A Fast Liquid Chromatographic Method for the Determination of Amino Acids

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Summary: A reversed-phase high performance liquid chromatographic (HPLC) procedure, using fluorescence detector, is outlined for the single and quantitative determination of trace amounts of amino acids in the ng l^{-1} by pre-column derivatisation. The optimisation of the operating conditions and possible separation mechanism for different amino acids in the chromatographic column are discussed. The fluorescence detection of the derivative has been a great advantage over the UV-detection system. The method can be used, however for the rapid determination of amino acids in complex biological samples.

Introduction

Proteins, amines and amino acids at nanogram level in complex and biological samples are required to be measured for various studies e.g. characterisation and structural elucidation of peptides and proteins. The detection of amino acids separated from a complex mixture poses a great problem. A solution to such problem is, however, normally achieved by separating amino acid mixtures using ion-exchange chromatography [1] followed by post-column derivatisation with a fluorigenic reagent. Bensen and Hare [2] in 1975 and Roth [3] in 1976 reported the post-column fluorigenic detection of amino acids in conventional amino acid analysis [2,3] and quantification of amino acids in natural water [4] based on *o*-phthalaldehyde (OPA) and 2-mercapto-ethanol (2-MERC) popular reaction.

With the advent of high performance liquid chromatography, various approaches using pre- or post-column derivatisation with OPA/2-MERC [5], fluorescamine [6] dansyl chloride [7] and phenyl isothiocyanate, PITC [8] have been described. Although all of these HPLC methods show encouraging results, none has been standardised sufficiently to allow for minute routine use in a variety of laboratories. Previous studies have further revealed that the OPA reaction product is not very stable [9-11]. Besides, the wavelength of emission of the OPA derivative is at 450 nm where there is a high back-ground emission from biological samples.

While most people employ OPA, the authors have carried out reversed-phase HPLC technique similar to that described by several workers [12-15] but substituted OPA by naphthalene-2,3-dicarbo-xaldehyde, NDA [16]. This new reagent [16] was synthesised in the author's laboratory and was found to offer several advantages over other methods, including ion-exchange. These advantages include not only specific reaction with primary amino group but reduced analysis time and greater stability of the derivative for easy fluorescence detection.

The present work is aimed to evaluate the applicability of NDA as a potential label for detection and quantitation of amino acids in a sample by the pre-column HPLC derivatisation.

Results and Discussion

Separation as a function of composition of mobile phase

The effect of solvent composition in the mobile phase was studied by using solvent system of varying compositions. Preliminary experiments demonstrated that separation of different amino acids on the column occurred by using a mixture of acetonitrile and water (65:35% v/v) as the mobile phase. Using this solvent system the fluorescent derivatives of amino acids with NDA/2-methyl-2-propanethiol were shown to be satisfactorily eluted from the column for all dilute amino acid solutions. Figure 1 shows the chromatogram which illustrates the resolution obtained under isocratic conditions for a mixture of 13 amino acids; this method may be extended to resolve a larger number of amino acids in a complex mixture by using gradient elution.

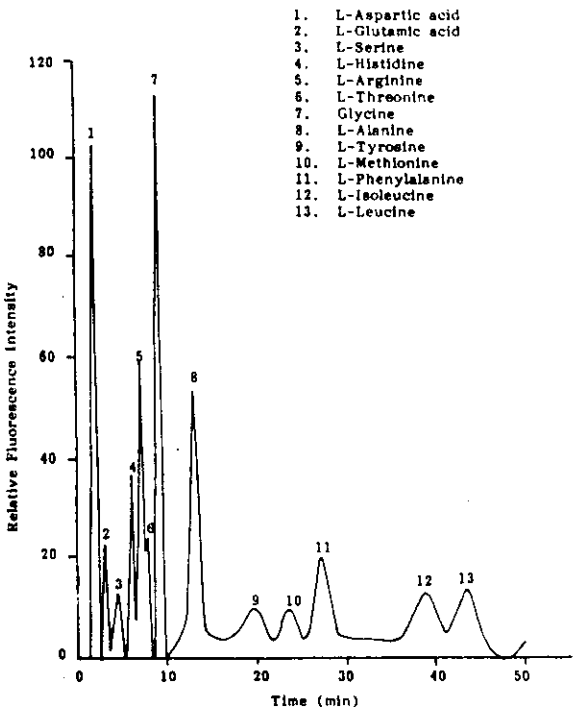

Fig. 1: Elution chromatogram of standard amino acid mixture.

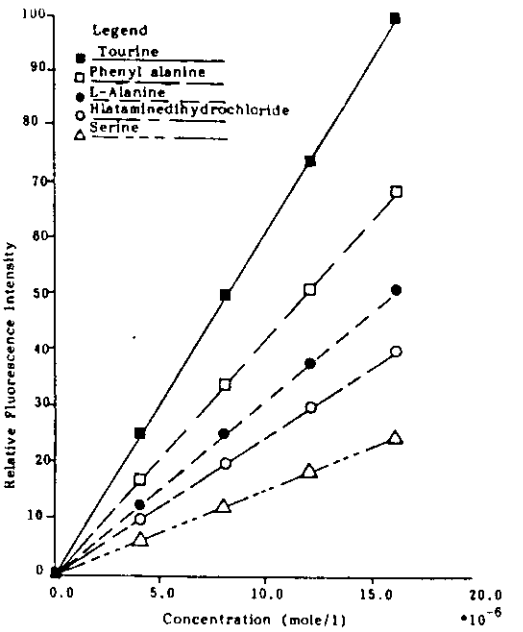

Fig. 2: Standard curves obtained with various amino acids using NDA as fluorigenic reagent.

Analytical application

In order to evaluate the usefulness of the naphthalene-2,3-dicarboxaldehyde (NDA) as a fluorescent label in the pre-column derivatisation, the linearity response was examined in this present work. Calibration graphs prepared from the results of injected standard solutions were linear from 1 to 24 n mol/ml amino acid (Figure 2). When measuring fluorescence using a Perkin-Elmer MPF-44B spectrofluorimeter, the fluorescence response decreased in the order glycine, L-taurine, L-alanine, L-methionine to L-arginine.

The high resolution and selectivity of the method allows one to distinguish, separate, detect and quantitate the amino acids present in the sample. A sample consisting of five different amino acids in mixture was derivatised, separated and estimated in this study.

Resolution versus derivatives structure relationship

Separation of different components is controlled by various factors and experimental conditions. Where the composition of a solvent system is an important controlling factor to enhance the column efficiency, the structures of the compounds equally play an important role in reducing or increasing the retention time. Most of the NDA-amino acid derivatives were separated isocratically on the reversed-phase column with acetonitrile-water (65:35 v/v) as eluant. The reaction time varied from 2-45 minutes. This variation of retention time may be due to wide range of polarities. Hydrophobic-hydrophilic balance of the fluorescent derivatives seems to influence the separation process. For example, a hydrocarbon stationary phase may retain on it hydrophobic parts of the molecule after it has been squeezed out of the mobile aqueous-acetonitrile phase. This, however, needs confirmation, NDA-amino adducts seem to offer an explanation to it.

The formation of the NDA-amino acid adduct lends a hydrophobic label to the amino acid, which facilitates for an easy separation.

As far as the order of elution is concerned, L-aspartic acid was eluted first, followed by L-glutamic acid, L-serine, L-histidine, L-threonine, glycine, L-alanine, L-tyrosine, L-methionine, L-phenylalanine, L-isoleucine, and L-leucine. This

elution order is in agreement with the order of the OPA-derivatives [15,17,18]. However, L-arginine is the only acid which was eluted unexpectedly before L-throenine in the elution order series.

Conclusion

The present investigation has demonstrated that NDA is a potential label for amino acids. Isocratic reversed-phase chromatography with fluorescence detection at 520 nm gave rapid, reproducible separation of the amino derivatives. More recently the NDA fluorigenic reaction has been modified [19] by replacing the thiol with sodium cyanide in the derivatisation reaction. The end product is a 1-cyano-2-substituted benzo-isoindole. This reaction has successfully been applied at pH 9.0, in a borate buffer, to determine catecholamine [20] using pre-column reversed phase high performance liquid chromatography (HPLC). The derivative exhibits fluorescence at 483 nm with excitation at 420 nm and is suitable for both fluorescence and chemiluminescence detection. These studies claimed that relative to the OPA/thiol system, the NDA method offered improved fluorescent product stability, enhanced sensitivity and the absence of apparent fluorescence quenching for primary amines containing L-amido functional groups.

Experimental

Instrumentation

The HPLC system used consisted of a Pye-Unicam X-PS pump, a Pye-Unicam PU4020 UV-detector and/or Perkin-Elmer 2000 fluorescence spectrophotometer with a flow cell. It also consisted of a 100 mm x 5 mm i.d. column pre-packed with 5 mm diameter ODS-hypersil. The analytical column was fitted with a silica pre-column before the injection valve and the injection were made using a Rheodyne 7125 valve fitted with a 20 µl loop. Fluorescence detection was carried out at 520 nm (excitation at 460 nm).

Chromatographic conditions

The flow rate of the carrier stream was maintained at 1.0 ml/min at a pressure of 3000 psi. Mobile phase contained a mixture of a acetonitrile and water (65:35% v/v).

HPLC pre-column derivatisation procedure

A dilute solution of the sample (0.2-0.3 ml) was treated with 1 ml of 10.9 mM B-cyclodextrin (B-CD). Borax-sodium hydroxide buffer (0.6 ml, pH 10.0), aqueous thiol solution (0.1 ml, 1% v/v) were then added followed by the addition of the suitable fluorigenic reagent in slight excess over the sample. The final volume was then made up to 5 ml with water. The derivative formed was thus ready for injection into the column.

References

1. D. H. Spackman, S. Moore and W. H. Stein, *Anal. Chem.*, **30**, 1190 (1958).
2. J. R. Bensen and P. E. Hare, *Proc. Nat. Acad. Sci., USA*, **72**, 619 (1975).
3. M. Roth, *J. Clin. Chem. Clin. Biochem.*, **14**, 361 (1976).
4. B. Josefsson, P. Lindroth and G. Ostling, *Anal. Chim. Acta.*, **89**, 21 (1977).
5. M. Roth, *Anal. Chem.*, **43**, 880 (1971).
6. S. Stein, et al., *Arch. Biochem. Biophys.*, **155**, 202 (1973).
7. H. Englehardt, et. al., *J. Biol. Chem.*, **45**, 336 (1974).
8. D. R. Koop, et al., *J. Biol. Chem.*, **257**, 8472 (1982).
9. Jr. S.S. Simons and D. F. Johnson, *J. Org. Chem.*, **43**, 2886 (1978).
10. Jr. S. S. Simons and D. F. Johnson, *Anal. Biochem.*, **82**, 250 (1977).
11. J. F. Stobaugh, Ph.D. Thesis, The University of Kansas (1982).
12. I. Molnar and C. Horvath, *Clin. Chem.*, **22**, 1497 (1976).
13. T. P. Davis, Jr. C.W. Gehrke, T. D. Cunningham, K. C. Kuo, K. O. Gerhardt, H. D. Johnson and C. H. Williams, *Clin. Chem.*, **24**, 1317 (1978).
14. D. C. Turnell and D. H. Cooper, *Clin. Chem.*, **28**, 527 (1982).
15. M. Griffin, S. J. Price and T. Palmer, *Clin. Chim. Acta*, **125**, 89 (1982).
16. J. W. Cook, L. Hunter and R. Schoental, *J. Chem. Soc. Suppl.*, 228 (1949).
17. B. N. Jones, S. Raabo and S. Stein, *J. Liq. Chromatogr.*, **4**, 565 (1981).
18. P. Lindroth and K. Mopper, *Anal. Chem.*, **51**, 1667 (1979).

19. B. Matuszewski, R. Givens, K. Srinivasachar and R. G. Carlson, in Abstracts, 10th International Symposium on Column Liquid Chromatography, San Francisco, California, pp. 1303 (1986).

20. T. Kawasaki, O. Wong, K. Imai, T. Hignchi, R. S. Givens, J. Stobugh and K. Kuwana, in Abstracts, 10th International Symposium on Column Liquid Chromatography San Francisco, California, pp. 3511 (1986).