Journal of Chromatography, 302 (1984) 21–29
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 417

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS IN URINE AND CEREBROSPINAL FLUID

STANLEY LAM*, HIDEHIKO AZUMAYA and ARTHUR KARMEN
Albert Einstein College of Medicine, The Bronx, NY 10461 (U.S.A.)

SUMMARY

Two different methods for analyzing amino acids by reversed-phase high-performance liquid chromatography (HPLC), both of which can separate D- and L-stereoisomers, have been used for studying the amino acid composition of cerebrospinal fluid (CSF) and urine. One method, by which Dns derivatives of amino acids are separated as mixed chelate complexes with Cu(II) and a single stereoisomer of a second amino acid, was used to analyze CSF. CSF contains *ca.* 10 μmole/l per amino acid, compared to 100 μmole/l in serum. The high sensitivity of fluorescence detection enabled complete analysis, starting with 50 μl of fluid.

The second method, which uses lower concentrations of both the copper and the second amino acid and detects amino acids by the change in absorbance of the copper complex, was used to measure the urine concentration of the lysine metabolite, pipecolic acid (piperidine-2-carboxylic acid), a secondary amino acid that is difficult to detect by the more usual detection methods. Our procedure involves passing urine through a cation-exchange column, collecting the fraction containing pipecolic acid, and chromatographing it on a reversed-phase HPLC column with a mobile phase containing L-aspartame and Cu(II). To assess the utility of the method, urine samples from a patient given loading doses of D- or L-isomers were analyzed. When either isomer was administered, both D- and L-isomers were detected, but in different proportions. Varying proportions and concentrations of both isomers were also detected in the urines of patients with hyperpipecolatemia from different metabolic abnormalities.

INTRODUCTION

Highly selective separation of amino acids can be accomplished by reversed-phase high-performance liquid chromatography (HPLC) of mixed chelate complexes of the analyte acids formed by reaction with Cu(II) and a second amino acid in the mobile phase. Enantiomers are resolved if a chiral Cu(II) complex is present in the mobile phase with which amino acids can form two diasteric ternary complexes of different stability:

$$\text{Cu(L-Ax)}_2 + \text{L-Ay} \rightleftharpoons \text{Cu(L-Ax)(L-Ay)}$$

$$\text{Cu(L-Ax)}_2 + \text{D-Ay} \rightleftharpoons \text{Cu(L-Ax)(D-Ay)}$$

0021-9673/84/$03.00

Many approaches to chromatography of mixed chelate complexes have been reported. Karger and co-workers[1,2] used the complex of Zn(II)–L-2-alkyl-4-octyldiethylenetriamine complexes; Hare and Gil-Av[3] the Cu(II)–proline, Weinstein *et al.*[4] the Cu(II)–N,N-di-*n*-propyl-L-alanine, Grushka and co-workers[5,6] the Cu(II)–aspartame and Cu(II)–aspartyl derivatives, Wernicke[7] the Cu(II)–phenylalanine and Lam and co-workers[8–10] the Cu(II)–proline, Cu(II)–arginine, Cu(II)–histidine and Cu(II)–histidine methyl ester complexes. In the work described here, we used two copper complexation methods for the analysis of the amino acid composition of body fluids and for the separation of the optical isomers of the lysine metabolite, pipecolic acid, in urine. This should enable us to study several examples of abnormal lysine metabolism.

EXPERIMENTAL

Reagents

Acetonitrile, distilled in glass, was bought from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). D- and L-Dns amino acids were purchased from Sigma (St. Louis, MO, U.S.A.) and Pierce (Rockford, IL, U.S.A.).

Instrumentation

The chromatograph consisted of two Altex 110A pumps and a Model 420 gradient microprocessor (Altex Scientific, Berkeley, CA, U.S.A.); a Rheodyne 7105 injection valve; analytical columns, 15 × 0.42 cm I.D., packed with Nucleosil C_{18} by the downward slurry technique for analysis of the Dns-amino acids; and 10 × 0.42 cm I.D. columns, similarly packed, for analysis of pipecolic acid. For pipecolic acid analysis, detection by UV absorbance was used with a Spectroflow 757 UV detector (Kratos, Ramsey, NJ, U.S.A.). Dns-amino acids were detected with a Fluoro-tec filter fluorometer (American Research Products, Kensington, MD, U.S.A.). The amplified detector signals were read out on a Model 4416 data system (Nelson Analytical, Cupertino, CA, U.S.A.) and a Model 56 chart recorder (Perkin Elmer, Norwalk, CT, U.S.A.).

Procedure

Pipecolic acid analysis. Patient urine was fractionated by the procedure of Dancis and Hutzler[11] on a 24 × 0.3 cm I.D. column, packed with cation-exchange resin (MR-201, Mark Instrument Co., Villa Nova, PA, U.S.A.), maintained at 60°C. The eluent was 0.2 *M* buffer, pH 2.9, prepared by adding 147 g of sodium citrate dihydrate, 32.5 g of Brij 35 detergent, 2.5 g of Na_2EDTA, 1 ml of octanoic acid, 0.25 ml pentachlorophenol (10% in isopropanol), and 10 ml thiodiglycol to 9.6 l of water. The eluent flow-rate was 0.33 ml/min. The fraction eluted at 68 min, which was found to contain the pipecolic acid, was collected and evaporated to dryness for injection into the HPLC column.

HPLC was performed on a reversed-phase column, through which buffer containing 295 mg L-aspartame (L-aspartyl-L-phenylalanine methyl ester) and 100 mg of copper sulfate in 1 l of water had been pumped overnight, prior to the analyses, to charge the stationary phase with these materials. The mobile phase was 50 ml of the above aspartame–copper sulfate solution, diluted 1:20 with copper sulfate (100 mg/l

water). Pipecolic acid was detected by the UV absorbance of the copper complex at 234 nm.

Dns derivatives of amino acids from body fluids were prepared by the method described by Tapuhi *et al.*[12]. The synthesis was performed in a polypropylene microcentrifuge tube by adding 50 μl of cerebrospinal fluid or amino acid standard to 25 μl of saturated lithium carbonate, then adding 50 μl of Dns chloride in acetonitrile (250 μg/ml) and heating the vial at 60°C for 15 min. For analysis, an aliquot of the reaction mixture was injected into the chromatograph without further treatment. The buffer was $5.0 \cdot 10^{-3}$ *M* of L-histidine methyl ester, $2.5 \cdot 10^{-3}$ *M* copper sulfate and 2.0 g of ammonium acetate, pH 5.5. The mobile phase was a stepwise gradient, blended with the buffer and a 45% acetonitrile solution of the same buffer.

RESULTS

Analysis of pipecolic acid in urine

The HPLC system described, which employed a lower-than-usual concentration of copper sulfate and second amino acid and detection by UV absorbance, resolved both D- and L-proline and D- and L-pipecolic acid (Fig. 1).

A urine sample, obtained from a volunteer subject who had injested 1 g of D-pipecolic acid, contained, as excepted, the D-isomer, which is believed not to be metabolized further but, as well as a smaller quantity of a compound with the retention time of the naturally occurring L-isomer (Fig. 2). Rechromatography of the presumed L-isomer by a second chromatographic procedure was consistent with the identification. The urine specimens from two patients with hyperpipecolatemia re-

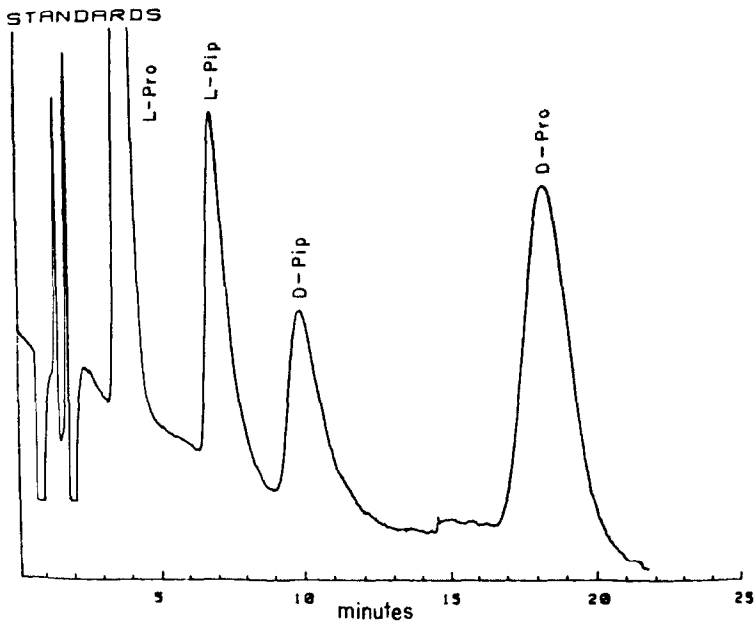

Fig. 1. Separation of stereoisomers of proline and pipecolic acid. The column was equilibrated with "loading buffer", containing 295 mg L-aspartame and 100 mg of copper sulfate in 1 l of water. Mobile phase: 100 mg of copper sulfate and 25 ml of loading buffer, diluted to 1 l with water. Flow-rate: 1.5 ml/min.

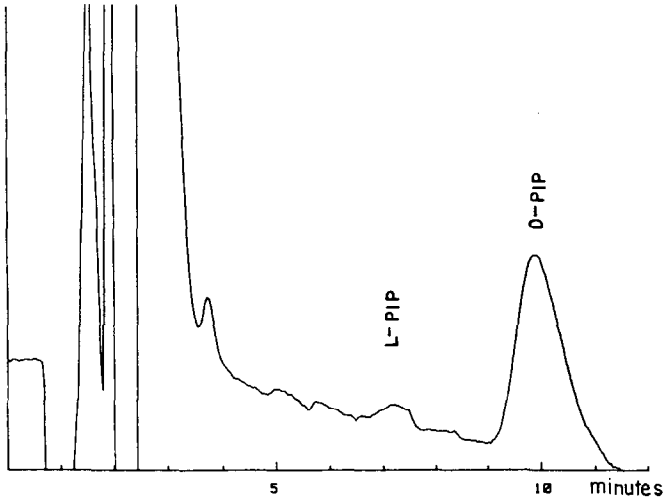

Fig. 2. Separation of pipecolic acid in urine of a volunteer who ingested 1 g of the D-isomer.

sulting from different enzyme defects had different distributions of isomers: one contained only the L-isomer (Fig. 3) while the other one contained both the D- and L-isomers (Fig. 4).

Analysis of amino acids in CSF

Amino acid profiling of CSF was carried out with the Cu(II)–L-histidine methyl ester complexing system we reported previously[10]. Complete assays of free, or non-protein amino acids could be obtained, starting with 50 μl of fluid, a volume which could be obtained as remainders of specimens analyzed in our clinical laboratory for glucose, protein, and cell content. More than 50 CSF samples have been analyzed. Most of them had amino acid profiles similar to that shown in Fig. 5, which was obtained from a patient with pneumonia and who was suspected of, but proved not

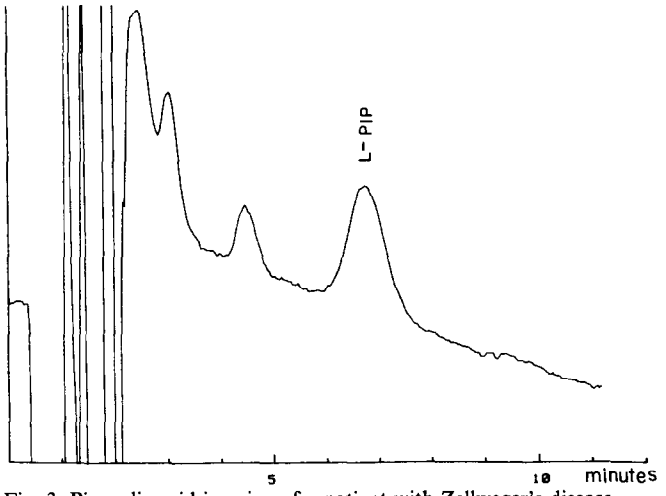

Fig. 3. Pipecolic acid in urine of a patient with Zellweger's disease.

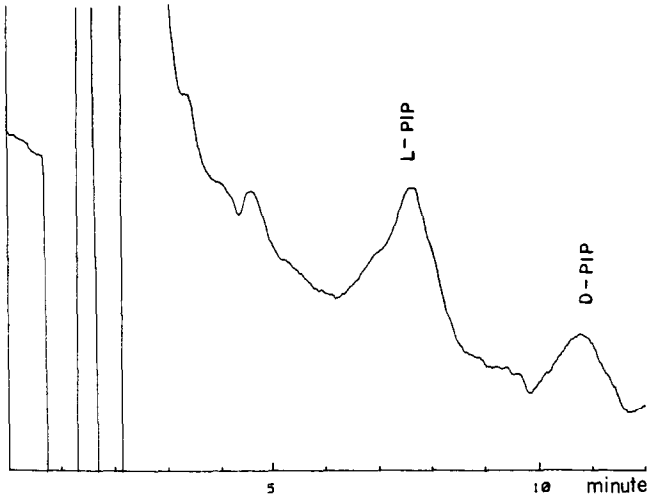

Fig. 4. Pipecolic acid in urine of a patient with hyperlysinemia.

to have, meningitis. The profiles obtained from samples from patients with a variety of other diseases, including disseminated cancer, were similar (Fig. 6). More obvious differences were observed in a sample from a patient with proven meningitis, which had different proportions of aromatic and aliphatic amino acids than the others (Fig. 7). A sample from a patient with systemic lupus erythematosus had markedly elevated ammonia and lower glutamic acid concentrations (Fig. 8). The retention times were quite reproducible, even though a gradient was employed. In analyses of four aliquots

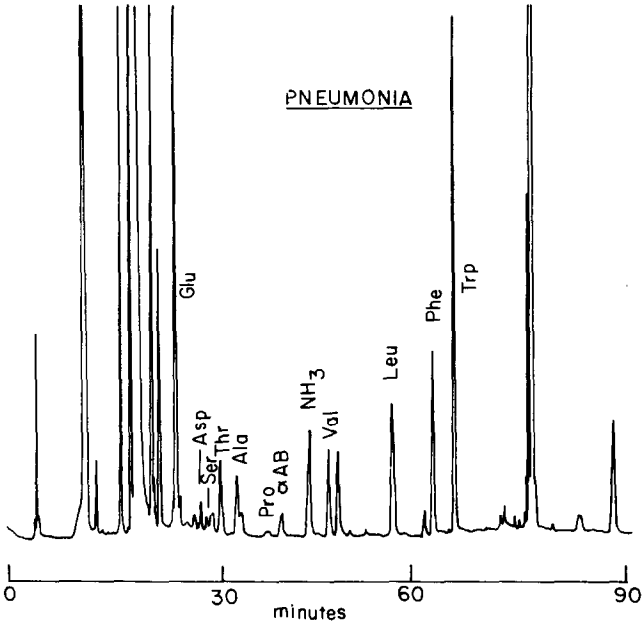

Fig. 5. Amino acid profile of CSF from a patient with pneumonia.

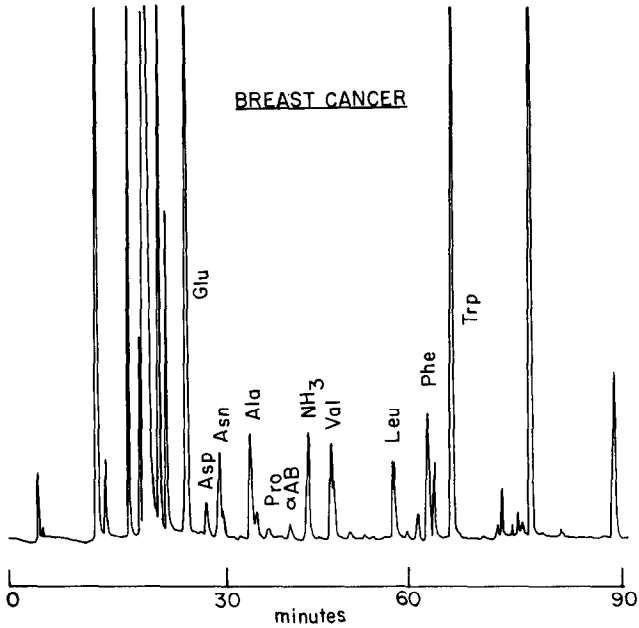

Fig. 6. Amino acid profile of CSF from a patient with breast cancer.

of a standard mixture, derivatized and chromatogrpahed separately, the retention times were reproducible within less than 0.5% relative standard deviation (R.S.D.). The peak areas were reproducible in most instance to within 5% (R.S.D.), except where peaks overlapped.

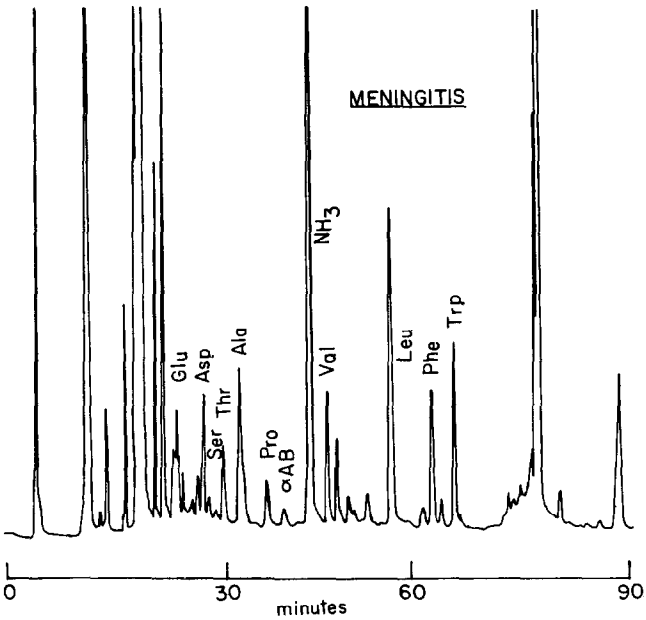

Fig. 7. Amino acid profile of CSF from a patient with meningitis.

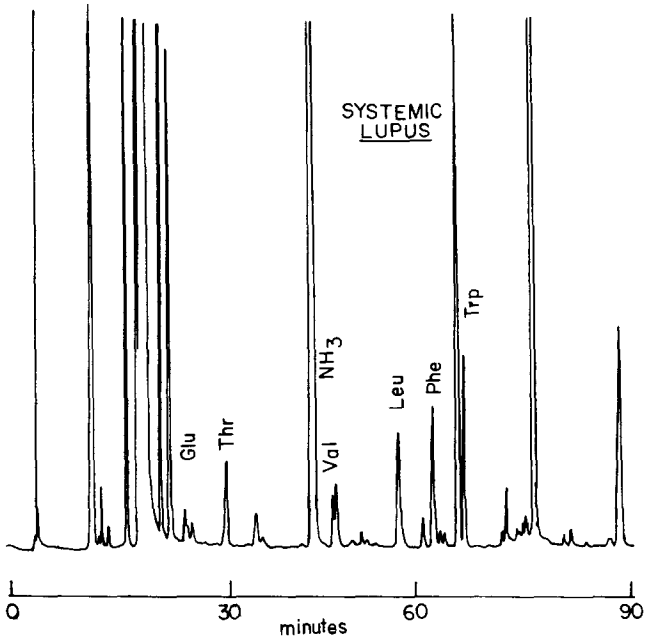

Fig. 8. Amino acid profile of CSF from a patient with systemic lupus.

DISCUSSION

We have previously described a methodology for the analysis of amino acids that employs HPLC of Dns derivatives and mixed chelate complexcation of the derivatives with Cu(II) and one of the pair of optical isomers of an amino acid[10]. With reversed-phase columns and mobile-phase gradients of acetonitrile in buffer containing the Cu(II)–histidine methyl ester, sixteen amino acid pairs are resolved in less than 2 h.

Interest in the amino acids of the CSF stems for the hypothesis that the ratio of aromatic to aliphatic acids may help predict mental status, particularly in patients with depressive disorders. Also of interest to us was the possibility that inflammatory disease of the central nervous system causes a change in the amino acid composition of the CSF. CSF contains *ca.* 10 μmole/l per amino acid, compared to 100 μmole/l in serum. The high sensitivity of fluorescence detection of Dns derivatives permitted the profile of amino acid composition of CSF to be carried out starting with 50 μl of sample, a quantity that is usually readily available from samples collected for more usual diagnostic testing. Of the more than 50 samples analysed, most had similar profiles. Several samples had easily discernible different composition, particularly five samples from a patient with bacterial meningitis and one from a patient with metastatic cancer of the breast. We were particularly interested in the possibility of detecting D-amino acids in CSF from patients with bacterial meningitis, since bacteria synthesise these isomers. Trace quantities of compounds with the retention times of D-isomers were detected. However, there generally were substantially greater numbers of compounds present than could be identified as amino acids by reference to the

standards available. Further work is required to confirm the identity of the compounds. The sensitivity of this method should facilitate study of the relationship between amino acid composition of the blood and that of the CSF.

The simultaneous detection and resolution of enantiomers of secondary amino acids is a more complex problem. N-dansylating results in highly fluorescent derivatives, but the optical isomers are not resolved by the complexation methods, possibly because the derivative, which contains a tertiary amino group, does not form the proper complex with the copper[1,9]. Analysis of underivatized amino acids with the copper system, followed by post-column reaction with O-phthalaldehyde for detection, results in clear separation of enantiomers, but the secondary amino acids, which do not form fluorescent derivatives with O-phthalaldehyde[3,4] are difficult to detect. The procedure proposed here, which uses low concentrations of Cu(II)–L-aspartame as the resolving agent, although not as sensitive as detection by fluorescence of the Dns derivatives, is a useful compromise. Both D,L-proline and D,L-pipecolic acid are resolved and detected.

Patients with at least three genetic diseases: Zellweger's disease, hyperpipecolatemia, and familial hyperlysinemia, excrete excessive quantities of pipecolic acid in their urine. In the metabolism of lysine in normal subjects, lysine condenses with α-ketoglutarate to give saccharopine, a reaction catalyzed by the enzyme, lysine–ketoglutarate reductase[13]. Saccharopine is subsequently oxidized to α-amino adipic acid, which eventually is converted to glutaryl CoA. Lysine also is metabolized to α-keto-ε-aminocaproic acid by cyclization to Δ^1-piperideine-2-carboxylic acid, which in turn is reduced to pipecolic acid. Lysine also enters yet other minor pathways by which homocitrulline and homoarginine are formed. In hyperlysinemia, pipecolic acid exretion increases because of overloading of the secondary pathway. The major metabolic pathway is blocked because of the absence of lysine–ketoglutarate reductase activity. In hyperpipecolatemia, the defect is presumed to be the deficiency in aminoadipic semialdehyde oxidase[13]. The mechanism of Zellweger's disease is even less well understood.

The pathway of metabolism of pipecolic acid can be of interest in defining the metabolic abnormality or enzyme deficiency in individual patients. Pipecolic acid found in the urine has been assumed to be the L-isomer. However, it has been postulated that the L-isomer may be racemized in one of the pathways. It is well known that the rabbit has a separate pathway to degrade the D-isomer. A stereoselective procedure for analyzing pipecolic acid would help in these studies.

D-Pipecolic acid, administered to a volunteer, was recovered in the urine. There was a suggestion of conversion of a small fraction of it to the L-isomer. Two patients with hyperpipecolatemia had different proportions of D- and L-isomers in their urine. A patient with Zellweger's disease excreted excessive quantities of L-pipecolic acid, while a patient with hyperlysinemia excreted both D- and L-isomers. We believe this is the first observation that the D-isomer of a natural amino acid is found in any excess amounts in man. The presence of excessive D-isomer may reflect overloading of the pipecolic pathway.

The procedure is as yet only semi-quantitative because of the limited stability of the system. Aspartame loaded on the column in relatively higher concentration is stripped off continuously by the more dilute mobile phase used to permit UV-absorbance detection. The retention of solutes therefore decreases with prolonged use

and, after about 8 h, the column requires regeneration and re-equilibration. Despite this limitation, this chromatographic system proved useful in the initial studies of pipecolic acid metabolism in individual patients reported here.

REFERENCES

1 J. N. LePage, W. Lindner, G. Davies, D. E. Seitz and B. L. Karger, *Anal. Chem.*, 51 (1979) 433.
2 W. Lindner, J. N. LePage, G. Davies, D. E. Seitz and B. L. Karger, *J. Chromatogr.*, 185 (1979) 323.
3 P. E. Hare and E. Gil-Av, *Science*, 204 (1979) 1226.
4 S. Weinstein, M. H. Engel and P. E. Hare, *Anal. Biochem.*, 121 (1982) 370.
5 C. Gilon, R. Leshem, Y. Tapuhi and E. Grushka, *J. Amer. Chem. Soc.*, 101 (1979) 7612.
6 C. Gilon, R. Leshem and E. Grushka, *Anal. Chem.*, 52 (1980) 1206.
7 R. Wernicke, *Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March 5–9, 1984*, Abstract 45.
8 S. Lam and A. Karmen, *J. Chromatogr.*, 239 (1982) 451.
9 S. Lam, F. Chow and A. Karmen, *J. Chromatogr.*, 199 (1980) 295.
10 S. Lam and A. Karmen, *J. Chromatogr.*, 289 (1984) 339.
11 J. Dancis and J. Hutzler, *Biochim. Biophys. Acta*, 675 (1981) 411.
12 Y. Tapuhi, D. E. Schjmidt, W. Lindner and B. L. Karger, *Anal. Biochem.*, 115 (1981) 325.
13 H. Ghadimi, in J. B. Stanbury, J. B. Wyngaarden and D. S. Fredrickson (Editors), *The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York, 3rd ed., 1972, p. 393.