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Automated method for the measurement of amino acids in urine by high-performance liquid chromatography

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Abstract

An automatic and sensitive HPLC method for the determination of primary and secondary amino acids included cystine and homocystine in urine samples is described. After a simple ultrafiltration, urine samples were subjected to reduction of disulfides, carboxymethylation of free thiols and double precolumn derivatization with *o*-phthalaldehyde–3-mercaptopropionic acid and 9-fluorenylmethyl chloroformate. All reactions were fully automated by means of an injector programme and were accomplished in 10 min. Since urine samples contain a large number of amino compounds, a good resolution was required. By optimization of the conditions, separation of 40 amino acids in 92 min was achieved. The recovery of amino acids ranged from 83% for TRP to 105% for CIT. The within-run and between-run R.S.D.s of urinary amino acid concentrations were below 10% for most amino acids except for HYL, LYS and ORN. The method was applied to the diagnosis of genetic disorders of amino acid metabolism.

Keywords: Amino acids

1. Introduction

The determination of amino acids in urine samples is essential for the diagnosis and follow-up of amino acid disorders, particularly when an amino acid transport disorder is suspected.

The determination of amino acids in urine samples is usually accomplished by ion-exchange chromatography followed by postcolumn derivatization with ninhydrin or *o*-phthalaldehyde (OPA)–2-mercaptoethanol (MCE) [1]. This method has been the method of choice since 1958 [2], allowing good resolution of most amino acids. However, although this method is accurate and precise, it has the disadvantage of a long analysis time (up to 4 h).

In recent years, several RP-HPLC procedures using precolumn derivatization for the determination of amino acids in biological samples, achieving shorter analysis times and increased sensitivity, have been described [3–14]. Among these, automatic on-line precolumn double derivatization with OPA–3-mercaptopropionic acid (3-MPA) and 9-fluorenylmethyl chloroformate (FMOC-Cl) [15] has been successfully applied for the determination of both primary and sec-

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ondary amino acids in protein hydrolysates [16] and plasma [17] with high sensitivity and reproducibility.

The aim of this work was to study and test a reliable method for the determination of most amino acids including free cystine (CYS2) and homocystine (HYC2) in urine samples using an automatic procedure involving reduction of dithiolic bonds, carboxymethylation of free thiols and double derivatization with OPA–3-MPA and FMOC followed by separation using RP-HPLC. The method should also have such characteristics as to be applicable to the diagnosis of hereditary and acquired disorders of amino acid metabolism.

2. Experimental

2.1. Materials

L-Amino acid crystalline salts, physiological basic, acidic and neutral amino acid calibration standards in 0.1 *M* HCl and iodoacetic acid were obtained from Sigma (St. Louis, MO, USA), POA and borate buffer from Pierce (Oud Beijerland, Netherlands), FMOC from Hewlett-Packard (Walbronn, Germany), 3-MPA and triethylamine (TEA) from Fluka (Buchs, Switzerland) and glacial acetic acid, sodium acetate, acetonitrile and methanol (HPLC gradient grade) from Merck (Darmstadt, Germany). Ultrafree-MC filters with low-protein-binding regenerated cellulose ultrafiltration membranes (10 000 molecular mass cut-off filters), used for centrifugal ultrafiltration of urine samples, were purchased from Millipore (Bedford, MA, USA). Water used for HPLC buffers was produced with a Millipore Milli-Q system.

A Hewlett-Packard HP 1090M HPLC apparatus, including cooled autosampler, DR5 solvent-delivery system, heated column compartment, HPLC ChemStation (DOS Series) and a Model 1046A programmable fluorescence detector, was used. A Nova-Pak C_{18} (4 μm) column (300 × 3.9 mm I.D.) was used (Waters, Milford, MA, USA) in conjunction with an ODS Hypersil (5 μm) guard column (20 × 4 mm I.D.) (Hewlett-Packard).

2.2. Methods

Aliquots of urine obtained from 24-h collection were immediately stored frozen at −20°C until analysis. Volumes of 80 μl of urine sample and 20 μl of internal standard solution containing 1 m*M* norvaline (NVAL) and 10 m*M* N-methylalanine (N-MALA) were ultrafiltered at 5000 *g* for 30 min using Ultrafree-MC filters. The ultrafiltrates obtained were subjected to an automatic procedure for reduction of S–S bonds using 3-MPA, carboxymethylation with iodoacetic acid and double derivatization using OPA–3-MPA and FMOC. The sample vials and reagent vials were stored in a cooled (4°C) rack and reactions were carried out in the needle of an autosampler at room temperature. The steps of the injection programme are given in Table 1. In the first step, to reduce dithiolic bonds, 5 μl of 3-MPA [0.5% (v/v) in 1 *M* borate buffer (pH 10.4)] were mixed with 6 μl of sample and then, to carboxymethylate free thiols, 1.5 μl of iodoacetic acid (120 m*M* in 0.14 *M* NaOH) were added. In the next step, to derivatize all primary amino acids including S-caroxymethylcysteine

Table 1
Injection programme for reduction of dithiolic bonds, carboxymethylation of free thiols and derivatization

Function[a]	Amount (μl)	Vial	Reagent
Draw	5	From vial 2	3-MPA
Draw	6	From vial *x*	Sample
Mix	6 × 3 cycles	In loop	
Draw	1.5	From vial 4	Iodoacetic acid
Mix	6 × 3 cycles	In loop	
Draw	5	From vial 0	OPA–3-MPA
Mix	7 × 5 cycles	In loop	
Draw	2	From vial 1	FMOC
Mix	5 × 5 cycles	In loop	
Draw	2.5	From vial 3	Acetic acid
Mix	3 × 2 cycles	In loop	
Inject			

[a] After each draw, the surface of the needle was washed with water in vial 100.

and S-carboxymethylhomocysteine, 5 μl of OPA–3-MPA solution [20 mg/ml OPA in 0.5 *M* borate buffer (pH 10.4) with 10% (v/v) of methanol and 2% of 3-MPA] were added, and to derivatize free secondary amino acids, 2 μl of FMOC were then added; finally, to adjust the pH, 2.5 μl of 1 *M* acetic acid were added and resulting mixture was injected. The separation of amino acids was accomplished by gradient elution according to the programme given in Table 2. The flow-rate was 0.8 ml/min, mobile phase A was 60 m*M* sodium acetate buffer (pH 6.86) containing 0.044% TEA and mobile phase B was 100 m*M* sodium acetate buffer (pH 5.45)–acetonitrile–methanol (21:74.5:4.5). The column temperature was 40°C. The excitation and emission wavelengths were changed in agreement with the times given in Table 3. For the detection of primary amino acids, $\lambda_{ex} = 340$ nm and $\lambda_{em} = 450$ nm were used and for the detection of secondary amino acids $\lambda_{ex} = 260$ nm and $\lambda_{em} = 315$ nm were used. Quantification was accomplished by the internal standard method, using a double internal standard technique (1 m*M* NVAL and 10 m*M* N-MALA).

Table 2
Gradient profile for the separation of amino acids

Time (min)	B (%)[a]	Flow-rate (ml/min)
0.0	0.0	0.8
1.00	5.6	0.8
7.00	6.2	0.8
9.00	6.2	0.8
21.00	7.7	0.8
28.00	7.7	0.8
35.00	8.0	0.8
39.00	9.2	0.8
42.00	9.5	0.8
48.00	16.0	0.8
49.00	16.0	0.8
50.00	18.0	0.8
70.00	22.0	0.8
77.00	28.0	0.8
85.00	32.0	0.8
94.00	100.0	0.8
99.00	100.0	0.8
100.00	0.0	0.8

[a] Mobile phase A = 60 m*M* sodium acetate buffer–0.044% TEA (pH 6.86); mobile phase B = 100 m*M* sodium acetate buffer (pH5.45)–acetonitrile–methanol (21:74.5:4.5, v/v/v).

Table 3
Programme for fluorescence detector

Time (min)	λ_{ex} (nm)	λ_{em} (nm)	Compounds
0.	340.0	450.0	Primary amino acids
79.50	260.0	315.0	HYP
81.00	340.0	450.0	LYS, ORN
87.00	260.0	315.0	Secondary amino acids

3. Results

A satisfactory separation of 38 amino acids and two internal standards (NVAL, N-MALA) in 92 min was achieved. Chromatograms obtained from a 400 μM standard solution and from a normal urine sample are shown in Fig. 1. The linear correlation between peak areas and concentrations was assessed in the range 10–400 μ*M* for the amino acids ASP, GLU, CM-CYS, AAD, ASN, CM-HCY, CIT, 1-MHIS, β-ALA, 3-MHIS, ANS, CAR. β-AIB, GABA, TYR, ABU, ETN, VAL, MET, CYSTA, TRP, ILE, HYL, LEU, HYP, LYS, ORN, SAR and PRO using four different concentrations (10, 100, 200 and 400 μ*M*) in triplicate. For the amino acids SER, GLN, HIS, GLY, TAU, ARG, ALA, THR and PHE the area/concentration correlation was assessed in the range 10–1000 μ*M* using six different concentrations (10, 100, 200, 400, 800 and 1000 μ*M*) in triplicate. The correlation coefficient was >0.999 for all amino acid. The detection limit for different amino acids ranged from 50 to 500 fmol with a signal-to-noise ratio of 3.

The precision of the method was assessed by evaluating the relative standard deviation (R.S.D.) of retention times and peak areas. To assess the within-run R.S.D., seven consecutive analyses of an amino acid standard solution at a concentration of 200 μ*M* (Table 4) were performed. The R.S.D. of areas ranged from 3% for PRO to 9% for LYS. LYS and ORN showed an

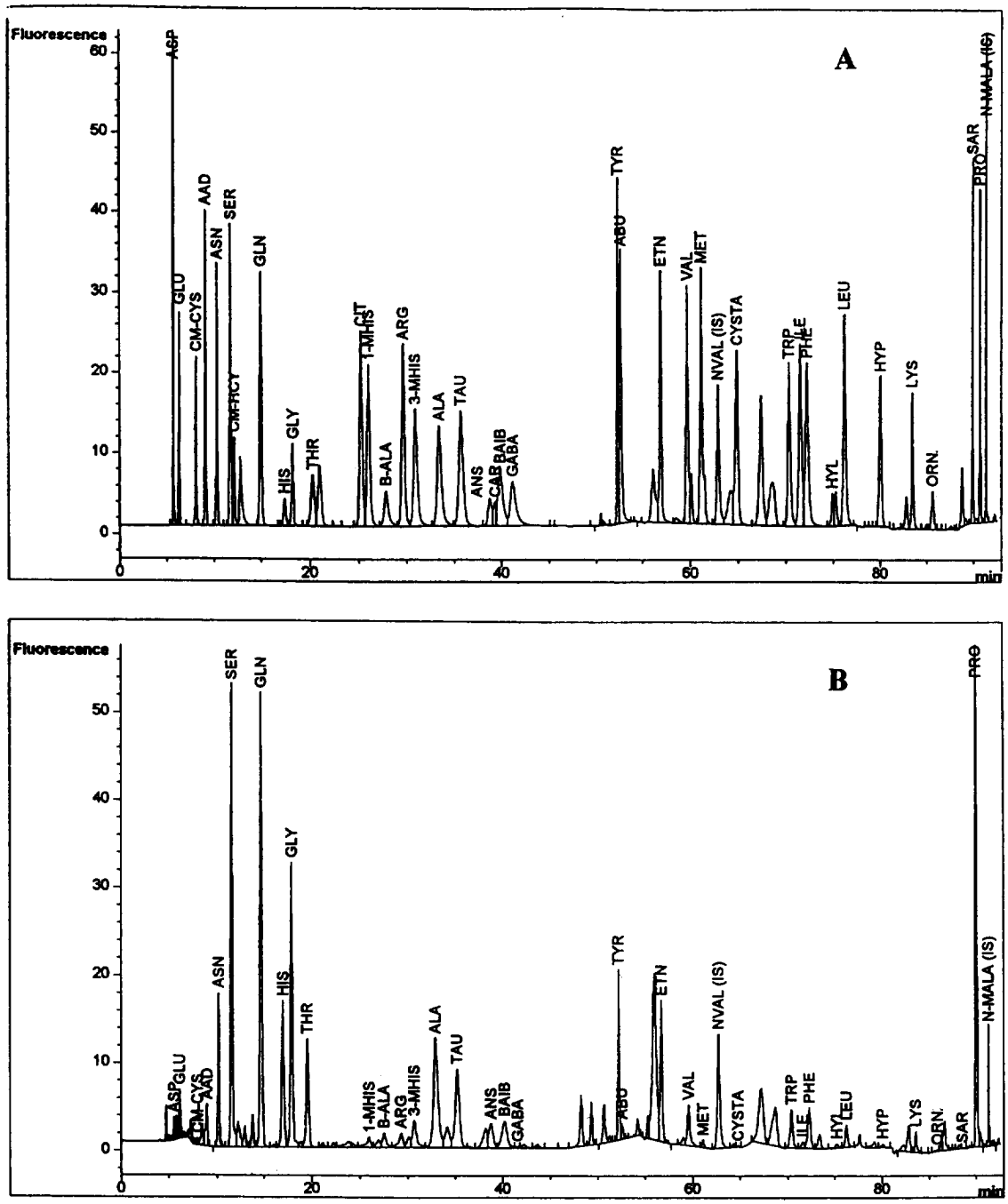

Fig. 1. Chromatograms of (A) 400 μM standard solution showing the separation of 38 amino acids and two internal standards (I.S.) and (B) a urine sample from a normal subject.

Table 4
Relative standard deviations for peak areas and retention times

Amino acid	R.S.D. (%)		Amino acid	R.S.D. (%)	
	Area	Time		Area	Time
ASP	4.62	0.31	BAIB	7.21	0.05
GLU	5.90	0.25	GABA	5.57	0.05
CM-CYS	4.10	0.19	TYR	4.07	0.01
AAD	4.02	0.19	ABU	4.09	0.01
ASN	4.10	0.17	ETN	6.48	0.01
SER	4.56	0.15	VAL	4.67	0.01
CM-HCY	5.05	0.41	MET	4.75	0.01
GLN	4.88	0.11	NVAL	4.01	0.01
HIS	7.22	0.35	CYSTA	3.68	0.36
GLY	4.26	0.10	TRP	6.23	0.02
THR	4.11	0.06	ILE	4.85	0.01
CIT	4.46	0.05	PHE	5.08	0.02
1-MHIS	5.34	0.11	HYL	6.71	0.01
BALA	4.37	0.08	LEU	4.76	0.01
ARG	5.05	0.05	HYP	4.82	0.01
3-MHIS	5.19	0.05	LYS	9.09	0.01
ALA	4.89	0.08	ORN	8.01	0.10
TAU	5.82	0.06	SAR	5.14	0.04
ANS	3.67	0.04	PRO	3.22	0.29
CAR	6.21	0.32	N-MALA	4.41	0.11

The precision of the method was evaluated by an R.S.D. based on seven consecutive analyses of a 200 μM amino acid standard solution.

R.S.D. of areas higher than those of most amino acids tested. The R.S.D. of retention times for all the amino acids were below 0.5%. The within-run and between-run precision based on analyses of a urine sample are shown in Table 5. The within-run R.S.D. ranged from 2 to 7% for most amino acids, with the exception of HYL, LYS and ORN, 11, 24 and 20%, respectively. The between-run R.S.D. ranged from 4 to 10% for most amino acids, again with the exception of HYL, LYS and ORN, 15, 20 and 27%, respectively.

The recovery was evaluated by adding different amounts of a standard mixture of various amino acids to a urine sample with a known amino acid concentration. The mean recovery for each amino acid was calculated at three different concentrations (100, 200 and 400 μM). The recovery did not vary significantly in the concentration range investigated. The mean recovery ranged from 83% for TRP to 105% for CIT.

The method was applied to the diagnosis of disorders of amino acids metabolism. Fig. 2 shows chromatograms of a urine sample from a patient with cystinuria and of a urine sample from a patient with phenylketonuria.

4. Discussion

OPA–3-MPA was chosen as the derivatizing reagent owing to the rapid reaction at room temperature and in aqueous solution, and the great possibility of automation. OPA–3-MPA forms highly fluorescent isoindoles with primary amino acids but it does not react with secondary amino acids. This problem was successfully overcome by the use of double precolumn derivatization with OPA–3-MPA and FMOC, allowing fluorescent detection of primary and secondary amino acids [15]. Further, the compounds formed by OPA with amino acids containing disulfides showed inadequate fluorescence, probably because of the formation of free thiolic groups in the reducing environment of the reaction. These groups compete with the thiolic reagent (3-MPA) required in the OPA reaction [18]. The reduction of disulfides and blockage of thiols by using iodoacetic acid before the OPA reaction has been shown to be an effective method to solve this problem [3]. The reduction agents generally used are 2-mercaptoethanol (MCE), sodium borohydride ($NaBH_4$) and dithioerythritol (DTE) [19]. We have found, however, that 3-MPA is useful for combined S–S bond reduction and OPA–3-MPA reaction in an automatic procedure. The use of 3-MPA instead of MCE in the OPA reaction increases the stability of the isoindoles formed [20]. The optimization of the conditions for the reduction, carboxymethylation [21] and derivatization reactions [22] are of great importance to maximize fluorescence. Different concentrations of reagent solutions and different times of reaction were tested to establish the optimum conditions and to set up a completely automatic procedure. The reduction with 3-MPA and reaction with iodoacetic acid were very fast

Table 5
Within-run and between-run precision of amino acid concentration

Amino acid	Within-run		Between-run	
	Mean concentration (μM)	R.S.D. (%)	Mean concentration (μM)	R.S.D. (%)
GLU	233.0	5.38	230.0	5.40
CM-CYS	119.0	4.88	133.0	6.63
AAD	43.0	4.34	50.0	5.31
ASN	252.0	5.43	280.0	7.93
SER	694.0	2.72	737.0	3.88
GLN	778.0	3.27	796.0	5.54
GLY	1844.0	3.63	1839.0	7.74
THR	243.0	7.17	284.0	8.12
ARG	34.0	3.09	35.0	5.75
3-MHIS	110.0	3.45	119.0	4.25
ALA	471.0	3.54	498.0	5.68
TAU	391.0	2.63	395.0	5.73
β-AIB	167.0	2.85	161.0	6.69
TYR	200.0	3.81	208.0	6.83
ABU	8.0	1.90	9.0	3.79
VAL	66.0	5.39	65.0	10.00
TRP	93.0	5.02	87.0	9.98
ILE	4.0	2.47	5.0	10.07
PHE	81.0	4.84	80.0	9.93
HYL	134.0	11.35	126.0	15.20
LEU	33.0	4.19	34.0	9.22
LYS	135.0	24.29	111.0	19.66
ORN	41.0	19.65	36.0	27.16

Aliquots of a urine sample stored at −20°C were analysed seven consecutive times in one day (within-run, $n = 7$) and twice on three different days (betwen-run, $n = 6$). Mean concentrations and R.S.D.s for the physiologically most important amino acids are shown.

and the overall time of the automatic procedure, including derivatization, was 10 min.

The precision and accuracy were high for most amino acids and, compared with the results obtained using RP-HPLC of phenylthiocarbamyl derivatives of urine samples [9], demonstrate a greater validity of the method. HYL, LYS and ORN showed R.S.D.s for peak areas and concentrations higher than those for other amino acids. A possible explanation is their weak fluorescence, observed by other investigators [5], which could be due to the formation of two fluorescent isoindole structures. While two peaks are evident in standard samples for HYL, they are not observed under the experimental conditions for LYS and ORN, possibly because of the wavelength change. Further research to overcome this problem is in progress.

While RP-HPLC methods for the measurement of plasma amino acids are currently used in clinical laboratories, the measurement of urinary amino acids is usually accomplished by ion-exchange chromatography. Other RP-HPLC methods to measure urinary amino acids [7,9] have shown problems of separation and precision. The method described here allows the analysis of complex amino acid mixtures such as in urine samples for the diagnosis of hereditary and acquired disorders of amino acid metabolism, with good precision and sensitivity, minimal

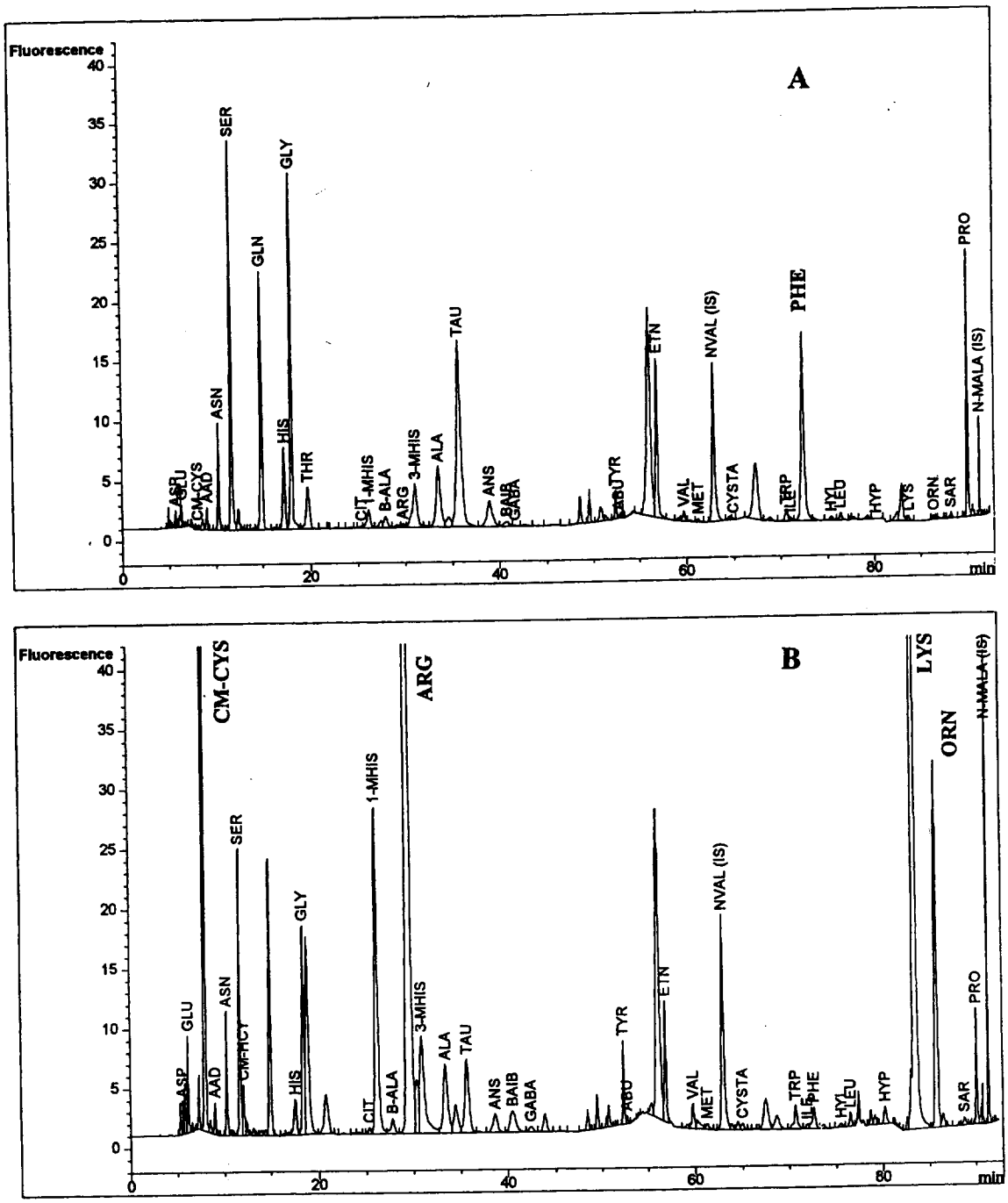

Fig. 2. Chromatograms of urine samples obtained from (A) a patient affected by phenylketonuria (PHE = 440 μM) and (B) a patient affected by cystinuria (CM-CYS = 2243 μM, ARG = 2054 μM, LYS = 4126 μM and ORN = 1507 μM).

manual sample handling and the advantage of a more versatile apparatus than ion-exchange analysers.

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