Journal of Chromatography, 164 (1979) 355—362
Biomedical Applications

CHROMBIO. 398

DETERMINATION OF PHENYLALANINE IN SERUM USING REVERSED-PHASE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

G.J. SCHMIDT, D.C. OLSON and W. SLAVIN

The Perkin-Elmer Corporation, Norwalk, Conn. 06856 (U.S.A.)

(Received May 1st, 1979)

SUMMARY

A liquid chromatography procedure is reported for determining phenylalanine in small volumes of serum. A 10-μl volume of serum was deproteinized with ethanol and an aliquot was derivatized with dansyl chloride reagent. The dansylated phenylalanine and the norleucine internal standard were separated using reversed-phase chromatography and measured with a fluorescence detector. Linearity was excellent over the range 50—800 mg/l. Within-run precision was better than 4%. Total analysis time including chromatography was approximately 40 min. As little as 300 pg of dansylated phenylalanine was detected.

INTRODUCTION

Phenylketonuria, an inborn error of metabolism, is characterized by incomplete hydroxylation of phenylalanine to tyrosine giving rise to abnormally high serum concentrations of phenylalanine. It is associated with varying degrees of mental retardation. The positive determination of serum phenylalanine concentrations is required as early as possible in the neonate in order to assess the need for proper nutritional therapy.

Several techniques have been utilized for determining serum phenylalanine concentrations. Classical ion-exchange column chromatography [1,2] has been used but generally requires large volumes of serum, which does not lend itself well to neonate screening. Primary screening procedures based on colorimetry [3,4], fluorimetry [5], and microbiological [6] techniques also require large volumes of serum and are not entirely specific for phenylalanine. Gas chromatography has also been used successfully for determining amino acid concentrations in physiological fluids [7]. Adams [8] reported a procedure using gas chromatography for determining phenylalanine in serum by forming the *n*-propyl N-acetyl derivatives. Gas chromatographic procedures that require the formation of a double derivative of the amino acids frequently entail lengthy pretreatment.

Modern liquid chromatography with fluorescence detection has more recent-

ly been used for determining amino acids [9—12] after chemical modification. These procedures have provided detection capability in the picogram range. The dansyl derivatives have been separated using both reversed-phase and normal-phase chromatography [12]. A procedure using these derivatives has been published very recently for determining the amino acid content of several protein hydrolysates [13]. These derivatives are particularly useful in that they may be prepared quickly and render the amino acids relatively non-polar, making reversed-phase chromatography with aqueous based mobile phases well-suited for their separation. We have recently published a procedure using these derivatives for determining ε-aminocaproic acid in small volumes of serum [14].

The procedure which we report here requires only minimal sample pretreatment and may be used with very small volumes of serum. The dansyl derivatives, which may be prepared in only 5 min, appear to be quite stable and may be measured very sensitively using a fluorescence detector.

MATERIALS AND METHODS

Apparatus

We used a Perkin-Elmer Model Series 2/2 liquid chromatograph equipped with a Model 100 column oven, a Rheodyne 7120 injection valve with a 20-μl injection loop and a Model 204A fluorescence spectrophotometer equipped with an LC flow cell (part No. 010-0456). An RP-8 reversed-phase column (part No. 258-1484, 0.46 × 25 cm, particle size 10 μm) was used. All chromatograms were recorded using a Perkin-Elmer Model 56 recorder. Special glassware included 5-ml conical centrifuge tubes, 16 × 100 mm PTFE-lined screw-capped tubes, 2-ml PTFE-lined capped sample vials and 10-μl, 20-μl, 50-μl and 1-ml disposable pipets. A bench-top centrifuge, an evaporation manifold, with a source of dry air, a heating block and a vortex-type mixer were also used. A Model W-185 sonifier, from Branson Sonic Power Co. (Danbury, Conn., U.S.A.) was used for degassing the mobile phase solutions.

Reagents and standards

Methanol and acetone, distilled in glass, were obtained from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). The acetronitrile, distilled in glass, UV grade, was also from Burdick and Jackson. Phenylalanine and norleucine standards were obtained from Sigma (St. Louis, Mo., U.S.A.). Dansyl chloride, 100 g/l in acetone, was obtained from Pierce (Rockford, Ill., U.S.A.); store at 4°. Sodium bicarbonate "analyzed reagent" was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Absolute ethanol was obtained from U.S. Industrial Chemicals Co. (New York, N.Y., U.S.A.). Sodium hydroxide, reagent grade, was obtained from J.T. Baker (Phillipsburg, N.J., U.S.A.).

Individual standard stock solutions were prepared for each amino acid by dissolving the amino acid in 0.01 *N* HCl to give a concentration of 1 mg/ml. The solutions were stored at 4°. The internal standard solution was prepared by diluting 1 ml of the norleucine stock solution with 4 ml deionized water to yield a concentration of 0.2 mg/ml. The solution was stored at 4°.

Dansyl chloride working solution was prepared by diluting 0.1 ml of dansyl chloride stock solution to a volume of 8 ml with acetone. This reagent was

prepared freshly each day. The buffer was prepared by adjusting the pH of a 0.1 mol/l sodium bicarbonate solution to 10.5 with 0.5 *N* NaOH. The buffer was filtered through a 0.45-μm filter before use.

The mobile phase solvents were prepared as follows. Pump A contained 50% acetonitrile in 0.1% phosphoric acid. Pump B contained 25% acetonitrile in 0.1% phosphoric acid. The deionized water used for preparing the mobile phase solvents was filtered before use through a Millipore 0.45-μm filter (Perkin-Elmer part No. 089-0839). Mobile phase mixtures were degassed using sonification at 50 W for 5 min.

Procedure

Add 10 μl of serum, 10 μl of internal standard working solution and 40 μl of ethanol to the bottom of a 5-ml conical centrifuge tube. Mix vigorously for 15 sec on a vortex-type mixer and centrifuge for 1 min at 2000 rpm (710 *g*). Transfer 20 μl of the supernatant to the bottom of a 2-ml sample vial and evaporate to dryness with a gentle current of air. Add 10 μl of pH 10.5 buffer followed by 50 μl of dansyl chloride working solution. Cap the vial tightly and mix vigorously for 15 sec. Heat the vial at 100° for 5 min in a block heater. Remove the vial and allow to cool. Inject 20 μl into the liquid chromatograph.

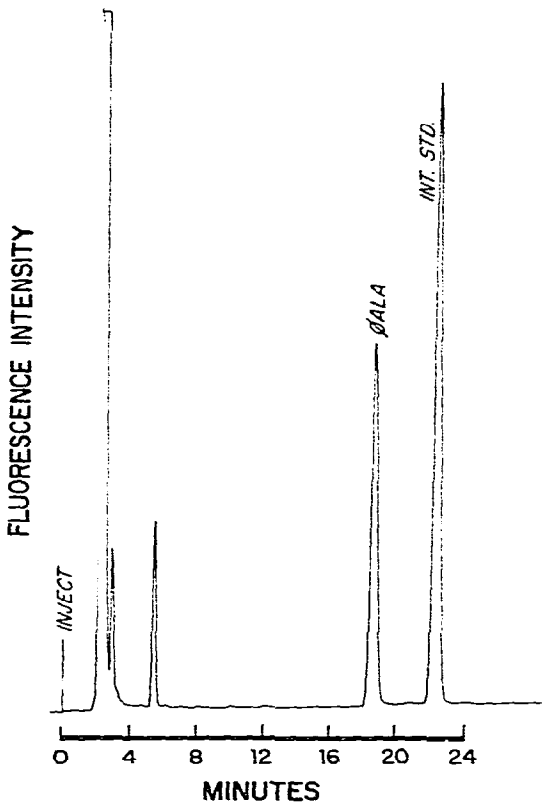

Fig. 1. Chromatogram showing the separation of dansylated phenylalanine (ϕALA) from the norleucine internal standard.

Chromatography

The mobile phase gradient was run from Pump B, containing 25% acetonitrile, to Pump A, containing 50% acetonitrile, at a rate of change of 3.6%/min. The mobile phase flow-rate was 1.5 ml/min and the column temperature was maintained at 60°. The fluorescence detector was set to an excitation wavelength of 320 nm and an emission wavelength of 522 nm. The bandwidths of both monochromators was set at 10 nm. Fig. 1 shows a chromatogram obtained from the injection of a 20-µl aliquot of a reaction mixture containing 1 µg each of phenylalanine and norleucine, which corresponds to approximately 300 ng of each dansylated amino acid on column. The retention times for the dansylated phenylalanine and norleucine were 18.7 and 22.5 min, respectively. It is important to run standards at the beginning of each working day to ensure that the chromatography conditions are satisfactory and to evaluate any long-term change in column efficiency.

Calibration

Calibration was accomplished by using the procedure on prepared serum standards to which varying concentrations of phenylalanine were added. Since serum pools contain amino acids at normal physiological concentrations, dialyzed serum was used to prepare the serum standards. To prepare the dialyzed serum, we took 20 ml of a serum pool and sealed it in a length of 7/8-in. dialysis tubing. The dialysis bag was placed in a beaker containing 2 l of deionized water and the beaker was put in the refrigerator. The water was replaced after 6 h with fresh deionized water and was refrigerated for an additional 16 h. At this time the amino acid concentration in the serum was insignificant.

From this dialyzed serum pool the serum standards containing phenylalanine were prepared over the concentration range of interest. Useful concentrations are 100, 250 and 500 mg/l. Before analyzing patients' sera, the serum standards were analyzed and the peak areas of the phenylalanine and norleucine peaks were measured. For each serum standard the peak area of the phenylalanine was divided by the peak area of the norleucine to obtain the peak area ratio. A working curve was prepared by plotting the peak area ratio of the phenylalanine against the concentration. The patients' sera were analyzed and the peak area ratios were calculated. The phenylalanine concentration in each serum was determined from the working curve.

RESULTS

Recoveries

We studied the recovery of phenylalanine from serum by adding the amino acid to dialyzed serum to yield a concentration of 250 mg/l. Nine samples were analyzed by the procedure and the area of the phenylalanine peaks were calculated and compared with the peak area of pure dansylated phenylalanine solutions. The average recovery was 98%. Recoveries approaching 100% are to be expected since the procedure does not require an extraction where losses might occur.

Linearity

The linearity of the procedure over the concentration range of interest was determined by adding known amounts of phenylalanine to dialyzed serum to give concentrations of 50, 100, 200 400 and 800 mg/l. Aliquots at each concentration were analyzed and the peak area ratios of the phenylalanine to the norleucine internal standard were calculated. A linear relationship of the peak area ratios existed over this concentration range.

Detection

We determined the detection limit for the dansylated phenylalanine by injecting known quantities of aqueous phenylalanine standards which had been previously dansylated. An injection aliquot containing 250 pg of the dansylated amino acid could just be detected using the criterion that the detection limit is equal to a signal that is twice the noise level. Normal physiological concentrations of serum phenylalanine are in the range of 3—22 mg/l. At a serum phenylalanine concentration of 3 mg/l, the lower limit of the normal concentration range, the amount of phenylalanine injected on column after processing a 10-μl serum sample according to the procedure, would be approximately 3 ng.

Precision

Within-run precision was estimated by analyzing nine 10-μl aliquots of a dialyzed serum standard containing added phenylalanine at a concentration of 250 mg/l. The results indicated that a precision of about 4% is obtained at this concentration.

Patient sera

Fig. 2A shows the chromatogram obtained from the analysis of a dialyzed serum standard containing added phenylalanine at a concentration of 300 mg/l. Fig. 2B shows the amino acid pattern of a normal serum sample. The concentration of phenylalanine was calculated to be 37 mg/l.

We analyzed about a dozen sera by this procedure from suspected phenylketonuric (PKU) patients. Fig. 3 shows the results obtained from two such sera. Chromatogram A illustrates an abnormal serum having an elevated phenylalanine concentration calculated to be 270 mg/l. Chromatogram B, likewise, is illustrative of another PKU serum, the phenylalanine concentration of which is 355 mg/l.

Interferences

We dansylated and chromatographed several other naturally occurring amino acids in order to assess them as possibly interfering with this analysis. Those compounds studied are shown in Table I. Leucine and cystine elute closest to the norleucine internal standard, but are well resolved and do not cause any interference. No amino acid tested eluted sufficiently close to the phenylalanine to be an interference. Previous work in our laboratory indicated that the other protein amino acids had retention times sufficiently different from either the phenylalanine or norleucine so as not to interfere.

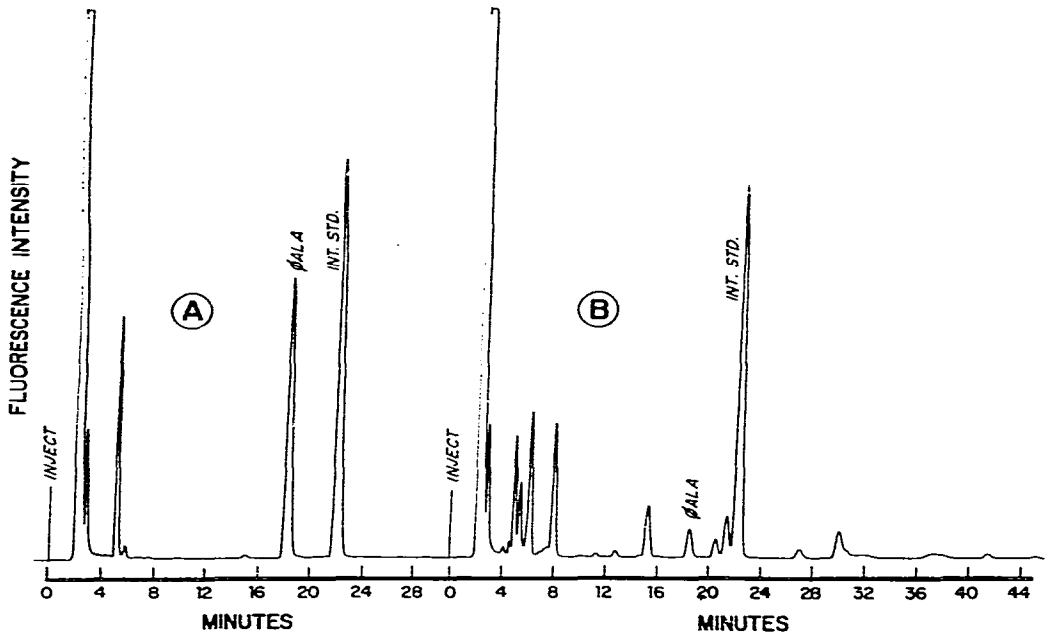

Fig. 2. (A) Chromatogram obtained from a serum standard containing added phenylalanine (ϕALA) at a concentration of 300 mg/l. (B) Chromatogram from a 10-μl aliquot of a normal serum sample.

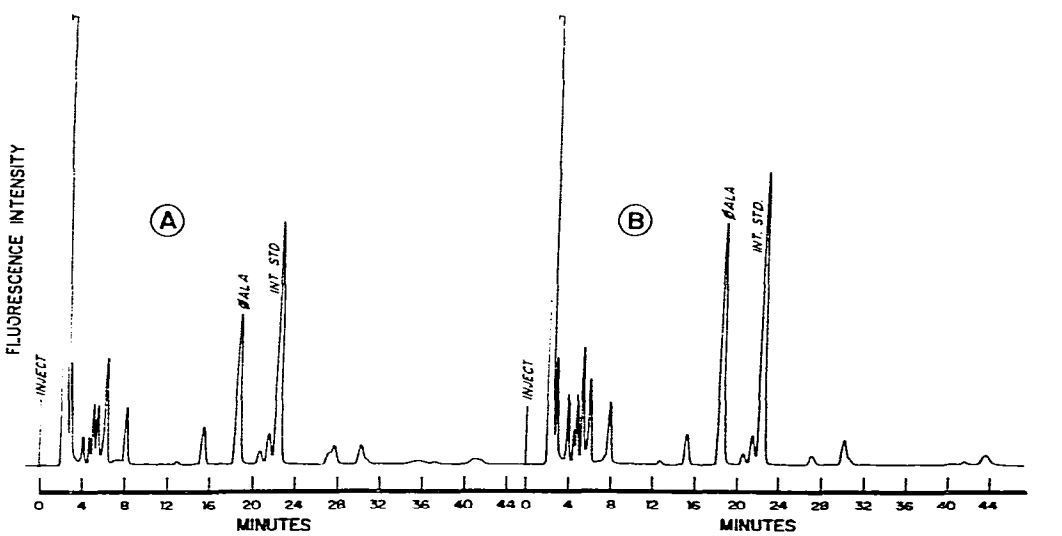

Fig. 3. Chromatograms of sera from patients with phenylketonuria. Chromatogram A shows a serum sample containing an elevated phenylalanine (ϕALA) concentration of 270 mg/l. Chromatogram B is of a patient serum containing phenylalanine at a concentration of 355 mg/l.

TABLE I

INTERFERENCE DATA

Compound	Retention time	Relative retention*
Methionine	14.9	0.636
Valine	15.3	0.655
Phenylalanine	18.7	0.820
Isoleucine	21.0	0.932
Leucine	21.4	0.951
Norleucine	22.4	1.000
Cystine	23.7	1.063
Tyrosine	42.3	1.966

*Norleucine = 1.00.

DISCUSSION

Liquid chromatography coupled with fluorescence detection is becoming an increasingly valuable technique for analyzing endogenous compounds at low concentrations. For those compounds which fluoresce weakly or not at all, derivatization to a suitable fluorophore may often be accomplished. Derivatization to the dansyl derivative is accomplished quickly and easily, and allows detection of picogram quantities.

We found the dansyl derivatives to be stable for at least two weeks when stored at 4°. The dansyl chloride reagent working solution was prepared fresh each day.

The dansylation of amino acids is most generally carried out at a reaction pH of about 9.5. However, we have used a pH of 10.5 in order to also derivatize phenolic hydroxyls, such as tyrosine. It is important to monitor the tyrosine concentration for this analysis because abnormal phenylalanine concentrations accompanied by high tyrosine concentrations may not be indicative of phenylketonuria but may rather reveal varying amino acid metabolism rates after birth. We did not observe any problem at the higher reaction pH due to increased hydrolysis of the dansyl chloride reagent.

The procedure provides very low detection limits for the dansylated amino acids which permits the quantitation of phenylalanine in 10-μl volumes of serum, an important consideration when testing for phenylketonuria in the neonate. Also, the chromatography conditions have been set to allow the separation of other amino acids as well. Thus, the procedure may also prove useful for screening other metabolic dysfunctions.

ACKNOWLEDGEMENTS

We wish to thank Dr. Peter Jatlow, Yale—New Haven Hospital, for supplying the PKU sera. We are also indebted to Elena Katz, our colleague at Perkin-Elmer, for her helpful advice.

REFERENCES

1 S.H. Jackson, W.B. Hanley, T. Geno and G.D. Gosse, Clin. Chem., 17 (1971) 538.

2 A. Mondino and G. Bongiovanni, J. Chromatogr., 67 (1972) 49.
3 H. Varley, Practical Clinical Biochemistry, Interscience Books, New York, 1967.
4 G.A. Jervis, Proc. Soc. Exp. Biol. Med., 81 (1952) 715.
5 M.W. McCaman and E. Robins, J. Lab. Clin. Med., 59 (1962) 885.
6 R. Guthrie and A. Susi, Pediatrics, 32 (1963) 338.
7 K. Blau, in H.A. Szymanski (Editor), Biomedical Applications of Gas Chromatography, Vol. 2, Plenum Press, New York, 1968, p. 1.
8 R.F. Adams, Clin. Chem. Newslett., 4 (1972) 39.
9 E. Johnson, A. Abu-Shumays and S.R. Abbott, J. Chromatogr., 134 (1977) 107.
10 M.R. Downing and K.G. Mann, Anal. Biochem., 74 (1976) 298.
11 J.R. Cronin and P.E. Hare, Anal. Biochem., 81 (1977) 151.
12 E. Bayer, E. Grom, B. Kaltenegger and R. Uhmann, Anal. Chem., 48 (1976) 1106.
13 J.M. Wilkinson, J. Chromatogr. Sci., 16 (1978) 547.
14 R.F. Adams, G.J. Schmidt and F.L. Vandemark, Clin. Chem., 48 (1977) 1226.