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DETERMINATION OF FREE AMINO ACIDS IN BIOLOGICAL SAMPLES: PROBLEMS OF QUANTITATION

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SUMMARY

An automatic on-line high-performance liquid chromatographic method was developed to study the effects of various precipitating agents and delayed deproteinization procedures on the estimation of plasma levels of amino acids. The optimized method for analysis is based on pre-column derivatization with *o*-phthalaldehyde in the presence of 2-mercaptoethanol. The separation of 25 amino acids is accomplished within 45 min on a 5-μm C_{18} column, using a multi-step gradient with two solvents. The method is sensitive and reproducible, and the relationship between the fluorescence intensity and concentration is linear for each amino acid over a wide concentration range.

INTRODUCTION

Amino acids (AAs) are considered to be the most important indicators of the nutritional requirements in various pathological states in humans [1–5].

In the past three decades, much information on the levels of free and total AAs in biological matrices has been accumulated by classical ion-exchange liquid chromatography (LC) based on post-column derivatization with ninhydrin, introduced by Spackman et al. [6] and later automated by Hamilton [7]. In the past 10–15 years, LC methods have undergone continual technical improvements. These have resulted in a reduction in analysis time from 24 h to 30 min through the use of small-particle-size resins, small-bore columns, high-pressure pumps and injection valves. The use of reversed-phase columns has greatly increased the sensitivity, reproducibility and ease of adaptability

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in research and routine laboratories for AA analysis. Various reagents, such as fluorescamine [8], *o*-phthalaldehyde (OPA) [9,10], dansyl chloride [11], dabsyl chloride [12], phenylthiohydanthion [13], 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) [14] and 9-fluorenylmethylchloroformate (FMOC) [15], have been introduced.

Despite the development of various LC systems and their continued application in biomedical sciences, there is no agreement as to what constitutes normal plasma AA levels in healthy subjects [16–22]. The observed discrepancies are probably due to the variation in specimens, sample preparation, clinical and nutritional conditions and timing of the sample collection.

There are only a few studies [23–28] dealing with sample preparation, despite its vital importance. It is known that the method of collection and treatment of biological specimens affects the outcome of the AA analysis [29].

In this study, an automatic on-line high-performance liquid chromatographic (HPLC) method, based on pre-column derivatization with OPA in the presence of 2-mercaptoethanol (2-ME), is developed. This optimized method is applied to a study of the effect of various precipitating agents and delayed deproteinization on plasma AA levels.

EXPERIMENTAL

Chemicals and reagents

Individual crystalline salts of L-amino acids (AMAC standard kit No. 20065), Brij (30%) and anhydrous OPA were obtained from Pierce Eurochemie (Oud-Beijerland, The Netherlands). Phosphoserine, 3-methylhistidine, homoserine, carnosine, cysteinesulphinic acid, α-, β- and γ-aminobutyric acid (ABA), taurine, α-alanine, homocysteine and 2-ME were obtained from Sigma (St. Louis, MO, U.S.A.). Methanol (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.). Anhydrous sodium dihydrogenphosphate, disodium hydrogenphosphate, 5-sulphosalicylic acid (SSA), perchloric acid, trifluoroacetic acid (TFA), ethanol, acetone, acetonitrile, boric acid, sodium hydroxide and hydrochloric acid were all analytical-reagent grade from Merck (Darmstadt, F.R.G.).

Individual 1 m*M* standard stock solutions of AAs were prepared in distilled water by the addition of a few drops of 0.1 *M* hydrochloric acid. A standard mixture containing 27 AAs at similar concentrations was prepared. This standard mixture was diluted to 5, 10, 20, 50, 75 and 100 μM to construct the calibration curve (fluorescence intensity versus the concentration of individual AAs). Water used for the preparation of buffers and standards was deionized (Milli-Q water purification system obtained from Millipore, Milford, MA, U.S.A.). All the standards were kept at $-70\,^{\circ}C$ when not in use. The OPA reagent was prepared according to ref. 21.

Apparatus

The chromatographic system consisted of two high-pressure delivery pumps (M45 and 6000A), a WISP 710B multiple sampler, a data module, a 730B system controller and a Model 420-AC fluorescence detector, with the monochromator set at 340 nm and a 450-nm cut-off filter, all supplied by Waters Assoc. (Milford, MA, U.S.A.).

Separation of AAs was performed on a 5-μm Microsorb C_{18} (150 mm×4.6 mm I.D.) column, obtained from Rainin Instruments (Woburn, MA, U.S.A.). A guard column (40 mm×4 mm I.D.) containing similar material was connected between the injector and the analytical column.

Chromatographic conditions

Sodium phosphate buffer (0.05 *M*, pH 6.7) was prepared by gradual addition of 0.05 *M* NaH_2PO_4 to 0.05 *M* Na_2HPO_4, and diluted to a concentration of 0.02 *M* with distilled water. Mobile phase A was phosphate buffer–methanol–tetrahydrofuran (98:1:1, v/v), and mobile phase B was phosphate buffer–methanol (35:65, v/v). Both solvents were filtered through a 0.45-μm filter membrane and sonicated for 10 min before use.

The gradient used is shown in Fig. 1 (%B). The flow-rate was maintained at 1.0 ml/min throughout except during the first 2 min, when it was increased

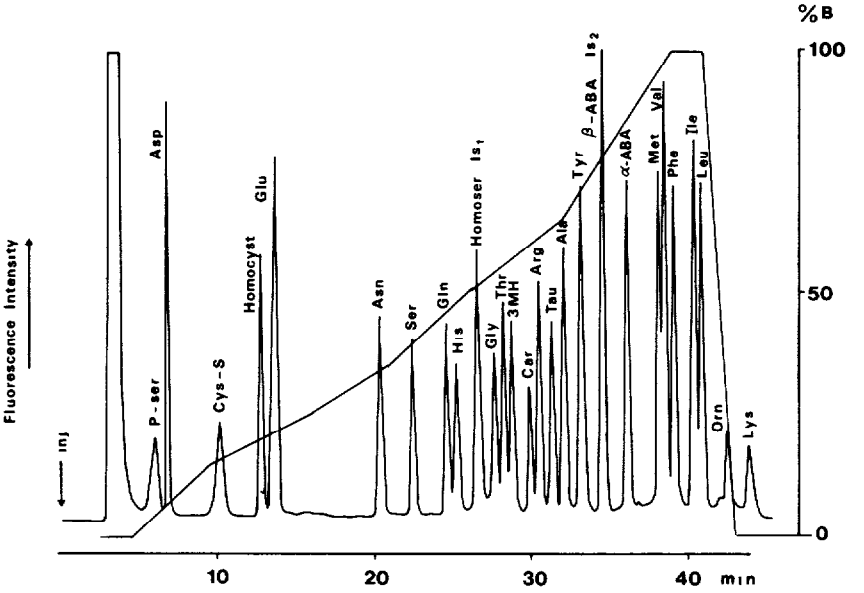

Fig. 1. Typical chromatogram showing the separation of 27 OPA–2-ME-derivatized standard AAs (10 μM). For chromatographic conditions see Experimental. Also shown is the mobile phase composition, expressed as the percentage of mobile phase B.

linearly from 0.2 to 1.0 ml/min. The column was equilibrated with mobile phase A for 7 min between injections.

Derivatization procedure

The derivatization of OPA with the sample or standard (25 μl each) was performed automatically for 2 min before injection onto the column. The details of this procedure have been published elsewhere [21].

Biological samples and their treatment

Venous blood samples were collected from eight healthy subjects (two females and six males aged 30–47 years) between 7 a.m. and 9 a.m. after overnight fast. Plasma was removed immediately after centrifugation of the blood samples at 2500 *g* for 15 min. Each plasma sample was divided into two equal portions of 0.5 ml, and 100 μl each of 200 μM β-ABA (I.S. 1) and homoserine (I.S. 2) were added before deproteinization with 100 μl of 30% SSA, 1 *M* perchloric acid, 20% TFA and 1 ml each of acetone, ethanol and acetoritrile separately. The total volume of each sample was adjusted to 2.0 ml with distilled water. These procedures removed 90–100% of sample proteins as determined by the modified Lowry method [30]. After centrifugation at 2500 *g*, the supernatant was separated and kept at $-70°C$ if not analysed immediately.

For the experiments on delayed deproteinization, to each 0.5-ml plasma sample, from eight healthy subjects, were added 100 μl of 100 μM each of I.S. 1 and I.S. 2. To this mixture, 100 μl of 30% SSA were added either immediately or after the plasma samples had been kept at room temperature for 30, 60, 120 or 180 min. The total volume was adjusted to 1.0 ml by addition of distilled water. The samples were then centrifuged at 1500 *g* for 15 min and the supernatants removed. The supernatants were kept at $-70°C$ if not analysed immediately.

RESULTS AND DISCUSSION

Any optimized method of analysis is judged by its separation of the substance of interest with reproducibility and sensitivity under specific experimental conditions. The goal in most clinical studies is to refine and improve the understanding of the effects of excessive or defective accumulation of the substances to be analysed. The desirable criteria for any new method are a good reproducibility with a coefficient of variation (C.V.) of 2–4% and a correlation coefficient (r^2) of unity for the regression line in the biological concentration range.

Fig. 1 shows the baseline separation of 10 μM of the 25 OPA-derivatized AAs where the whole separation is completed within 45 min. Fig. 2 shows the separation of plasma AAs under identical experimental conditions.

Table I shows the reproducibility of the data in terms of retention time and

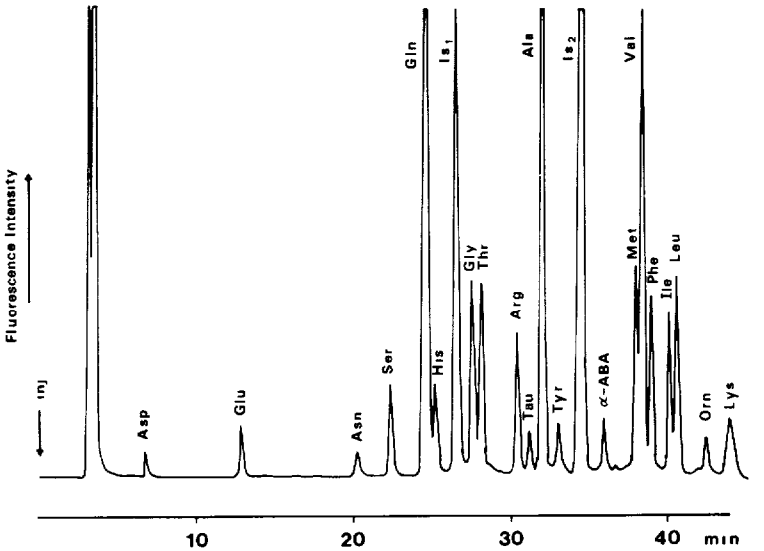

Fig. 2. Chromatogram of plasma amino acids from a healthy volunteer under identical experimental conditions as in Fig. 1.

integrated area for each AA. Most of the AAs show highly reproducible results in terms of retention times, with C.V. values less than 1%. For integrated areas, most AAs show reproducible results with C.V. values between 1 and 4%, apart from Cys-S (5.24%), Orn (5.44%) and Lys (5.52%). The regression data for all AAs in the concentration range 5–100 μM gave r^2 equal to unity.

In dealing with biological samples, the proteins should be completely removed from the sample prior to analysis. Plasma proteins are known to adsorb to the ion-exchange resin and to clog the column [31]. Similarly, direct injections onto reversed-phase columns are known to affect the retention behaviour of the solutes and cause premature column failure by irreversible adsorption of proteins onto the stationary phase [32]. Various procedures such as ultrafiltration, liquid–liquid and liquid–solid extraction, use of a column-switching technique and chemical precipitation are commonly used to remove the proteins from biological samples. With ultrafiltration, the amount of free AAs is dependent on the volume of the sample taken and it is a time-dependent procedure. It is also known that certain AAs degrade during the process [33]. In liquid–liquid extraction, the solvent should be of equal or weaker solvent strength compared with the mobile phase to overcome deleterious affects on peaks shape and resolution. In addition, the solvent should afford a high degree of recovery for the analytes, which is difficult to achieve since AAs differ in their physicochemical characteristics, such as pK_a values and hydrophibicity. An evaporation step could be introduced to eliminate the presence of the solvent but the stability of AAs could be a problem.

TABLE I

REPRODUCIBILITY OF RETENTION TIMES AND INTEGRATED AREAS

Obtained by the HPLC method based on pre-column derivatization of amino acids with OPA-2-ME. All parameters are calculated from seven analyses with the concentration of amino acids corresponding to 5 μM. S.D. = relative standard deviation.

Amino acid	Retention time (t_R)			Area ($A \times 10^6$)		
	Mean (min)	S.D. (min)	C.V. (%)	Mean	S.D.	C.V. (%)
Asp	6.89	0.04	0.52	3.37	0.06	1.80
Cys-S	11.15	0.08	0.74	1.00	0.03	5.24
Glu	13.48	0.03	0.25	3.16	0.03	1.01
Asn	20.74	0.02	0.12	2.11	0.04	1.72
Ser	22.76	0.02	0.11	2.21	0.05	2.53
Gln	24.90	0.02	0.10	1.89	0.06	3.37
His	25.50	0.01	0.06	1.75	0.05	2.82
Gly	27.84	0.00	0.00	2.02	0.04	2.05
Thr	28.29	0.03	0.09	2.46	0.05	2.06
3-MH	28.70	0.02	0.08	2.10	0.03	1.52
Car	29.86	0.01	0.05	1.62	0.06	3.90
Arg	30.53	0.03	0.09	2.16	0.05	2.24
Tau	31.31	0.03	0.09	2.33	0.06	2.71
Ala	32.13	0.02	0.07	2.87	0.05	1.78
Tyr	33.13	0.02	0.07	3.18	0.04	1.43
β-ABA	34.52	0.03	0.08	5.09	0.07	1.31
α-ABA	35.98	0.03	0.10	3.28	0.06	1.75
Met	37.91	0.03	0.08	3.38	0.07	2.10
Val	38.20	0.03	0.09	4.22	0.05	1.12
Phe	38.80	0.03	0.08	3.12	0.05	1.54
Ile	40.02	0.05	0.12	3.55	0.05	1.38
Leu	40.51	0.06	0.15	3.27	0.06	1.9
Orn	42.26	0.07	0.16	1.08	0.06	5.44
Lys	43.73	0.11	0.24	1.35	0.07	5.52

Column switching [34] or the use of a column packing material with external and internal surfaces specially designed for a plasma sample [35] could be an appropriate procedure. Even the use of a micellar mobile phase has been proposed, to keep the plasma proteins solubilized and to protect the analytical column [36]. However, up to now no attempt has been made to use these methods for the analysis of AAs in biological matrices, and the reliability of the analytical column after a number of injections has so far not been evaluated.

Among these procedures, chemical precipitation is still one of the most suitable methods, but it is slow. In this strategy, various acids, bases and organic solvents are used. In selecting any such deproteinization procedures, the main

factors to be considered are the amount of protein to be removed, the thermal and chemical stability and solubility of the analytes and cost and time.

Table II shows the effect of various deproteinizing agents on plasma AA levels. Among these agents, the use of 30% SSA and 1 *M* perchloric acid showed consistent results with high recoveries for most AAs. The variation in recovery is large with organic solvents. Asp and Glu increased by 44–54%, whereas Asn and Gln decreased by 12–28% in organic solvents, compared with perchloric acid and SSA. Asp and Glu are hydrolysis products of Asn and Gln, and the increase in the former AAs is the consequence of the decreased concentrations of the latter AAs [24,37,38]. Among the AAs, only Thr, Phe, Ile and Orn are stable in all precipitating media. The degradation of AAs is less in acids such as perchloric acid and SSA than in organic solvents. Various factors, such as chemical instability, hydrolysis, degradation and oxidation, could account for these variations in AA levels.

Table III shows the effect of delayed proteinization on plasma AA levels. Most of the AAs remained stable for up to 30 min at room temperature, except

TABLE II

PLASMA LEVELS OF FREE AMINO ACIDS IN EIGHT HEALTHY SUBJECTS

Values, obtained after deproteinization with 30% SSA (I), 1 *M* perchloric acid (II), 20% TFA (III), acetonitrile (IV), ethanol (V) and acetone (VI).

Amino acid	Concentration (mean ± S.D. $n=8$) (μM)					
	I	II	III	IV	V	VI
Asp	13± 4	12± 3	10± 4	18± 6	17± 4	20± 5
Glu	33± 5	32± 4	33± 4	47± 6	40± 4	49± 5
Asn	68± 7	68± 6	63± 8	56± 9	62± 7	49± 9
Ser	92±12	92±11	87±12	83± 9	83±10	80±12
Gln	498±40	482±37	483±49	453±48	440±43	436±46
His	93±11	92±10	87±11	74± 8	79±12	73±11
Gly	232±26	236±31	215±27	219±32	230±32	214±28
Thr	121±19	123±17	114±21	118±18	116±21	111±19
3-MH	8± 4	8± 4	6± 3	6± 4	5± 3	5± 3
Arg	118±27	110±21	105±26	89±21	93±25	82±20
Tau	60± 5	58± 3	55± 5	48± 5	48± 5	37± 4
Ala	396±37	400±42	406±32	366±36	382±39	372±31
Tyr	69± 8	59± 9	51±10	63±11	62±11	51±11
Met	29± 5	29± 4	28± 4	27± 5	25± 4	27± 5
Val	268±41	260±36	217±27	232±31	257±31	230±33
Phe	66±13	64±10	65±11	66±12	63±10	62±11
Ile	72±16	74± 9	68±12	72±11	70± 9	71±12
Leu	166±29	163±25	165±25	142±21	152±27	137±26
Orn	54± 7	48± 6	47± 6	51± 5	53± 6	50± 6
Lys	223±38	212±33	202±38	199±33	201±36	187±24

TABLE III

PLASMA LEVELS OF FREE AMINO ACIDS IN EIGHT HEALTHY SUBJECTS

Samples were deproteinized with SSA immediately (I) and after keeping the samples for 30 (II), 60 (III), 120 (IV) or 180 (V) min at room temperature (21 ± 2 °C).

Amino acid	Concentration (mean ± S.D.) (μM)				
	I	II	III	IV	V
Asp	13 ± 4	15 ± 3	21 ± 4	26 ± 5	18 ± 4
Glu	33 ± 15	34 ± 7	48 ± 8	70 ± 9	42 ± 9
Asn	68 ± 7	63 ± 8	44 ± 7	32 ± 6	25 ± 4
Ser	92 ± 12	92 ± 11	82 ± 9	76 ± 9	61 ± 11
Gln	498 ± 40	470 ± 33	446 ± 38	386 ± 29	228 ± 32
His	93 ± 11	82 ± 9	73 ± 10	57 ± 7	34 ± 8
Gly	232 ± 26	243 ± 28	256 ± 30	206 ± 32	180 ± 31
Thr	121 ± 19	119 ± 20	115 ± 17	87 ± 16	73 ± 16
3-MH	8 ± 4	6 ± 3	3 ± 2	N.D.[a]	N.D.[a]
Arg	118 ± 27	94 ± 18	64 ± 16	41 ± 13	23 ± 8
Tau	60 ± 5	60 ± 4	58 ± 3	54 ± 4	51 ± 2
Ala	396 ± 37	394 ± 38	378 ± 36	301 ± 28	178 ± 14
Tyr	69 ± 8	70 ± 7	70 ± 7	64 ± 5	49 ± 5
Met	29 ± 5	24 ± 3	20 ± 3	14 ± 2	8 ± 2
Val	268 ± 41	276 ± 39	279 ± 38	199 ± 23	115 ± 12
Phe	66 ± 13	65 ± 11	61 ± 8	55 ± 8	44 ± 6
Ile	72 ± 16	71 ± 14	68 ± 11	58 ± 10	38 ± 6
Leu	166 ± 29	167 ± 27	158 ± 27	141 ± 21	93 ± 14
Orn	54 ± 7	56 ± 7	64 ± 7	48 ± 8	40 ± 5
Lys	223 ± 38	220 ± 34	209 ± 31	166 ± 31	132 ± 16

[a]N.D. = not detected.

Asp, Glu, Gly, Ala and Orn, which increased by 15, 3, 5, 3 and 4%, respectively, while Asn, Gln, His, 3-MH, Arg and Met decreased by 6, 12, 23, 20 and 17%, respectively. A previous study [26] showed similar tendencies for Gln, Glu, Asp and Asn, even when stored at −20 °C. In our study, most AAs degraded with time when the plasma samples were kept at room temperature, except Asp and Glu, which increased for the first 2 h and then decreased. These increases were probably due to hydrolysis of Asn and Gln, and the subsequent decrease is attributed to degradation similar to other AAs.

Apart from the above factors, the time of day, nutritional status of the subject, shift in baseline, overlap of one or more AAs, contamination by platelets and leukocytes, physical activity by subjects before blood collection, menstrual cycle, sex and age differences could result in variations in the plasma AA profile. Hence, all these factors should be controlled to assess with certainty the clinical data under various pathological conditions.

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