendrell J, Aviles XF (1986) J Chromatogr 358: 401–413
Winkler G, Heinz XF, Kunz C (1984) J Chromatogr 297: 63–73
Wilkinson JM (1979) J Chromatogr Sci 16: 547–552

Authors' address: Prof. V. Stocchi, Istituto di Chimica Biologica "Giorgio Fornaini", Università degli Studi di Urbino, Via Saffi, 2, I-61029 Urbino, Italy.

Received August 16, 1991

Verleger: Springer-Verlag KG, Sachsenplatz 4–6, A-1201 Wien. – Herausgeber: Prof. Dr. Gert Lubec, The Hospitals for Sick Children, Great Ormond Street, London WC 1N3YH, U.K. – Redaktion: Sachsenplatz 4–6, A-1201 Wien. – Satz und Umbruch: Asco Trade Typesetting Limited, Hong Kong; Reproduktion und Offsetdruck: Novographic, Ing. W. Schmid, Maurer-Lange-Gasse 64, A-1238 Wien. – Verlagsort: Wien – Herstellungsort: Wien. – Printed in Austria.