Journal of Chromatography, 242 (1982) 145–152
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,779

MICROCHROMATOGRAPHIC ANALYSIS OF Dns-AMINO ACIDS WITH A SENSITIVITY OF 10^{-13} MOLE*

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(First received September 2nd, 1981; revised manuscript received January 28th, 1982)

SUMMARY

A method for the analysis of Dns-amino acids at the level of 10^{-13} mole/h based on microcolumn hydrophobic chromatography with fluorimetric detection has been developed. The separation of all components was achieved by obtaining gradients of pH and acetonitrile concentration in the eluent. Ultra-high sensitivity of analysis is ensured by using capillary microcolumns ($d_c = 0.05$ cm) and a fluorimeter with a cell volume of 1.3 μl. When high-speed (high-temperature) dansylation is used, this method is also suitable for the analysis of free amino acids with the same sensitivity.

INTRODUCTION

Dansyl (Dns) (1-dimethylaminonaphthalene-5-sulphonate) derivatives of amino acids are widely used for the determination of amino acid sequences in proteins and peptides by Edman's method modified by Gray[1]. Owing to high sensitivity of this method ($1 \cdot 10^{-12}$–10^{-10} mole) in the identification and determination of N-terminal amino acids in the form of Dns derivatives, it became very popular in the manual stepwise degradation procedure for the determination of the primary structures of proteins.

Thin-layer chromatography (TLC) on polyamide[2] or silica gel[3] is a classical method for the analysis of Dns-amino acids. The limiting sensitivity of photographic recording is $1 \cdot 10^{-12}$ mole of Dns-amino acids.

Methods for the analysis of Dns-amino acids by high-performance liquid chromatography (HPLC) have been described. However, the results are much inferior to those obtained, for example, in the analysis of phenylthiohydantoin (PTH)-amino

* Presented at the *3rd Danube Symposium on Chromatography, Siófok, August 31–September 3, 1981.* The majority of the papers presented at this symposium has been published in *J. Chromatogr.*, Vol. 241, No. 1 (1982).

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acids[4]. Dns-amino acids have been separated on Spherisorb 5-ODS[5], Bondapak C_{18}[5] and LiChrosorb RP-8 (refs. 6, 7), *i.e.*, by hydrophobic (reversed-phase) chromatography. However, in these methods the separation of some pairs of Dns-amino acids is unsatisfactory and, moreover, the sensitivity of analysis is greatly inferior to that of TLC. It should be borne in mind that hydrophobic chromatography should be carried out in an aqueous–organic medium (water–methanol or water–acetonitrile) and water is known to quench the fluorescence of Dns-amino acids[2]. Hence, the limiting sensitivity achieved was $1 \cdot 10^{-10}$ mole with fluorimetric detection. On the other hand, hydrophobic chromatography of Dns-amino acids has been carried out[5,7] by using buffer solutions at high pH (7–8), but this was unfavourable for the selective separation of Dns-amino acids because at this pH their carboxylic groups are highly ionized whereas the maximum selectivity of the sorption of acids on hydrophobic material is observed at pH $< pK$.

The aim of this work was to develop a highly sensitive method for the hydrophobic chromatography of Dns-amino acids ensuring adequate separation of all components.

To attain a high sensitivity it is desirable to carry out chromatographic analysis on capillary microcolumns with corresponding decreases in the free volumes of the injector, connection and detector cell, as has already been proposed[8–10]. An n-fold decrease in column diameter leads to an n^2-fold increase in the analytical sensitivity and also makes it possible to decrease n^2 times the amount of sorbent in the column and the volume of the eluting liquid, and the cost of analysis can therefore be greatly reduced. There are other advantages of microcolumns that permit the development of very high-speed and very efficient chromatographic analysis[11]. It has been shown recently[12,13] that when a microcolumn 1 mm in diameter is used it is possible to attain the same efficiency as on a common column.

Microcolumns also suffer from some drawbacks. There is a great danger of leakages in the hydraulic system and increasing extra-column volumes (injector, connectors and detector sample cell), which results in an increase in extra-column spreading. Therefore, these extra-column volumes should be decreased to the same extent as that of the column, *i.e.*, n^2 times, but this is particularly difficult with the detector sample cell. In this instance the detector response can also decrease; that of the spectrophotometric detector decrease proportionally to the length of the optical path and that of the fluorimeter (flow detector) decreases proportionally to the decrease in the amount of substance being analysed, *i.e.*, if its concentration is maintained, proportionally to a decrease in sample volume in accordance with a decrease in the volume of the measuring cell. When a fluorimetric detector is used, the problem seems to be insoluble: an n-fold decrease in column diameter will allow an n^2-fold decrease in the amount of the substance to be analysed but will require an n^2-fold decrease in the sample cell volume. As a result, it is necessary to increase the sample concentration n^2 times in order to maintain the same mass sensitivity of the detector. Hence, when microcolumns are used, it is impossible to increase the analytical sensitivity of the fluorimetric analysis by this method.

Fortunately, there are other methods for increasing detector sensitivity: increasing the power and efficiency of the radiation source, minimizing losses in the exciting and luminescent light and the suppression of optical and electric noise.

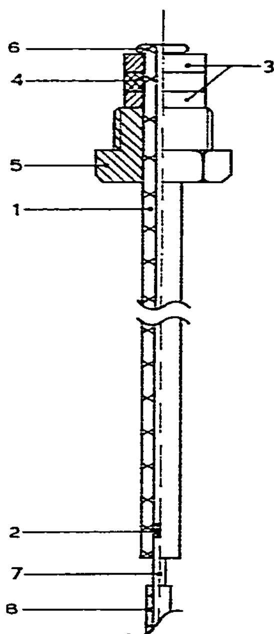
EXPERIMENTAL

Apparatus

A Kh Zh-3301 microchromatograph (Special Design Bureau of Analytical Instruments, Academy of sciences of the U.S.S.R., Leningrad, U.S.S.R.) was used for the analysis. It was equipped with syringe pumps with 2-ml cells and an eluent flow-rate of 30–4000 μl/h, a fluorimetric and a spectrophotometric detector with sample cells of 1.3 and 0.5 μl, respectively, an injector and a gradient assembly with a mixer. The latter makes it possible to obtain a composition gradient of the eluted solution of any desired shape by using programmed changes in the frequency of turning on the two syringe pumps.

Column design and packing

A home-made fluoroplastic or glass column (250 × 0.5 mm I.D.) packed with Silasorb-300-C_{18} sorbent (d_p = 7 μm) (Lachema, Brno, Czechoslovakia) was used. (Figs. 1 and 2).



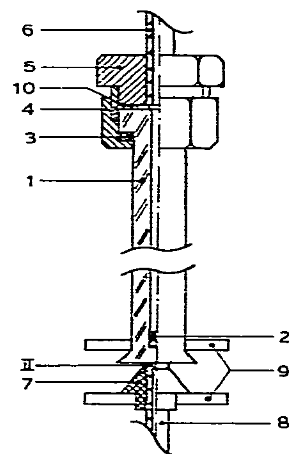


Fig. 1. Construction of fluoroplastic column. 1 = Microcolumn made from fluoroplastic capillary, I.D. 0.5 mm, O.D. 2 mm; 2 = porous titanium filter, diameter 0.6 mm; 3 = steel washer, I.D. 2 mm; 4 = fluoroplastic sealing washer; 5 = eye bolt; 6 = expansion of the fluoroplastic capillary; 7 = section of steel capillary, I.D. 0.3 mm, O.D. 0.6 mm, length 2 cm; 8 = polyethylene capillary for connection with the detector cell. The microcolumn is screwed directly into the injector and is sealed by seals 3, 4 and 5.

Fig. 2. Construction of glass column. 1 = Microcolumn made from glass capillary, I.D. 0.5 mm, O.D. 5 mm; 2 = porous fluoroplastic of polyamide filter, length 2 mm; 3 = fluoroplastic washer; 4, 5 = threaded steel seals; 6 = fluoroplastic capillary, I.D. 0.5 mm, O.D. 1 mm; 7 = fluoroplastic cone; 8 = polyethylene capillary, I.D. 0.3 mm, O.D. 1 mm (for connection with the fluorimeter cell); 9 = alligator clip; 10 = expanding end of the fluoroplastic capillary; 11 = expanding end of the polyethylene capillary.

A 40–60% sorbent slurry in a 5% detergent solution (Brij-35; polyoxylauric alcohol) in propanol–water (1:1) was prepared. The slurry was degassed under vacuum. The column was then filled with the dispersion medium by a syringe pump and the slurry was introduced into a fluoroplastic or metal capillary (100–120 cm × 1 mm I.D.). The capillary with the slurry was connected to the column and the pump for feeding the dispersion medium was turned on. At the beginning of packing the slurry was introduced into the column at the maximum micropump speed (10 ml/h). The flow-rate of dispersion medium was gradually decreased as the length of the column of settling sorbent increased until the limiting pressure for the given system, 100–120 kg/cm^2, was attained. If the system is assembled correctly and the slurry concentration is appropriate, the slurry should enter the column continuously. The packed column was disconnected when the pressure in the system dropped to zero. The detergent was removed by washing the column with water–propanol (1:1) for 30 min at a rate of 2 ml/h and the column was equilibrated by using the initial eluent.

When the reproducibility of elution volumes of Dns-amino acids decreased, the column was regenerated by successive washing with methanol, chloroform and the initial eluent.

Reactants

Buffered aqueous acetonitrile solutions were used as eluents. Chromatographically pure aceonitrile in ampoules with a transmission coefficient of $T^{1\,cm}_{260\,nm} \geq 95\%$ was used. Other reactants used to prepare the eluents were of C.P. grade. Model mixtures of Dns-amino acids were prepared from materials supplied by Serva (Heidelberg, G.F.R.), Calbiochem (Los Angeles, CA, U.S.A.) or Reanal (Budapest, Hungary).

Preparation of reference mixtures and eluents

Acetonitrile concentration and pH gradients were obtained by mixing two eluted solutions: (A) acetonitrile (25%)–0.01 *M* sodium formate solution, pH = 3.5 (75%), and (B) acetonitrile (60%)–0.01 *M* phosphate buffer, pH = 7.0 (40%) (0.005 *M* of Na_2HPO_4 + 0.005 *M* of NaH_2PO_4).

Before use the eluents were filtered through a Whatman GF/F glass membrane and degassed under vacuum. To ensure a narrow starting zone solutions of Dns-amino acids were prepared as 10% acetonitrile solutions in 0.1 *M* hydrochloric acid.

RESULTS AND DISCUSSION

As already mentioned, for the separation of more polar Dns-amino acids eluted from the column first it is desirable to carry out elution at pH 3.5 in order to increase their capacity factors and relative retentions. It should be mentioned, however, that the introduction of an acid into an aqueous solution of Dns-amino acid leads to additional quenching of luminescence[2]. Nevertheless, the resultant loss in detection sensitivity (F) is not great: F(pH 7)/F(pH 3.5) = 1.8 (for Dns-serine).

Fig. 3 shows the chromatogram of a group of polar Dns-amino acids obtained under normal or isocratic conditions. It can be seen that at pH 3.5 the separation of a group of hydrophilic Dns-amino acids can be substantially improved (compared with that reported in refs. 5 and 7). At the same time, in spite of luminescence quenching in

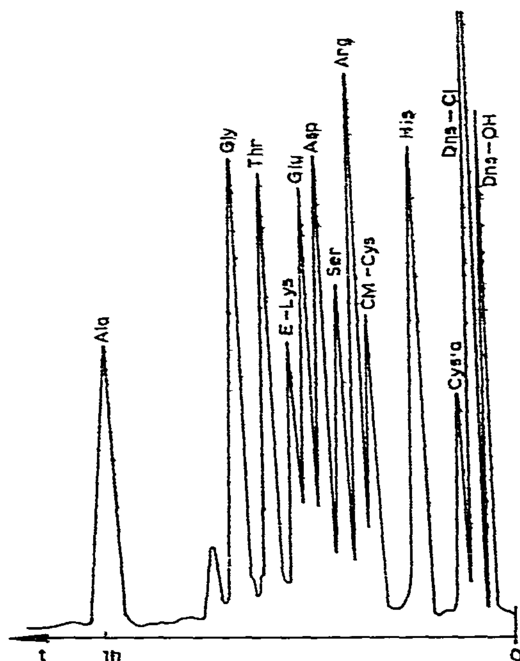


Fig. 3. Chromatogram of the separation of a mixture of polar Dns-amino acids. Elution rate, 500 μl/h; column, 250 × 0.5 mm I.D.; sorbent, Silasorb-300-C_{18}, d_p = 7 μm; eluent, acetonitrile (25%)–0.01 *M* sodium formate (75%), pH = 3.5; amount of sample, $2 \cdot 10^{-13}$ mole of each amino acid.

aqueous solution, fluorimetric detection of Dns-amino acids guarantees at least a 100-fold higher sensitivity than photometric detection.

When the eluent is programmed, the situation in microcolumn chromatography is very complex because it is difficult to attain a good mixture of two flows delivered from pumps operating at different velocities with very low flow-rates.

Our experiments showed that after passing to a column less than 1 mm in diameter the T-shaped mixer does not ensure adequate mixing of the liquids. Good mixing leading to reproducible gradients was attained only if a micromixer with forced mixing (Fig. 4) was used.

Fig. 5 shows the chromatogram of Dns-amino acids obtained under the conditions of gradient elution and the corresponding gradient shape. The arrow shows the position of elution of *o*-Dns-tyrosine (sensitive analysis of this Dns-amino acid is impossible because when it is irradiated with UV light at 365 nm, it emits yellow-orange luminescence). The use of this method ensures reproducibility of the volume with a relative standard deviation (σ) of 1.7%, of reduced peak heights (ratio of peak height to the sum of heights of all peaks) with σ = 2.30% and of the areas under the peaks (obtained by weighing) with σ = 2.85%.

Fig. 5 shows that when not only the acetonitrile concentration gradient but also the pH gradient was used it was possible to improve appreciably the quality of separation of Dns-amino acids, and when microcolumn HPLC was used it was possible to carry out an analysis at the level of 10^{-13} mole of each Dns-amino acid in 1 h.

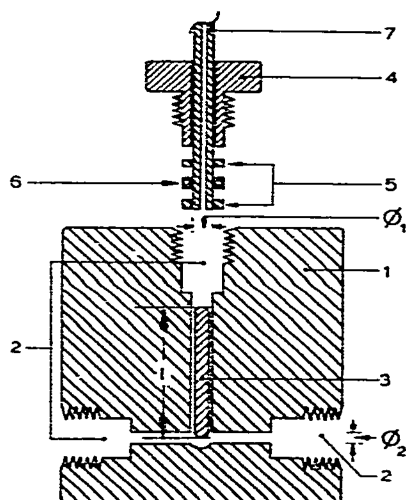


Fig. 4. Mixer for gradient assembly. 1 = Titanium body of the mixer; 2 = threaded opening for capillary connections; 3 = rod-like steel stirrer in a polyethylene coating 10 mm in length and 1 mm in diameter; 4 = connector; 5 = steel rings; 6 = fluoroplastic ring; 7 = supply capillary. The diameter of the mixing channel is 2 mm and that of the entrance channel is 0.3 mm. The mixer is placed on a magnetic stirrer.

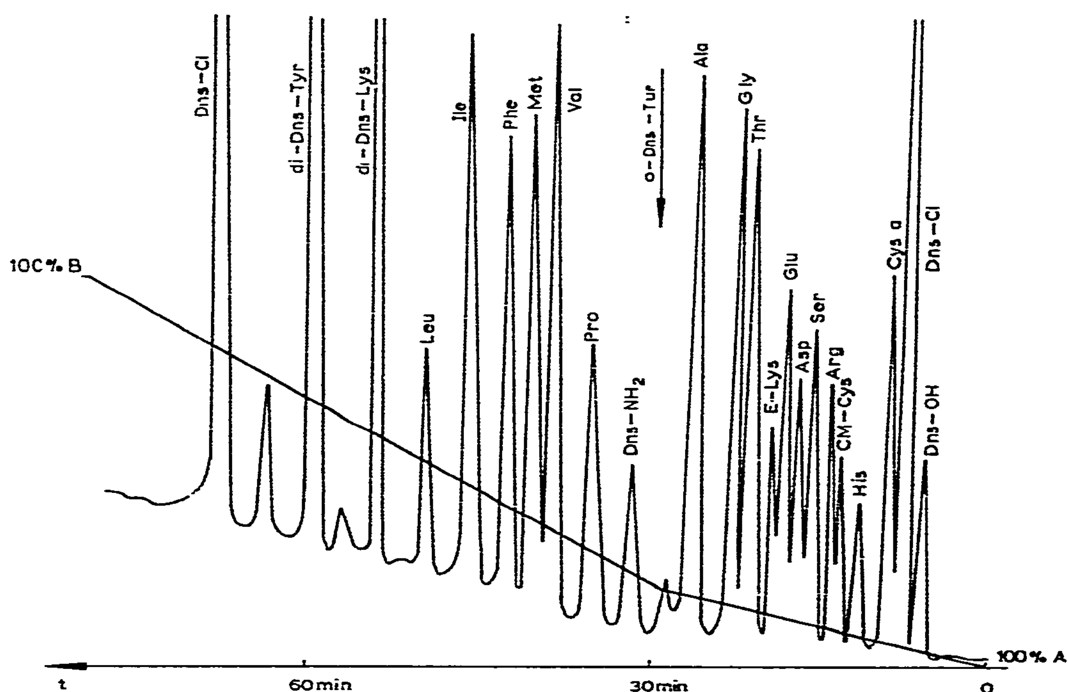


Fig. 5. Chromatogram of the separation of a mixture of Dns-amino acids under the conditions of gradient elution. Column, 250 × 0.5 mm I.D.; sorbent, Silasorb-300-C_{18}, d_p = 7 μm; elution rate, 1 ml/h. Eluents: (A) acetonitrile (25%)–0.01 *M* sodium formate, pH = 3.5 (75%); (B) acetonitrile (60%)–0.01 *M* phosphate buffer, pH = 7.0 (0.005 *M* NaH_2PO_4 + 0.005 *M* Na_2HPO_4) (40%). Amount of sample, $2\text{–}5 \cdot 10^{-13}$ mole of each amino acid.

The problems of a further increase in the sensitivity of analysis of Dns-amino acids should now be considered. Because in microcolumn chromatography (column diameter, $d_c \leqslant 0.5$ mm) a detector cell of volume less than 1 μl should be used to prevent zone spreading in the detector, which prevents separation, it is almost impossible to obtain a fluorimeter cell thickness greater than 2 mm, which considerably decreases the sensitivity of luminescence recording. Hence, the only possibility of attaining a sensitivity of recording of Dns-amino acids sufficient for microcolumn chromatography was to choose exciting and emission light filters that would permit the maximum effective cell illumination by the exciting emission of a deuterium lamp. A UFS-1 light filter (1 mm) was used to pass the UV light and a ZhS-12 light filter (2 mm) was employed to cut off the luminescent light. This combination if filters ensures maximum sensitivity of detection of Dns-amino acids with the fluorimeter of the Kh Zh-3301 chromatograph. The sensitivity of analysis of Dns-amino acids according to the above method is $22 \cdot 10^{13}$% of the scale per mole ($2 \cdot 10^{-13}$ mole of Dns-glycine gives a peak height of $\geqslant 45$% of the recorder scale height). Thus, the limiting sensitivity of analysis is about 10^{-14} mole. This value is much higher than those reported for the previously described methods[5-7] and higher than that in which the analysis of Dns-amino acids is carried out on silica gel in an organic eluent[5], *i.e.*, under the conditions of maximum quantum yield of luminescence of Dns-amino acids. In the latter work the limit of detection sensitivity (with a signal-to-noise ratio of 2:1) was 10^{-13} mole.

It should be emphasized that the method of microcolumn hydrophobic chromatography of Dns-amino acids described here provides great possibilities for increasing sensitivity (to the femtomole level) by improving the fluorimeter design. The following approaches are possible: (1) increase in the power of the UV light source; (2) collection of luminescent light from all of the surface of the sample cell; (3) use of a hydrodynamic cell with luminescence excitation by a laser beam with a changing frequency[14]; and (4) use of chemiluminescent reaction for the excitation of the fluorescence of Dns-amino acids[15].

As already stated, the method of high-speed (2 min) dansylation[6] permits the use of this procedure not only for the analysis of amino acid protein sequences with a sensitivity of about 10^{-13} mole, but also for the amino acid analysis of protein hydrolysates at the $1 \cdot 10^{-13}$–$1 \cdot 10^{-12}$ mole level.

It should be noted that in microcolumn chromatography post-column derivatization with the formation of coloured or luminescent products in the reactor is undesirable. When the method of detection generally employed in amino acid analysers is used for analysis on microcolumns, the contribution of extra-column spreading to the chromatogram becomes much more pronounced. Hence, the proposed analysis of amino acids with pre-column derivatization with the formation of Dns-derivatives is an optimum variation of microchromatographic amino acid analysis. An alternative approach is pre-column derivatization with *o*-phthalic dialdehyde[16].

In practice, the ultramicroanalysis of amino acids at this level depends on the development of a procedure for the hydrolysis of nanogram amounts of protein and the dansylation of hydrolysates of volume 5–10 μl. The investigation of this problem is in progress.

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