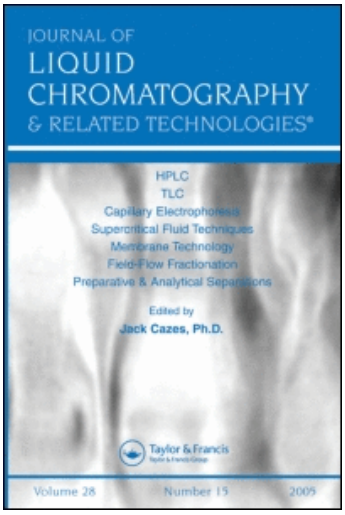

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Practical Approach to Improve the Resolution of Dansyl-Amino Acids by High-Performance Liquid Chromatography

Antonio R. Martins[a]; Afonso P. Padovan[a]
[a] Departamento de Farmacologia Faculdade de Medicina de Ribeirão Prěto, Universidade de São Paulo, São Paulo, Brasil

To cite this Article Martins, Antonio R. and Padovan, Afonso P.(1996) 'A Practical Approach to Improve the Resolution of Dansyl-Amino Acids by High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 19: 3, 467 — 476
To link to this Article: DOI: 10.1080/10826079608001228
URL: http://dx.doi.org/10.1080/10826079608001228

J. LIQ. CHROM. & REL. TECHNOL., 19(3), 467–476 (1996)

A PRACTICAL APPROACH TO IMPROVE THE RESOLUTION OF DANSYL-AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Antonio R. Martins, Afonso P. Padovan

Departamento de Farmacologia
Faculdade de Medicina de Ribeirão Prêto
Universidade de São Paulo
14049-900 Ribeirão Prêto, São Paulo, Brasil.

ABSTRACT

We describe an HPLC procedure for the separation of dansyl (Dns)-derivatives of all common amino acids present in polypeptide hydrolyzates. A linear gradient of solvent B [methanol: water, 70:30, (v/v)] in solvent A [30 mM sodium phosphate buffer, pH 7.4, containing 5 mL methanol and 6.5 mL tetrahydrofuran (THF)], from 0 to 100% B in 30 min, at 1.0 mL/min and 25 °C, was used to elute the Dns-amino acids from a 4 μm NovaPak C_{18} column with good resolution. The Dns-amino acids were detected fluorometrically at 338 nm wavelength excitation and 455 nm emission.

The concentration of sodium phosphate buffer was critical for the resolution of the dansyl-derivatives of: Arg from Ser and Thr, NH_3 from Val and Met and of cysteic acid from Asp and Glu. THF mainly improved the resolution of the dansyl-derivatives of: Arg from Ser and Thr, NH_3 from Val, and Leu from Ile and Trp.

The effects of sodium phosphate buffer and THF on the column capacity factor provided a convenient and reproducible manner to adjust elution conditions for Dns-amino acid separation when using new columns.

Using this procedure, we showed that Asx is the N-terminal amino acid residue of human pancreatic secretory trypsin inhibitor, in agreement with sequence data.

INTRODUCTION

A general aspect of HPLC methodology is the necessity of adjusting a given elution protocol to each new column to be used, to obtain a good resolution.[1] This is particularly valid for the HPLC separation of 1-dimethylamino-naphthalene-5-sulfonyl- (dansyl, Dns) amino acids. The separation of Dns-amino acids requires the use of application-selected columns[2] and presents difficulties to resolve some Dns-amino acids, such as: Glu/cysteic acid, Thr/Gly, Ile/Leu, Trp/Ile/Leu, Ile/Phe.[3,4]

None of the methods for the separation of Dns-amino acids[2,4-7] that we have tried yielded good resolution with 10 μm μBondapak C_{18} and 4 μm NovaPak C_{18} (Waters), and with 5 μm Spherisorb C_{18} (Applied Science Division) columns. In addition to be able to resolve Dns-amino acids with good efficiency and peak simmetry, it is also convenient that such analytical procedure can be adjustable for use with new columns, by predictable changes in solvent composition.

To address this issue, we have developed an HPLC procedure for the separation of the dansyl-derivatives of all the common amino acids present in polypeptide hydrolyzates. The procedure is based on a linear gradient of increasing methanol and decreasing tetrahydrofuran (THF) and sodium phosphate concentrations.

The effects of THF and sodium phosphate concentrations on column capacity factors (k') of the Dns-amino acids described here have been used to develop a new procedure for the separation of Dns-amino acids that fullfills the above requirements. The applicability of this procedure was evaluated using it to identify the N-terminal amino acid residue of homogeneous human pancreatic secretory trypsin inhibitor (hPSTI).

MATERIALS AND METHODS

Equipment

The liquid chromatograph used here (Waters Associates, Milford, MA) consisted of: two Model 6000A pumps, a Model 660 programmer, a Model U6K Universal injector and a Model 420-AC fluorescence detector fitted with a F4T5 BL lamp, and 338 and 455 nm wavelength excitation (band-pass) and emission (long-pass) filters, respectively. Chromatograms were recorded using a RB201 recorder (ECB, São Paulo, SP). pH values were determined using a Micronal (São Paulo, SP) model B-222 digital pH-meter.

Standards and Reagents

Dansyl-chloride was from Sigma, St. Louis, MO. LC-grade solvents and all other reagents were from Merck (Darmstadt, DFR). Distilled water was purified using a Milli-Q apparatus (Millipore, Bedford, MA). Homogeneous hPSTI was a gift from Dr. L. J. Greene, Laboratório de Química de Proteinas, Faculdade de Medicina de Ribeirão Prêto, Universidade de São Paulo.

A stock solution of L-amino acids was prepared either by diluting Amino Acid Standard H (containing, per mL, 1.25 μmoles L-cystine and 2.50 μmoles of: ammonia, L-Ala, L-Arg, L-Asp, L-Glu, L-Gly, L-His, L-Ile, L-Leu, L-Lys, L-Met, L-Phe, L-Pro, L-Ser, L-Thr, L-Tyr, L-Val; Pierce, Rockford, IL) or dissolving weighed amounts of each L-amino acid (Pierce) in 0.1 N hydrochloric acid to provide a final concentration of 50 nmol/mL. The stock solutions were stored at -20 °C. A given amount of the amino acid mixture was taken by volume into a pyrolyzed[8] glass vial and evaporated to dryness. The dry sample was dansylated as described below. Standard Dns-amino acids were from Pierce. Nor-leucine (Ahx, 2-aminohexanoic acid) was used as an internal standard.

Mobile Phase Preparation

Solvent A was prepared by mixing: 30 mL 100 mM sodium phosphate buffer, pH 7.4, 5 mL methanol and 6.5 mL tetrahydrofuran in a volumetric flask and adding water to 100 mL, unless otherwise stated. Solvent B was prepared by mixing methanol and water, 70 : 30 (v/v). Mobile phases were degassed by sonication under reduced pressure before use.

Chromatographic Conditions

Dansyl-amino acids and reaction by-products were separated by gradient elution chromatography (0 to 100% B in 30 min, curve 6), at a flow rate of 1.0 mL/min and at 25 °C, using a stainless steel 4 μm NovaPak C_{18} column (3.9 mm x 150 mm). A 5 cm Bondapak precolumn (37-50 μm C18/Corasil packing; Waters) was placed between the injector and column. The effluent was monitored fluorometrically. The retention time of Dns-Asp, eluted with 100 % methanol, was taken as t_0.

At the end of a run, initial conditions were restored by running a reversed gradient in 10 min, at 1.0 mL/min. Then, about 10 min were required for column equilibration. This procedure was found necessary for maintaining optimal column performance.

The overnight shut down procedure consisted in washing the column with about 35 mL water, followed by 20 mL solvent B, at 1 mL/min. Washing the buffer pump, tubing and the column with water was found essential because solvent A contained phosphate buffer.

Dansylation Procedure

Dansylation was carried out by a modification[2] of the procedure described by Tapuhi et al.[9] Samples (up to 20 nmol total NH_2) were transfered to pyrolyzed (4.4 mm I.D. x 6 mm) glass tubes, dried under reduced pressure and dissolved in 40 μL 40 mM lithium carbonate buffer, pH 9.5. Then, 20 μL of 1.5 mg/mL (5.56 mM) Dns-Cl solution in acetonitrile were added to the sample, shaken for 2 min, and the mixture was incubated for 40 min at 37 °C in the dark. The reaction was stopped by adding 2 μL 8.9 M ethylamine hydrochloride, and further incubating for 10 min at 37 °C. The derivatization mixture was evaporated to dryness under reduced pressure, at room temperature. The dry sample was either stored in the dark at -20 °C for up to one week or diluted in 100 μL solvent A immediately before injection.

N-terminal Determination of Human Pancreatic Secretory Trypsin Inhibitor

hPSTI (200 pmol) and nor-leucine (Ahx, 200 pmol) were transfered to a hydrolysis tube, dried under reduced pressure, redissolved in 40 μL 40 mM lithium carbonate buffer, pH 9.5, and dansylated. After drying the

derivatization mixture under reduced pressure, 100 μL constant boiling HCl were added to the tube. The material was incubated at 110 °C for 18 h, evaporated over solid NaOH and dissolved in 100 μL solvent A. A 50 μL aliquot of the hydrolyzate was used for the HPLC analysis of dansyl-amino acids. Control tubes in which hPSTI, Ahx or both substituted for water were processed in parallel.

RESULTS AND DISCUSSION

HPLC Separation of Dansyl-Amino Acids

Fig. 1 shows the resolution, within 35 min, of a mixture of Dns-amino acids, including dansyl sulfonic acid and the derivatives of: ammonia, the rection quencher ethylamine,[9] and the internal standard nor-leucine. The k's for the dansyl derivatives of: cysteic acid, Lys at the e-amino group (eK), Nor-valine (Avl, 2-aminovaleric acid), Nor-leucine, Trp, methylamine, ethylamine and n-propyl-amine were: 1.8, 11.5, 12.9, 15.5, 15.6, 14.7, 16.6 and 18.7, respectively.

Under the derivatization conditions used here Trp, His, Lys, cystine and Tyr are converted to their di-Dns derivatives.[5,9] Besides ethylamine, either methyl- or n-propyl-amine can be used as a reaction quencher. The quencher has been selected such that its Dns-derivative does not interfere in the chromatogram. Avl is an alternative internal standard to Ahx.

The limit of sensitivity attained here was about 2 pmol, for a signal-to-noise ratio of 5:1 .

When the guard column was changed after 100-120 analyses, Dns-amino acids were well separated for at least 600 analyses per column.

Effect of the Concentration of Sodium Phosphate Buffer, pH 7.4, on Resolution

Capacity factor lines of Dns-amino acids did not cross, except those of cysteic acid, Arg and ammonia (Fig. 2). Thus, for example, at a sodium phosphate buffer concentration of 10 mM in solvent A, Arg coeluted with Ala and after Ser, Thr and Gly. At a buffer concentration of 30 mM, Arg eluted between Ser and Thr and was well resolved from them (see also Fig. 1).

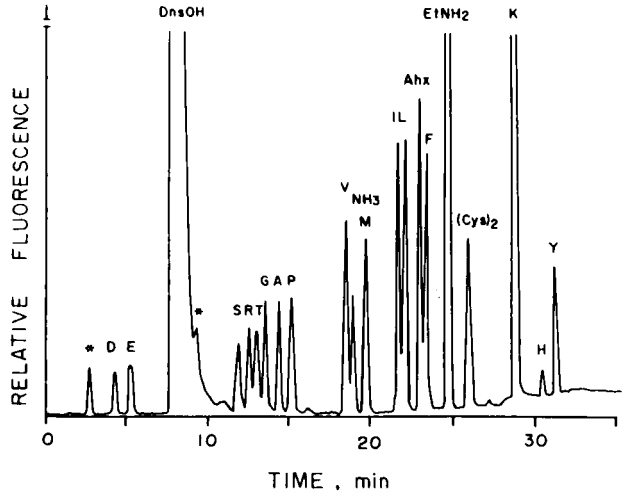

Figure 1. Reverse phase HPLC of a mixture of dansyl-amino acids. A sample containing: amino acid standard H (1 nmol of ammonia and of each amino acid, except L-cystine whose amount was 0.5 nmol) and norleucine (1 nmol) was derivatized and *ca.* 60 pmol of each dansyl-derivative was injected. Chromatographic conditions were as in Methods. Fluorometer gain, 64. Amino acids are abbreviated by the one-letter system. Ahx, norleucine; DnsOH, dansyl sulfonic acid; $EtNH_2$, ethyl-amine; *, unidentified peak.

Dns-derivatives of Asp, Arg and ammonia are usually difficult to resolve from those of cysteic acid/Glu, Ser/Thr/Gly and Met/Val, respectively.[3,4] The changes in sodium phosphate buffer concentration shown here have provided a way to optimize the separation of these Dns-amino acids.

Effect of Tetrahydrofuran Concentration on Resolution

The capacity factors of the Dns-amino acids decreased with increasing THF concentration in solvent A (Fig. 3). As expected, this effect was more intense for the earlier eluting derivatives, whereas those eluting by the end of the run were less affected. The differential effect of THF concentration on the separation of the Dns derivatives of: Arg, Thr and Gly; Ala and Pro; Val, ammonia and Met; Leu and Trp, was usefull for the separation of these compounds. Taken together, the data in Figs. 2 and 3 exemplify a systematic

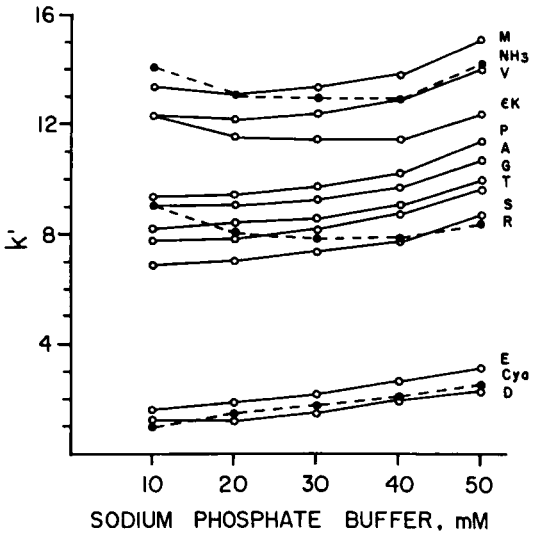

Figure 2. Changes in k' as a function of the concentration of sodium phosphate buffer, pH 7.4, in solvent A. A series of gradient runs using as solvent A: 10 to 50 mM sodium phosphate buffer, pH 7.4, containing 5 mL methanol and 6.5 mL tetrahydrofuran per 100 mL solvent A, and as solvent B: metanol and water, 70 : 30 (v/v), were performed. k's are the averages of three independent determinations, which did not differ by more than 2.2 %. Dashed lines are for: Cya, R and NH_3.

approach for the adjustment of the elution protocol proposed here to each new column to be used for the separation of Dns-amino acids.

Determination of the N-terminal Amino Acid Residue of Human Pancreatic Secretory Trypsin Inhibitor

The N-terminal amino acid identifyied here (Fig. 4), Asx, agreed with the known N-terminal of hPSTI, Asp.[10] Free Asp and Asn have not been detected in unhydrolyzed control samples of hPSTI. The ε-Dns-Lys derivative was also formed and did not interfere in the HPLC analysis.

In conclusion, we have proposed here a new method for the separation of Dns-amino acids that is fast, sensitive, and applicable to the determination of the N-terminal amino acid residue of small samples of polypeptides. When using new columns, the separation of Dns-amino acids can be conveniently

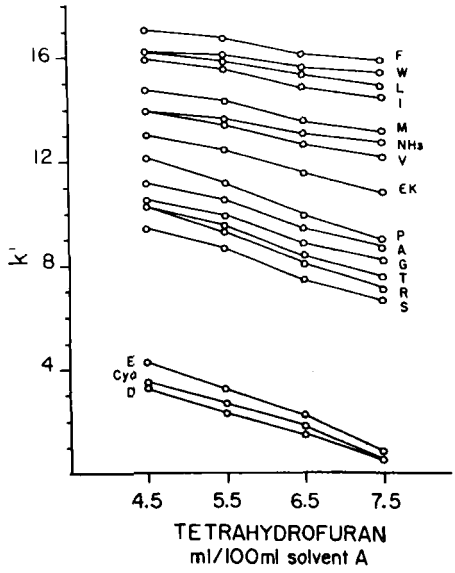

Figure 3. Changes in k' as a function of tetrahydrofuran concentration in solvent A. A series of gradient runs using as solvent A: 30 mM sodium phosphate buffer, pH 7.4, containing 5 mL methanol and 4.5 to 7.5 mL tetrahydrofuran per 100 mL solvent A, and as solvent B: metanol and water, 70 : 30 (v/v), were performed. k's are the averages of three independent determinations, which did not differ by more than 2.5 %.

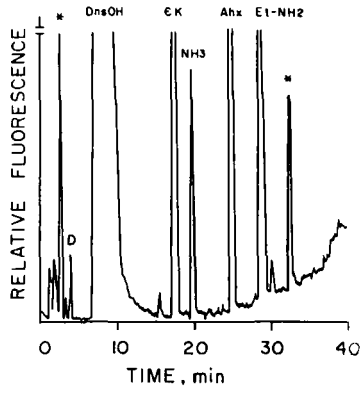

Figure 4. Identification of the N-terminal amino acid residue of a 200 pmol sample of hPSTI by the present HPLC method. Chromatographic conditions were as in Methods except gradient time (35 min) and the use of a new NovaPak C_{18} column. *, unidentified peak. Fluorometer gain, 128.

tailored by changes in the concentration of sodium phosphate buffer and THF, and by changes in gradient time.

ACKNOWLEDGEMENTS

The technical assistance of Mr. J. C. Rosa is greatly appreciated. We thank Drs. J. R. Giglio and F. M. A. Corrêa, Departamentos de Bioquímica and Farmacologia, Faculdade de Medicina de Ribeirão Prêto, Universidade de São Paulo, for Dns-amino acid standards and for helpfull comments on this manuscript, respectively. This work was supported by grants FAPESP 88/3884-3 and CNPq 502297/91-3.

REFERENCES

1. B. N. Jones, "Amino Acid Analysis by o-Phthaldialdehyde Precolumn Derivatization and Reverse Phase HPLC," in **Methods of Protein Microcharacterization**, J.E. Shively, ed., The Humana Press Inc., Clifton, NJ. 1986, pp.121-151.

2. C. De Jong, G. J. Hughes, E. van Wieringen, K.J. Wilson, J. Chromatogr., **241**, 345-359 (1982).

3. B. Orey, H. S. Lu, R. W. Gracy, J. Chromatogr., **270**, 253-266 (1983).

4. F. J. Márquez, A. R. Quesada, F. Sánchez-Jiménez, I. Núñez de Castro, J. Chromatogr., **380**, 275-283 (1986).

5. Y. Tapuhi, N. Miller, B. L. Karger, J. Chromatogr., **205**, 325-337 (1981).

6. S. Weiner, A. Tishbee, J. Chromatogr., **213**, 501-506 (1981).

7. P. Fürst, L. Pollack, T. A. Grase, H. Godel, P. Stehle, J. Chromatogr., **499**, 557-569 (1990).

8. W .R. Gray: "End-Group Analysis Using Dansyl Chloride," in **Methods in Enzymology, vol. XXVB (Enzyme Structure, part B)**, C. H. W. Hirs, S. N. Timasheff, eds., Academic Press Inc., New York, 1972, pp. 121-138.

9. Y. Tapuhi, D. E. Schmidt, W. Lindner, B. L. Karger, Anal. Biochem., **115,** 123-129 (1981).

10. D. C. Bartelt, R. Shapanka, L. J. Greene, Arch. Biochem. Biophys. **179,** 189-199 (1977).

Recieved July 28, 1995
Accepted August 15, 1995
Manuscript 3935