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HIGH-SPEED LIQUID CHROMATOGRAPHIC DETERMINATION OF PHENYLPYRUVIC ACID

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SUMMARY

A high-speed liquid chromatographic method has been developed for the determination of urinary phenylpyruvic acid. This acid is converted by treatment with naphthalene-2,3-diamine into 3-benzyl-2-hydroxybenzoquinoxaline, which is extracted into carbon tetrachloride for separation. 2-Mercaptoethanol is a useful stabilizer, and 2-chlorothioxanthone is a suitable internal standard. The method is specific, and the results are not affected by the presence of such 2-oxo-acids as pyruvic acid, 2-oxobutyric acid, 2-oxoglutaric acid, and 4-hydroxyphenylpyruvic acid.

INTRODUCTION

Phenylketonuria is an inherited disease that may cause mental deficiency if not properly treated. The disease is characterized primarily by a metabolic error that prevents phenylalanine in the body from being converted into tyrosine. As the amount of phenylalanine increases, the amino acid is de-aminated to phenylpyruvic acid (PPA), the blood concentration and urinary excretion of which become excessive. The determination of PPA offers a useful technique for differentiating between phenylketonuria and hyperphenylalaninaemia.

Hitherto, PPA has usually been determined by colorimetric methods depending on a reaction with ferric chloride[1,2] or 2,4-dinitrophenylhydrazine[3]; these methods, however, lack specificity. Although the enol–borate complex method[4–6] is more sensitive and selective, it is affected by the presence of 4-hydroxyphenylpyruvic acid, which is usually formed at increased levels in tyrosinaemia.

This paper describes the development of a high-speed liquid chromatographic determination of PPA; the method is based on the formation of 3-benzyl-2-hydroxybenzoquinoxaline (BHQ) by a reaction between PPA and naphthalene-2,3-diamine.

EXPERIMENTAL

The apparatus used in this work was a Shimadzu–Du Pont liquid chromatograph 840 equipped with a UV absorption detector (254 nm). The separation was

carried out on a 1-m column of Zipax Permaphase ETH (Shimadzu Seisakusho, Kyoto, Japan).

PPA, naphthalene-2,3-diamine and 2-chlorothioxanthone were obtained from Tokyo Organic Chemicals (Tokyo, Japan). The other reagents and organic solvents used were of reagent grade.

Preparation and purification of naphthalene-2,3-diamine

Naphthalene-2,3-diamine was stable as its sulphate, which was prepared by adding 50% aqueous sulphuric acid to an ethanol solution of the diamine; the product was recrystallized from 2% aqueous sulphuric acid.

Preparation of BHQ

BHQ was prepared by interaction of PPA (500 mg) and naphthalene-2,3-diamine (450 mg) in methanol (20 ml) and 2 *N* aqueous hydrochloric acid (20 ml) at about 40° for 1 h. The product was filtered off, washed with water, dried and recrystallized from ethyl acetate; its m.p. was 270 ± 1°. Analysis: (calculated for $C_{19}H_{14}ON_2$) C, 79.79%, H, 4.93%, N, 9.80%; found C, 79.77%, H, 4.92%, N, 9.68%.

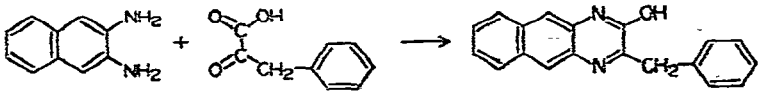
Procedure

To 100 μl of an aqueous sample solution were added 5 ml of 2.5 *N* aqueous hydrochloric acid containing 3 mg of naphthalene-2,3-diamine sulphate, 10 μl of 2-mercaptoethanol and 3 μg of 2-chlorothioxanthone in 100 μl of methanol. The mixture was warmed in a water-bath at about 80° for 2 h, then cooled, and the BHQ formed was extracted into 10 ml of carbon tetrachloride. The organic phase was washed with 10 ml of 3 *N* aqueous hydrochloric acid, the carbon tetrachloride was evaporated in a rotary evaporator, the residue was dissolved in a few drops of NN-dimethylformamide, and 10 μl of this solution were subjected to high-speed liquid chromatography under the conditions shown in Fig. 1.

RESULTS AND DISCUSSION

Hinsberg[7] reported originally that 2-oxo-acids react with *o*-phenylenediamine to form quinoxaline derivatives in aqueous acidic media; this reaction has been applied to the fluorimetric[8] or gas chromatographic determination[9] of 2-oxo-acids. In our work, such a reaction is used to prepare a derivative of PPA for high-speed liquid chromatographic separation.

As the result of preliminary experiments on a few types of aromatic *o*-diamines, naphthalene-2,3-diamine was chosen as a suitable reagent.



This derivatization of PPA is considered to result in a large increase in its affinity for non-polar solvents. Various polar compounds occur in biological samples, and, accordingly, a combination of this derivatization with reversed-phase chromatography offered a convenient method for the determination of PPA in such samples.

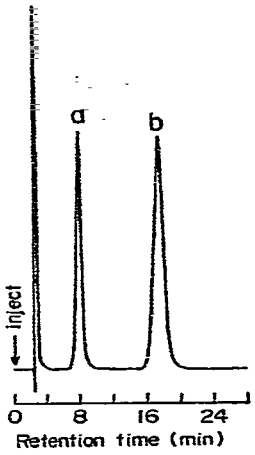


Fig. 1. Liquid chromatogram of a standard mixture of BHQ (peak a) and 2-chlorothioxanthone (internal standard; peak b). Operating conditions: column, 1 m of Permaphase ETH; mobile phase, water–acetonitrile (18:7); column temperature, 40°; flow-rate, 0.5 ml/min; detector, UV photometer.

For this purpose, a 1-m column of Zipax Permaphase ETH was used. Fig. 1 shows a typical chromatogram obtained from a standard mixture of BHQ and the internal standard (2-chlorothioxanthone). A good separation is attained in only 20 min, even without a gradient-elution technique.

As a result of an investigation into the effect of hydrochloric acid concentration in the reaction mixture, 2.5 *N* was selected as a suitable concentration. Fig. 2a shows a chromatogram obtained by allowing PPA to react with naphthalene-2,3-diamine in 2.5 *N* hydrochloric acid. A peak possibly attributable to a by-product can be seen ahead of the peak for BHQ. The ratio of these two peaks varied with slight changes in the reaction conditions, and the results were significantly affected. Spikner and Towne[8] reported that sulphuric acid was the preferred medium for a similar reaction, because

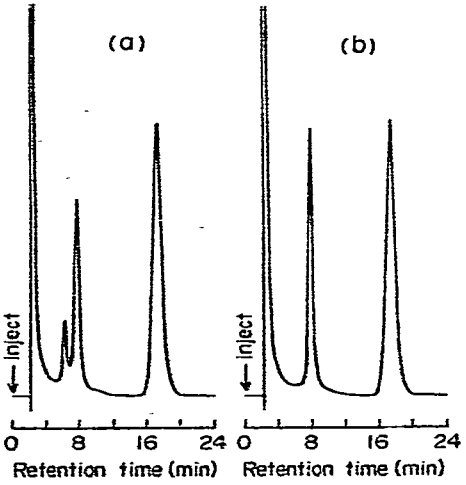
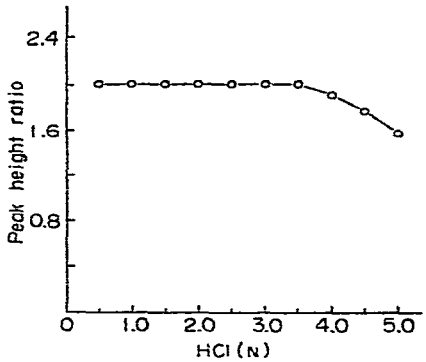


Fig. 2. Effect of 2-mercaptoethanol on the reaction.

aromatic *o*-diamines and the quinoxaline products were stabilized against oxidation in this medium. We attempted to carry out the reaction under similar conditions, but did not obtain good results; however during this work, it was fortunately discovered that formation of the by-product was depressed by addition of 2-mercaptoethanol. As shown in Fig. 2b, the addition of 10 μl of 2-mercaptoethanol to the reaction mixture suppressed formation of the by-product almost completely. The other reaction conditions, *e.g.*, temperature, time and concentration of naphthalene-2,3-diamine were also investigated.

Next, the extractability of BHQ was studied. This compound could be easily extracted from the aqueous layer with such varied organic solvents as carbon tetrachloride, dichloromethane, diethyl ether, chloroform and ethyl acetate; carbon tetrachloride was chosen as being the most suitable, as it separated clearly from the aqueous phase as a lower layer, which was convenient for the separation procedure. Fig. 3 shows the relationship between the extractability of BHQ into carbon tetrachloride and the hydrochloric acid concentration in the aqueous layer; BHQ was completely extractable at hydrochloric acid concentrations below 3 *N*. After the BHQ had been extracted into carbon tetrachloride, the extract was washed with 3 *N* hydrochloric acid in order to remove as much as possible of the excess of naphthalene-2,3-diamine. Under these extraction conditions, the internal standard 2-chlorothioxanthone was also almost completely extracted.



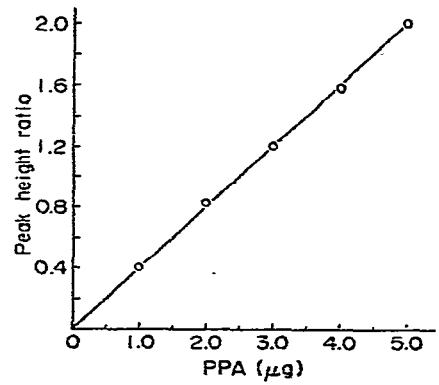


Fig. 3. Relationship between extractability of BHQ into carbon tetrachloride and hydrochloric acid concentration in the aqueous layer.

Fig. 4. Calibration graph for determination of PPA.

Fig. 4 illustrates the calibration graph obtained by the recommended procedure. The relative peak-height ratios of BHQ to internal standard were plotted against the amount of PPA in the solution; the relationship was rectilinear, at least in the concentration range shown.

In this method, the results were not affected by the presence of such 2-oxo-acids as pyruvic, 2-oxobutyric, 2-oxoglutaric and 4-hydroxyphenylpyruvic acids.

In recovery tests, the method was applied to normal human urine to which had been added 3.00 μg of PPA per 100 μl; the reproducibility was determined by carrying out eleven identical analyses, with the results shown in Table I. The chromatogram obtained in the recovery experiments is shown in Fig. 5; no interfering

TABLE I
RECOVERY OF PPA FROM URINE (100 μl)

Amount added (μg)	*Amount found* (μg)	*Recovery* (%)
3.00	2.96	98.7
3.00	3.07	102.3
3.00	3.09	103.0
3.00	2.96	98.7
3.00	3.06	102.0
3.00	3.04	101.3
3.00	3.00	100.0
3.00	3.10	103.3
3.00	3.10	103.3
3.00	3 04	101.3
3.00	2.96	98.7
		($\sigma_{rel.}$ = 1.85%)

peak was observed. Normal urine was used directly in these experiments, but the urine from a phenylketonuric patient should first be appropriately diluted with water, owing to the large amount of PPA in such urine[6].

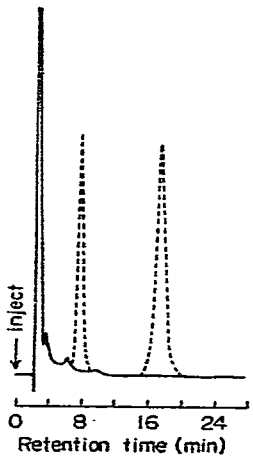


Fig. 5. Liquid chromatogram obtained in recovery experiments from urine: ———, urine blank.

CONCLUSIONS

A method for the high-speed liquid chromatographic determination of PPA in urine has been developed. It is more sensitive, selective and reproducible than are previously reported methods, such as those involving use of 2,4-dinitrophenylhydrazine, ferric chloride or the enol–borate complex.

We are currently studying the application of this method to serum.

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