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SENSITIVE DETECTION OF AMINO ACIDS IN HUMAN SERUM AND DRIED BLOOD DISC OF 3 mm DIAMETER FOR DIAGNOSIS OF INBORN ERRORS OF METABOLISM

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SUMMARY

Sensitive high-performance liquid chromatographic determination of amino and imino acids in human serum (5 μl) and dried blood (2.6—2.8 μl) on a paper disc (3 mm diameter) of normal and abnormal newborns with inborn errors of metabolism (phenylketonuria, maple syrup urine disease and tyrosinosis) is described. Amino and imino acids in the biological specimens were extracted with ethanol and derivatized with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole at 60°C and at pH 8.0 for 1 min. The fluorescent derivatives were separated on μBondapak C_{18} and detected fluorometrically (530 nm/470 nm). The method was about one order of magnitude more sensitive than the similar method using *o*-phthalaldehyde. The amino acid contents obtained by the proposed method were comparable to those obtained by the amino acid analyser with use of *o*-phthalaldehyde.

INTRODUCTION

Various derivatives have been employed to enhance amino acid detection using either pre- or post-column techniques in high-performance liquid chromatography (HPLC). Post-column derivatization with ninhydrin is commonly adopted after separation of amino acids using ion-exchange chromatography [1]. Recently, *o*-phthalaldehyde (OPA) [2], a fluorogenic reagent for primary amines, has been used for such a purpose as a pre- or post-column derivatization reagent.

It has been revealed that 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F), a new fluorogenic reagent for both primary and secondary amines [3], is useful as a pre- and post-column derivatization reagent of amino and imino acids in HPLC [4—6].

The purpose of the present investigation was to develop a rapid and sensitive

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method for determination of amino and imino acids in human serum (5 μl) and dried blood on a paper disc (3 mm diameter) for diagnosis of inborn errors of metabolism.

EXPERIMENTAL

Reagents and chemicals

All chemicals were analytical reagent grade, unless otherwise noted. NBD-F was synthesized in our laboratory [7]. Standard amino acid solution was purchased from Ajinomoto (Tokyo, Japan). Dried blood on a paper disc from normal and abnormal newborns was kindly supplied by Dr. H. Naruse of the National Center for Nervous, Mental and Muscular Disorders (Tokyo, Japan). Methanol and tetrahydrofuran were of HPLC grade. Water was deionized and doubly distilled.

HPLC conditions and apparatus

An HPLC instrument (Waters Assoc., Milford, MA, U.S.A.) consisting of two Model 6000A pumps, a Model 600 solvent programmer and a U6K injector was used. All chromatographic runs were made at ambient temperature using a flow-rate of 2.0 ml/min. μBondapak C_{18} (30 cm × 3.9 mm I.D., 10 μm) (Waters Assoc.) was used. The column effluent was monitored by a Hitachi 650-10S spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan) with an 18-μl flow-cell with excitation at 470 nm and emission at 530 nm, fitted with a Shimadzu R-1A integrator and a Hitachi 056 one-pen recorder.

The following three solvent systems were used: Solvent A, methanol—tetrahydrofuran—0.1 *M* phosphate buffer (pH 6.0, Na^+) (3.75:1.6:94.65); Solvent B, methanol—tetrahydrofuran—0.1 *M* phosphate buffer (pH 6.0, Na^+) (25:15:60); Solvent C, methanol—water (40:60). The elution programme was as follows: (1) isocratic elution by Solvent A for 24 min, (2) linear gradient from Solvent A to 100% Solvent B over 30 min, (3) isocratic elution by Solvent B for 6 min, and (4) isocratic elution by Solvent C for 12 min. All mobile phases were degassed daily with an ultrasonic bath under vacuum prior to use.

Extraction and derivatization procedure for serum samples

A 5-μl aliquot of serum and 15 μl of ϵ-aminocaproic acid solution (internal standard, 167 μM) were transferred to a 400-μl conical centrifuge tube. Then 50 μl of ethanol were added and the two phases were mixed thoroughly. The mixed solution was centrifuged at 8000 *g* for 5 min and 20 μl of the supernatant were transferred to a 500-μl reaction vial. 20 μl of 0.1 *M* borate buffer (pH 8.0, Na^+) and 12 μl of 83 m*M* NBD-F in ethanol were added and the mixture was heated at 60°C for 1 min. After cooling on ice—water, 150 μl of 0.5 m*M* hydrochloric acid were added to the reaction mixture, and 10 μl of the final solution were subjected to HPLC. Quantitation was made by calculation of the peak area ratio of each amino and imino acid to the internal standard.

Extraction and derivatization procedure for dried blood samples

One sheet of a paper disc (3 mm diameter) spotted with blood was soaked

in 40 μl of 70% ethanol containing 500 pmol of ε-aminocaproic acid and extracted at 5° C for 12 h. A 10-μl aliquot of the extract, 10 μl of 0.1 *M* borate buffer (pH 8.0, Na^+) and 6 μl of 83 m*M* NBD-F in ethanol were well mixed in a 500-μl reaction vial and heated at 60° C for 1 min. Then, 14 μl of 0.1 *M* hydrochloric acid were added to the reaction mixture, and 10 μl of the final solution were subjected to HPLC.

RESULTS AND DISCUSSION

Quantitative determination of amino acids in biological fluids usually involves separation by ion-exchange chromatography and detection by post-column derivatization with ninhydrin [1]. Substitution of ninhydrin by a fluorogenic reagent such as OPA [2] or fluorescamine [8], which react only with primary amines, has recently led to an increase in sensitivity of the method. Elimination of the post-column reactor yields a less expensive and more versatile system where high sensitivity can be achieved. Reversed-phase HPLC of amino acids of dansyl [9], phenylthiohydantoin [10] and OPA [11] derivatives has been performed.

NBD-F has been developed as a fluorogenic reagent for amines [3] with which it is more reactive than NBD-Cl [12]. Of particular importance is the fact that only one peak was observed for each amino and imino acid derivatized with NBD-F. Thus it has been used successfully for fluorometric determination of amino and imino acids and other amines with the pre- and post-column derivatization technique [4—6, 13]. Pre-column derivatization with NBD-F at pH 8.0 and at 60° C for 1 min and separation on HPLC afforded the detection limits of 10—100 fmol of amino and imino acids [4, 5]. Another advantage is that no interference from other compounds in biological samples would occur because the fluorescent excitation (470 nm) and emission (530 nm) of NBD-amino and -imino acid derivatives are in the long-wavelength region of the spectrum.

No significant change in the fluorescence intensities of NBD-amino and -imino acid derivatives, such as NBD-alanine [14], -glycine [15] and -proline [6] was observed during a 5-h period under dark conditions. Thus the average deviation of 10 pmol of each amino and imino acid was good (2.78%, $n = 5$). However, 30% of each derivative was degraded after 3 h exposure to indoor light, but NBD-Tyr, which is the most sensitive to light, completely disappeared under the same conditions.

In the present study, the application of the method to the analysis of amino and imino acids in biological fluids is demonstrated and discussed. Firstly, sera obtained from adults were subjected to amino acid analysis. Ethanol was chosen as deproteinizing agent instead of acidic agents such as perchloric and trichloroacetic acid, because it is preferable in the reaction of amino and imino acids with NBD-F, and its concentration was tentatively decided as 70% on the basis of the method of Ohura [16]. A chromatogram thus obtained is shown in Fig. 1.

In this experiment the same gradient elution system of 0.1 *M* sodium phosphate buffer (pH 6.0), methanol and tetrahydrofuran was adopted that had been used for hydrolysates of a few μg of proteins such as rabbit pyruvate

kinase M_1, rabbit aldolase A and papain [5]. The total analysis time for serum including the deproteinization and derivatization step is less than 80 min. All the amino and imino acid derivatives were well separated except that separations of serine (peak 3) from aspargine, glycine (peak 5) from glutamine, and threonine (peak 8) from taurine are difficult under the present conditions. When amino and imino acid standards were derivatized and chromatographed, the relationship obtained between peak area ratio to the internal standard (ϵ-aminocaproic acid) and amount of amino and imino acid was linear in the range 0.5—100 pmol.

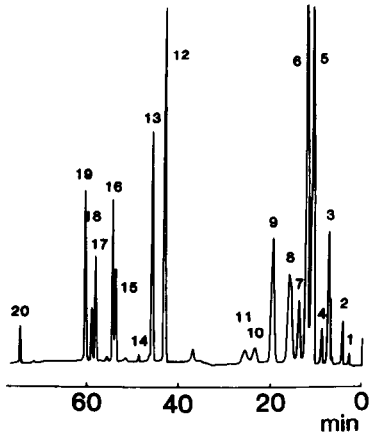

Fig. 1. Elution profile of a human serum. Procedures for deproteinization, derivatization and HPLC conditions are cited in the text. Peaks: 1 = NBD-Asp, 2 = NBD-Glu, 3 = NBD-Ser + NBD-Asn, 4 = NBD-His, 5 = NBD-Gly + NBD-Gln, 6 = NBD-OH, 7 = NBD-Arg, 8 = NBD-Thr + NBD-Tau, 9 = NBD-Ala, 10 = NBD-Pro, 11 = NBD-NH_2, 12 = NBD-ϵ-aminocaproic acid, 13 = NBD-Val, 14 = NBD-Met, 15 = NBD-Ile, 16 = NBD-Leu, 17 = NBD-Phe, 18 = NBD-Orn, 19 = NBD-Lys, 20 = NBD-Tyr.

TABLE I

AMINO AND IMINO ACID CONCENTRATIONS (μM) IN SERUM OF SEVEN NORMAL MALES

Amino acid	1	2	3	4	5	6	7
His	89	53	91	89	72	88	101
Arg	101	89	88	75	110	77	52
Ala	430	380	419	325	370	335	450
Pro	190	203	230	234	211	193	252
Val	221	250	240	237	252	205	232
Met	31	20	31	35	23	32	19
Ile	66	88	89	90	78	73	54
Leu	153	121	135	141	128	150	160
Phe	51	58	66	35	51	53	65
Orn	51	107	108	77	93	65	81
Lys	176	182	135	185	198	170	181
Tyr	45	53	40	72	38	40	63

Repetitive analysis of the same serum showed that the variations in area of all peaks compared to the area of the internal standard were small (coefficient of variation, C.V., is in the range 0.8—9.5%, n = 5). Thus the precision of the present method involving extraction and pre-column derivatization with NBD-F may be slightly inferior to that obtained by ion-exchange chromatography. Part of this deviation is due to pipetting errors that may occur during the extraction and derivatization procedure. The average recovery of amino and imino acids by addition of amino and imino acid standard solution to serum was 98.9% (n = 5).

The data obtained in this experiment (Table I) are comparable to those obtained using the other method [17]. The sensitivity of the method is about one order of magnitude higher than that described in a recent report [18] for 50 μl of serum using OPA as fluorogenic reagent.

Since this method allows the detection of amino and imino acids in extremely small amounts of serum, we tried to measure amino and imino acid contents in whole blood obtained from newborns.

A newborn blood sample (2.6—2.8 μl) applied on a paper disc (3 mm diameter) was extracted, as for serum, with 70% ethanol, derivatized with NBD-F and analysed by the proposed method. Fig. 2 shows a representative chromatogram of amino and imino acids in whole blood of a normal newborn. The variation in the ratio of peak area of each amino and imino acid to that of the internal standard was within C.V. = 8.4% (n = 4). A major part of this deviation might be caused by the sample preparation, especially by punching out of the paper.

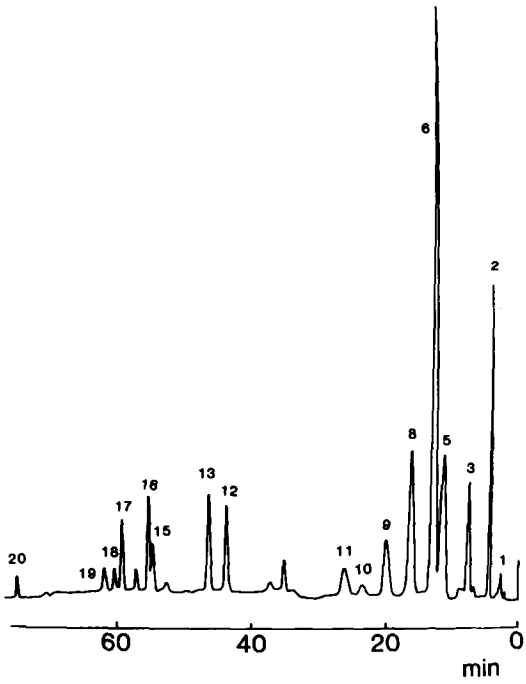

Fig. 2. Elution profile of a normal blood disc. Procedures for extraction, derivatization and HPLC conditions are cited in the text. Numbers are the same as in Fig. 1.

A summary of the results obtained from the whole blood of five normal newborns is made in Table II. The concentrations of each amino and imino acid in whole blood were comparable to the values reported by others using an amino acid analyser [19].

Fig. 3 shows chromatograms of amino and imino acid derivatives from blood of newborns with inborn errors of metabolism (phenylketonuria, maple syrup urine disease and tyrosinosis). Large peaks of amino acids [phenylalanine (peak 17) in Fig. 3a, valine (peak 13), isoleucine (peak 15) and leucine (peak 16) in Fig. 3b, and tyrosine (peak 20) in Fig. 3c] appear in these chromatograms. It is known that a large amount of alloisoleucine would arise in maple syrup urine disease, but in this system alloisoleucine and isoleucine co-eluted. The concentrations of amino and imino acids related to the diseases obtained by the proposed method and by an amino acid analyser using OPA as post-column derivatization reagent are shown in Table III, suggesting a good correlation between the two. In conclusion, the present method should be useful in the diagnosis of hereditary inborn errors of metabolism and would also be applicable to the detection of hyperprolinaemia [20].

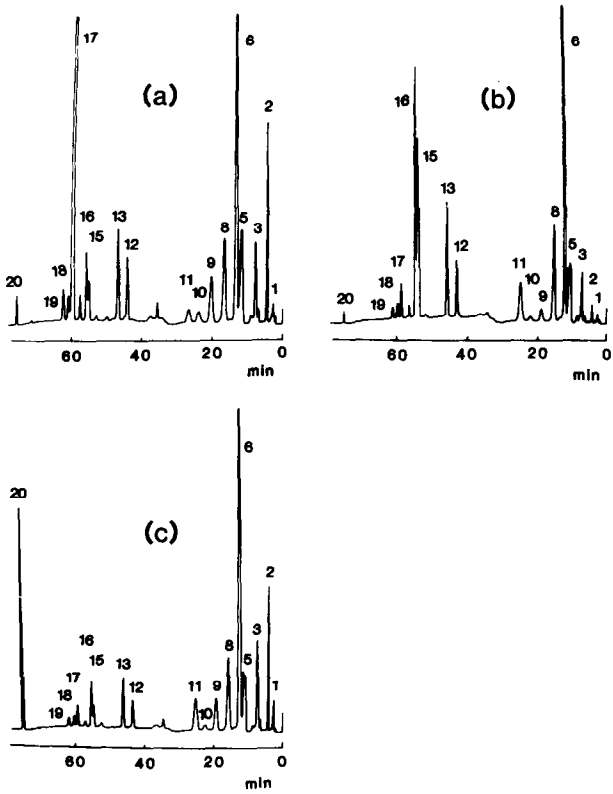

Fig. 3. Elution profiles of abnormal blood disc obtained from newborns with phenylketonuria (a), maple syrup urine disease (b) and tyrosinosis (c). Procedures for extraction, derivatization and HPLC conditions are cited in the text. Numbers are the same as in Fig. 1.

TABLE II

AMINO AND IMINO ACID CONCENTRATIONS (μM) IN DRIED BLOOD DISCS FROM FIVE NORMAL NEWBORNS

Amino acid	1	2	3	4	5
His	—	15	30	—	23
Ala	335	294	435	362	306
Pro	210	199	250	243	255
Val	208	270	190	185	235
Ile	95	83	70	85	65
Leu	126	154	160	129	177
Phe	51	33	55	80	71
Orn	46	59	76	110	57
Lys	164	188	130	139	155
Tyr	53	41	36	55	59

TABLE III

ABNORMAL AMINO ACID CONCENTRATIONS (μM) IN DRIED BLOOD DISCS OBTAINED FROM INBORN ERRORS OF METABOLISM: COMPARISON OF THE DATA FROM THE TWO METHODS

Disease	Amino acid	Sample	NBD-F*	OPA**
Phenylketonuria	Phe	1	2800	2720
		2	2100	2280
		3	1550	1510
Maple syrup urine disease	Val	4	400	420
		5	410	400
		6	380	360
	Leu	4	1200	1330
		5	1650	1690
		6	570	560
Tyrosinosis	Tyr	7	2010	2140
		8	930	960
		9	1200	1250

*Present method.
**Post-column derivatization with OPA.

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