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DIRECT DETERMINATION OF PHENYLALANINE IN SERUM EXTRACTS OF PHENYLKETONURIA PATIENTS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ZHANG LEFENG*, YU YUNLU and YANG RUIYU
Dalian Institute of Chemical Physics, Academia Sinica, Dalian (China)

SUMMARY

A method for direct determination of phenylalanine (Phe) in serum extract by reversed-phase high-performance liquid chromatography with an octadecylsilane column has been devised. Phe is monitored at 220 nm with a UV detector. The mobile phase is a mixture of methanol and water (10:90) with added phosphoric acid and potassium dihydrogen phosphate and has a pH value of 4.3. The minimum detectable amount is 6 ng. The average contents of Phe found for healthy adults and children are 12 and 15 μg/ml respectively. Phe contents of sera of three phenylketonuria children are about 10 to 15 times higher than these values.

INTRODUCTION

It is known that for phenylketonuria (PKU) patients the normal metabolism of phenylalanine (Phe) to tyrosine (Tyr) is inhibited to some extent. Instead, phenylpyruvic acid is formed from Phe and is excreted in the urine. It is very important to determine the PKU in new-born children in order to combat this disease at the earliest opportunity. Some screening methods have already been established for this purpose. However, sometimes more accurate determinations of the Phe concentration in patient sera are required for clinical purpose. Therefore, it was necessary to devise a direct method for its measurements.

Numerous chromatographic methods, including thin-layer chromatography[1], gas chromatography[2–4] and high-performance liquid chromatography[5–9] have been developed for the analysis of amino acids. However, most authors have focused their attention on the separation of all commonly encountered amino acids by derivatization. The ever-growing improvements in the technique of reversed-phase high-performance liquid chromatography (RPLC) have solved many analytical problems in biomedical and other fields. Hancock *et al.*[9] reported the separation of free amino acids by RPLC, but this was accomplished only for a synthetic mixture of pure amino acids.

We intended to use this technique for the direct and fast measurement of Phe in serum. For this purpose, only the analysis of one amino acid is required and the interferences in separation from other amino acids and extraneous substances can be

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avoided by the selection of a suitable monitoring wavelength of the UV detector, based on the difference in the chemical structures of other components present in serum.

The minimum detectable amount by this method is 6 ng. The sensitivity is high enough for the analysis of both healthy and PKU sera.

EXPERIMENTAL

Apparatus

A Hitachi 635 AM high-performance liquid chromatograph with multi-wavelength UV detector was used. The column was a 250 × 4 mm tube, packed with YWG-ODS of 10-μm particle size, obtained from Tianjing Second Chemical Reagent Plant. A 250 × 2.6 mm column was also used in a few tests.

Chemicals

All chemicals used in this work were AR grade, except mandelic acid and *p*-hydroxybenzoic acid which were CP chemicals. Deionized water was used for preparing the mobile phase and other solutions.

Preparation of samples

To a centrifuge tube containing 0.2 ml serum, 0.2 ml ethanol and 0.4 ml chloroform were added. The tube was shaken vigorously and then centrifuged at 4500 rpm for 30 min. The upper layer was the ethanol solution containing the Phe to be determined and could be injected directly into the column. The volume of the upper layer was still 0.2 ml after extraction. Therefore, the Phe content of the sample could be expressed directly in terms of its concentration in the ethanol layer. The lower layer was a chloroform solution, which was discarded after extraction. There were some pale yellow viscous substances in the interface of the two layers.

Conditions for chromatography

The mobile phase used was prepared by mixing methanol and water in a ratio of 10:90 with phosphoric acid and potassium dihydrogen phosphate at concentrations of $1 \cdot 10^{-4}$ *M* and 0.01 *M* respectively. The final pH was 4.3. Elution was carried out at a rate of 0.6 ml/min. A rate of 0.4 ml/min was also used in a few tests.

RESULTS AND DISCUSSION

Choice of mobile-phase composition

The commonly encountered amino acids containing a phenyl group in serum are Phe and Tyr. Interference from other amino acids can easily be avoided by the choice of a suitable monitoring wavelength for the UV detector. At a certain range of wavelengths, they give little or no response. The wavelength of 220 nm was chosen in this work. In studying the separation of Phe from other phenyl group-containing substances which may be present in serum, the composition and pH value of the mobile phase were changed to observe the effect on the retention of Phe and several other aromatic acids.

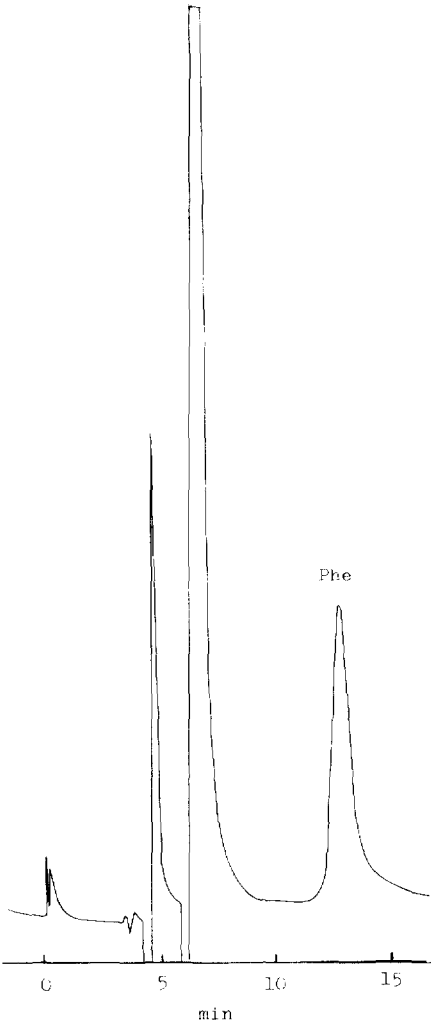
A neutral mobile phase (mixture of methanol and water only), eluted the aro-

TABLE I

EFFECTS OF MOBILE-PHASE COMPOSITION ON k' OF ACIDS

Column: 2.6 × 250 mm. Flow-rate: 0.4 ml/min. MA = Mandelic acid; DHBA = 3,4-dihydroxybenzoic acid; PHBA = *p*-hydroxybenzoic acid; BA = benzoic acid.

Mobile phase			k'					
H_3PO_4	KH_2PO_4	*pH*	*MA*	*DHBA*	*PHBA*	*BA*	*Phe*	*Tyr*
0	0	7.0	0.30	0.56	0.74	1.13	2.48	—
1×10^{-4} *M*	0.01 *M*	4.1	3.35	5.04	9.87	26.5	2.57	0.74
1×10^{-3} *M*	0.01 *M*	3.1	6.17	6.26	12.3	43.1	2.74	—



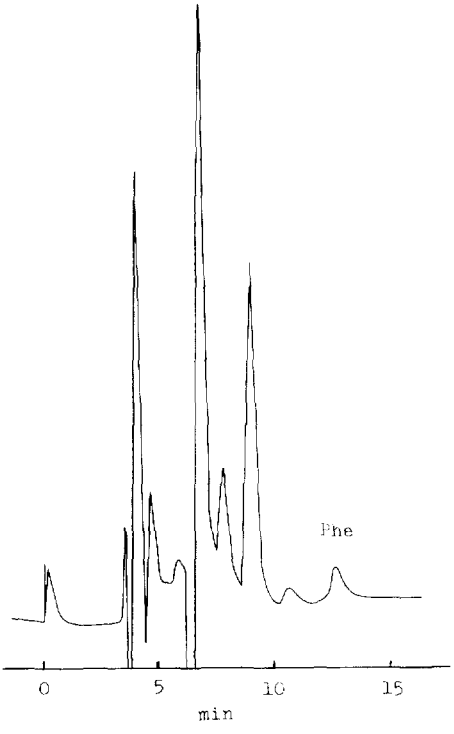


Fig. 1. Chromatogram of standard Phe solution (100 μg/ml). Column: 4 × 250 mm. Flow-rate: 0.6 ml/min.
Fig. 2. Chromatogram of serum extract of healthy adult. All conditions as in Fig. 1.

matic acids earlier than Phe, and their capacity factors, k', were near or less than 1 (Table I). Obviously, under such conditions practically no retention was of these aromatic acids taking place. By adding orthophorphoric acid and potassium dihydrogen-phosphate to the mobile phase, the retention values of the aromatic acids increased greatly. This did not significantly affect the retention value of Phe. When the pH value of moble phase was changed from 7.0 to 3.1, the k' of benzoic acid changed from 1.13 to 43.1 while that of Phe changed only from 2.48 to 2.74. This can be interpreted as due to the differences in the degree of ionization of Phe and other aromatic acids at various pH values of the mobile phase. By decreasing the pH value of the mobile phase, the degree of ionization of these aromatic acids is decreased and the retention of acidic molecules by the stationary phase is greatly increased. The ionization of Phe is not significantly affected by the pH value of the mobile phase due to the structure of the molecule. The adjustment of the pH value

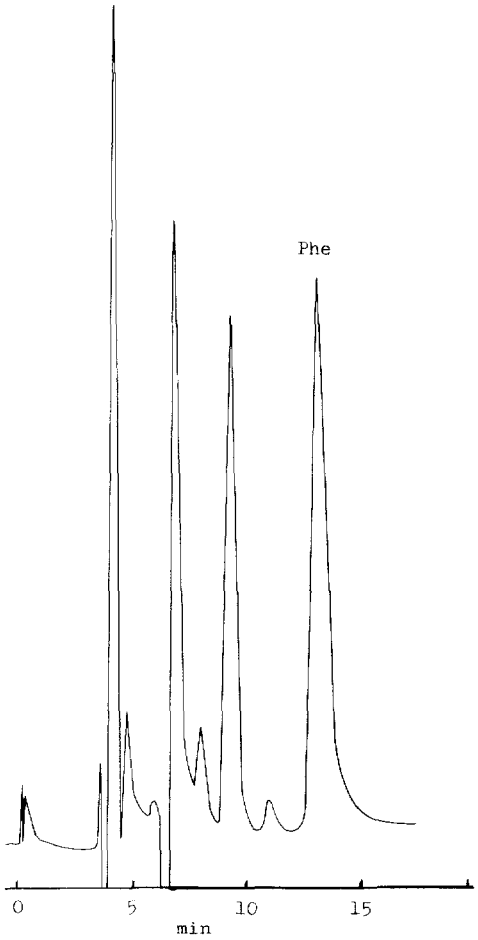


Fig. 3. Chromatogram of serum extract of PKU child. All conditions as in Fig. 1.

TABLE II

EFFECTS OF MOBILE-PHASE COMPOSITION ON RETENTION TIME t_R

Column: 4 × 250 mm. Flow-rate: 0.4 ml/min.

Mobile phase			t_R *(sec)*					
H_3PO_4 *(M)*	KH_2PO_4 *(M)*	*pH*	*Standard Phe*	*Serum peak number*				
				1	*2*	*3*	*4*	*5*
$1.4 \cdot 10^{-3}$	0.1	4.1	1184	251	466	762	1043	1181
$1.4 \cdot 10^{-4}$	0.01	4.3	1101	238	450	684	971	1097
$1.4 \cdot 10^{-4}$	0.1	4.5	1082	240	454	644	839	1081
0	0.01	4.9	1020	–	441	610	780	1020

can easily eliminate the overlap of the peaks of such aromatic acids with that of Phe, if any.

In practice, the pH value of the mobile phase was 4.3, at which no interference was observed in routine analysis. Figs. 1–3 illustrate the chromatograms of standard Phe solution, normal serum and PKU serum.

Peak identification

With authentic Phe, slightly different retention values at various pH values of the mobile phase were obtained. A peak, corresponding to such retention values, was found in the sample analysis. Table II shows that with the pH value changing from 4.1 to 4.9, the retention time of the peak decreased from 1181 sec to 1020 sec, which is in good agreement with that of the authentic peak at four values. The maximum and average deviations were 4 and 2 sec respectively. This indicates that the peak can be definitely identified as Phe.

Another proof of the peak purity was obtained by comparing the ratio of peak heights of authentic Phe and of the identified peaks at different wavelengths. Table III shows that the contribution at 210 nm was nearly four times higher than that at 220 nm. The ratio for the sample was 3.84, which was in good agreement with 3.90 for pure Phe. This means there was no significant contribution to the peak height from constituents other than Phe in the serum at the selected wavelength. The constituents not separated from Phe, if there are any, were not considered in this work,

TABLE III

PEAK HEIGHT RATIOS AT 210 AND 220 nm

Wavelength (nm)	*Peak height (mm)*	
	Standard Phe	*Phe in serum**
210	121	146
220	31.0	38.0
Ratio 210/220	3.90	3.84

* Serum obtained from child patient.

since they gave no response at the stated detector conditions. This much simplifies the method for routine analysis.

Further evidence of Phe peak in serum can also be obtained by comparing the chromatograms obtained from the sera of healthy persons and those of PKU patients. Only this peak changed more than 10-fold. The analytical results were in agreement with the clinical diagnosis.

Calibration curve and minimum detectable amount

Peak height was used for the quantitative measurement of Phe in serum. The relationship of peak height *versus* sample concentration is linear up to 250 μg/ml. For a 1-μl injection, the minimum detectable amount of Phe was 6 ng and corresponded to a concentration of 6 μg/ml of serum. The average Phe content in serum is 12–15 μg/ml. Therefore, this method is sensitive enough even for the serum of healthy persons.

TABLE IV

Phe CONTENTS IN SERA OF HEALTHY ADULTS AND CHILDREN

μg Phe per ml serum	
Adults	*Children*
16.3	11.4
11.7	10.3
14.2	12.0
13.8	13.0
12.9	19.6
10.8	19.6
11.9	13.0
10.7	13.6
12.3	13.0
14.5	10.4
12.9	6.8
11.9	10.9
11.5	12.5
11.5	14.6
12.7	14.6
9.8	17.2
16.0	10.9
9.4	8.3
11.5	9.7
10.7	12.2
	26.8
	30.4
	32.1
	17.2
	20.7
	8.6
	17.2
Average 12.4	15.1

TABLE V

Phe CONTENTS IN SERA OF PKU CHILDREN AND THEIR FAMILY MEMBERS

Sample source	*μg Phe per ml serum*
Boy aged 7 years	150
His father	7.3
His mother	7.8
His brother	10.3
Boy aged 4 years	178
His father	17.0
His mother	9.8
Boy aged 6 months	212
His mother	22.3

Recovery

Recoveries from a single extraction were 72% to 94% for concentrations from 25 μg/ml to 200 μg/ml in serum. Some loss in low-content sera might be due to occlusion by the viscous substances at the interface of layers. However, the fluctuation of actual contents for healthy persons are greater than the loss in this step. Repeated extractions are time-consuming and not considered to be necessary for routine analysis.

Applications

The method has been used for the determination of more than 50 samples from healthy adults and children. The average contents of Phe in serum for healthy adults and children were 12 and 15 μg/ml respectively. The sera from three PKU patients, all boys of aged 7 years, 4 years and 6 months, were found to contain 150, 178 and 212 μg/ml Phe, respectively. These values are about 10 to 15 times higher than those of the average healthy adults and children. The sera of some family members of the patients were determined and it is interesting to note that all of them were found to be normal. All the application data are given in Tables IV and V.

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