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SENSITIVE ANALYSIS OF ASPARAGINE AND GLUTAMINE IN PHYSIOLOGICAL FLUIDS AND CELLS BY PRECOLUMN DERIVATIZATION WITH PHENYLISOTHIOCYANATE AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The analytical methodologies for the determination of free amino acids in plasma, serum, erythrocytes and leukemic cells are described. Deproteinization of the sample by methanol or organic acids is followed by derivatization with phenylisothiocyanate to form stable phenylthiocarbamylamino acid derivatives. The derivatives are separated by reversed-phase high-performance liquid chromatography in 80 min using a 5-μm C_{18} column (250 × 4 mm I.D.) and monitored by ultraviolet detection at 254 nm. Twenty physiological amino acids are resolved and quantified in plasma and erythrocyte samples. The resolution and sensitivity of the analytical method permitted unequivocal quantification of very low asparagine and glutamine levels in leukemic cells and growth media following treatment with asparaginase and glutaminase enzymes despite the presence of high aspartic and glutamic acid levels.

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INTRODUCTION

The amino acid analysis method first developed by Moore et al. [1, 2] is not suitable for the separation and quantitation of asparagine and glutamine in some physiological samples. These amino acids appear as a triplet with glutamate that is incompletely resolved and highly pH-dependent. More recent new methods for amino acid analysis have sought to achieve a number of objectives: (1) low level detection (quantification of 1–10 pmol); (2) short analysis times with standard high-performance liquid chromatographic (HPLC) techniques; (3) detection of secondary as well as primary amino acids; (4) linear detector response; and (5) simple and rapid sample preparation. A number of precolumn derivatization techniques have been described using *o*-phthalaldehyde (OPA) [3, 4], dansyl chloride [5–7], dimethylaminoazobenzene-4′-sulfonyl chloride (DABS) [8] and phenylisothiocyanate (PITC) [9, 10].

The use of PITC for peptide sequencing was first introduced by Edman in 1950 [11]. These derivatives are usually cyclized and rearrange under acid conditions to form phenylthiohydantoin derivatives. Recently, Heinrikson and Meredith [9] introduced precolumn derivatization using PITC to form phenylthiocarbamyl (PTC) derivatives of amino acids present in protein hydrolyzates. The derivatives were separated by reversed-phase HPLC and monitored by UV detection at 254 nm. They found that PITC reacts quantitatively with all amino acids including proline and hydroxyproline to form derivatives that are stable, produce a linear detector response and have high molar absorptivities permitting the analysis at 1–10 pmol levels. The method is also characterized by high reproducibility of elution times and quantification. The potential for separation of both dicarboxylic amino acids from asparagine and glutamine made these derivatives particularly interesting for physiological samples and our studies with amidase enzymes.

In our laboratories we have developed methods for amino acid analysis of physiological fluids and cells using precolumn derivatization with PITC and separation by reversed-phase HPLC. Twenty physiological amino acids are resolved within 80 min with a sensitivity of 10 pmol. A comparison of sample extraction methods using methanol, perchloroacetic acid (PCA) and 5-sulfosalicylic acid (SSA) is also presented.

EXPERIMENTAL

Materials

Amino acids were purchased from Schwartz-Mann (Orangeburg, NJ, U.S.A.) and Sigma (St. Louis, MO, U.S.A.). *S*-Carboxymethyl-L-cysteine and L-α-aminoadipate were also obtained from Sigma. Phenylisothiocyanate and 5-sulfosalicylic acid (Sequanal grade) were from Pierce (Rockford, IL, U.S.A.). Triethylamine (99+%) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile and ethanol were HPLC grade from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Derivatization

Standards were prepared as follows: 1 m*M* aqueous solutions of Asp, Glu,

Ser, Gly, Asn, Gln, Thr, Ala, His, Pro, HPro, Arg, Tyr, Val, Met, Cys, Ile, Leu, Phe, Trp, Lys, α-aminoadipate and carboxymethylcysteine were prepared. A 100-μl volume (100 nmol) of each was added to a borosilicate test tube and lyophilized to obtain a dry powder. The residue was dissolved in 25 μl glass-distilled, deionized water, 25 μl of triethylamine and 50 μl ethanol, then evaporated to dryness under high vacuum (0.5 h at room temperature, < 0.2 mm Torr). The residues were dissolved in 70 μl ethanol, 10 μl water, 10 μl triethylamine and 10 μl PITC, allowed to react for 20 min at room temperature, then evaporated to dryness under high vacuum (1 h at room temperature, < 0.2 mm Torr). The PTC-amino acids were stored dry in the freezer or dissolved in a solution containing 5% acetonitrile and 95% 7 m*M* disodium hydrogen phosphate, adjusted to pH 7.4 with phosphoric acid (buffer C) and frozen or kept on ice until the analysis. Samples (10 μl) containing 10 pmol to 10 nmol of each amino acid were analyzed by reversed-phase HPLC as described below. PTC derivatives of individual amino acids were also prepared and analyzed in order to establish the identities of each peak.

Chromatography

Samples were injected onto a 250 × 4 mm Bio-Sil ODS-5S column (Bio-Rad, Richmond, CA, U.S.A.) and resolved by a two-step gradient elution. The first step consisted of a 40-min linear gradient from 100% of buffer A (0.05 *M* ammonium acetate adjusted to pH 6.8 with phosphoric acid) to 92% buffer A and 8% of buffer B [0.1 *M* ammonium acetate, pH 6.8—acetonitrile (50:50)]. The second step consisted of a 40-min linear gradient from 92% A plus 8% B to 40% A plus 60% B. The flow-rate was kept constant at 1.0 ml/min and the effluent monitored at 254 nm (Spectroflow 773 variable-wavelength UV detector, Kratos, Ramsey, NJ, U.S.A.). The column was washed with 100% buffer B for 15 min, then reequilibrated with buffer A for 10 min prior to the next injection. Peak integration was performed using an M730 data module (Waters Assoc., Milford, MA, U.S.A.). Prefilters of Solvicon (Whatman, Clifton, NJ, U.S.A.) preceded the sample injector and the column.

Sample preparation of serum or plasma

Samples (50 μl) of serum or plasma were added to 450 μl of cold methanol containing 10 μl of 1 m*M* L-α-aminoadipate (internal standard) in a 1.5-ml microfuge tube. The mixture was vortexed and centrifuged at 8700 *g* for 1 min in a microfuge (Beckman, Palo Alto, CA, U.S.A.). The supernates were transferred to test tubes and evaporated to dryness using a Speed Vac concentrator (Savant Instruments, Hicksville, NY, U.S.A.) (1 h, oven temperature 45°C). The residues were derivatized as described above, dissolved in 50 μl buffer C and 10-μl samples analyzed (absorbance range = 0.05 a.u.f.s.).

Sample preparation of erythrocytes

Methanol extraction. A 50-μl aliquot of packed, unwashed erythrocytes (approx. $4 \cdot 10^8$ cells, 12.5 mg hemoglobin) was extracted and analyzed as described above except the extracts were sonicated for 1 min prior to centrifugation.

SSA extraction. A 50-μl aliquot of packed, unwashed erythrocytes was

added to 0.5 ml of 10% SSA and 10 μl of a 1 m*M* solution of internal standard in a 1.5-ml microfuge tube. The mixture was sonicated in an ice bath for 2 min and centrifuged at 8700 *g* for 1 min. The pellets were suspended in 0.4 ml of 5% SSA and centrifuged for 1 min at 8700 *g*. This last procedure was repeated and the supernatants were combined. The pH was adjusted to 2.6– 2.8 with 2 *M* lithium hydroxide and the extracts diluted to 3 ml with a lithium citrate buffer (12.8 g citric acid in 1 l, pH adjusted to 2.6 with 1 *M* lithium hydroxide). The samples were lyophilized to obtain dry powders which were dissolved in 0.5 ml of 0.01 *M* acetic acid and applied to 2.5 × 0.5 cm Dowex 50W-X8 columns equilibrated with 0.01 *M* acetic acid. After washing with three column volumes of 0.01 *M* acetic acid, amino acids were eluted with three column volumes of 1 *M* ammonium hydroxide. These were lyophilized, derivatized and dissolved in 50 μl of buffer C prior to analysis (10-μl injection, absorbance range = 0.05 a.u.f.s.).

PCA extraction. A 50-μl aliquot of packed, unwashed erythrocytes was added to 0.5 ml phosphate-buffered saline containing 10 μl of 1 m*M* internal standard in a 1.5-ml microfuge tube; then 0.5 ml of 0.8 *M* PCA were added, the tubes vortexed and put on ice for 5 min. After a 1 min centrifugation at 8700 *g*, the pellets were resuspended in 0.5 ml of 0.4 *M* PCA and centrifuged for 1 min. The supernatants were combined, neutralized with potassium hydroxide, centrifuged for 1 min at 8700 *g* and the supernatants were lyophilized. In order to remove residual salts, the PCA extracts were dissolved in 0.5 ml of 0.01 *M* acetic acid, adjusted to pH 3 with 1 *M* hydrochloric acid, and the samples applied to cation-exchange columns as described above. Samples were derivatized and analyzed as described above. Controls for each extraction method were performed using water instead of physiological fluid or cells.

Sample preparation of leukemic cells

L5178Y cells and growth media were extracted with SSA as described elsewhere [12]. The supernatants were adjusted to pH 2.6– 2.8 with lithium hydroxide and stored frozen. Aliquots of 0.5 ml were applied to cation-exchange columns as described for erythrocyte SSA extracts, lyophilized, derivatized and dissolved in 25 μl of buffer C prior to analysis. Samples (10 μl) were injected and eluted by a linear gradient from 100% buffer A to 93% A plus 7% B over 35 min. This system maximized the resolution of the early eluting amino acid derivatives.

RESULTS

Separation of physiological amino acids

Fig. 1 illustrates a separation of a synthetic mixture containing 5 nmol of each amino acid reference compound. Resolution of all twenty amino acids normally present in physiological samples is achieved; in particular, asparagine, glutamine and glutamate are separated with baseline resolution. In addition, α-aminoadipate, hydroxyproline and carboxymethylcysteine are resolved. Elution times are approximately twice those required for separation of amino acid hydrolysates [9]. The internal standard, α-aminoadipate, had an average

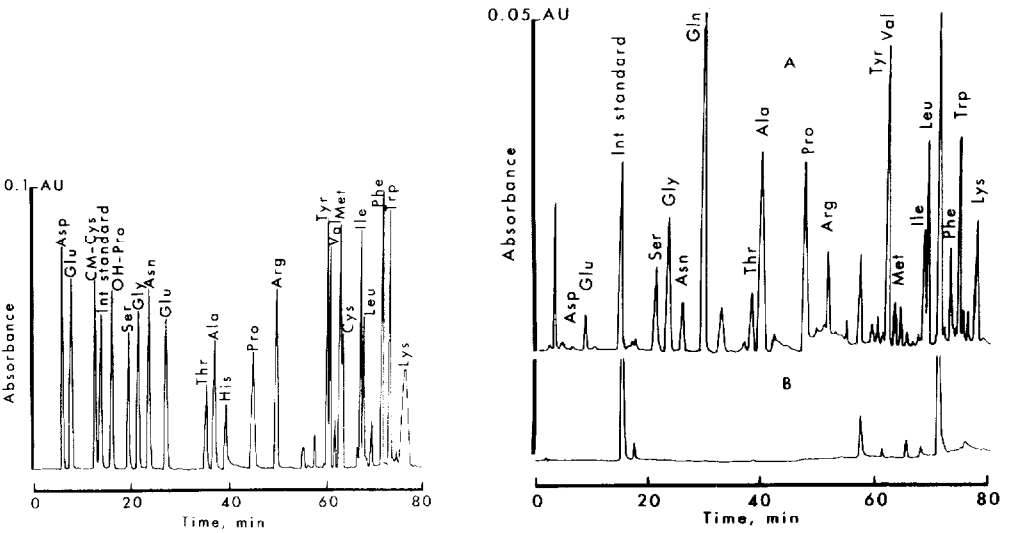

Fig. 1. Chromatographic separation of 5 nmol each of an amino acid mixture after derivatization with PITC. α-Aminoadipate was used as internal standard. Detection was at 254 nm (0.1 a.u.f.s.).

Fig. 2. (A) Chromatographic separation of amino acids in a 50-μl sample of human plasma after methanol extraction and derivatization with PITC. One fifth of the sample was injected on the column. The internal standard was α-aminoadipate (2 nmol). Detection at 254 nm (0.05 a.u.f.s.). Tyr and Val were not separated on this particular chromatogram. (B) Separation of water blank plus internal standard derivatized and analysed at the same time and conditions as the plasma sample.

peak area of 3.0 ± 0.02 · 10^5 μV · sec per nmol (n = 5). For six analyses of 0.1– 10 nmol of each amino acid, the average ratio of the amino acid peak area to internal standard peak area per nmol was 1.0 ± 0.08 (S.D.) (n = 16) for all amino acids except threonine (0.63 ± 0.04), histidine (0.68 ± 0.06), cysteine (0.64), phenylalanine (1.3 ± 0.08), tryptophan (1.3 ± 0.12) and lysine (1.8 ± 0.36). Plots of peak area versus nmol of amino acid are linear from 10 pmol to 10 nmol with the exception of histidine which appears to have a lower detector response at lower amounts.

Fig. 2A shows a chromatogram of a 50-μl sample of human plasma extracted with methanol. The results were identical for serum. Fig. 2B is a water blank extracted and derivatized under the same conditions. α-Aminoadipic acid, the internal standard, is seen in both analyses. Unidentified peaks are present in the control at about 18, 57, 61, 65, 68 and 71 min; these may represent products of PITC and amine impurities in the solvents. They could not be eliminated by reaction of the triethylamine with ninhydrin and redistillation, but they were clearly separated from the amino acid derivatives.

Table I shows the calculated average concentrations for four analyses of the same sample of human plasma. These concentrations agree well with literature values obtained by amino acid analysis with ninhydrin post-column derivatization and with *o*-phthalaldehyde precolumn derivatization [13– 15].

Fig. 3 shows the separation of methanol extracts of erythrocytes. Sonication was required to achieve maximal extraction efficiency. PCA and SSA extraction procedures produced an unidentified peak that co-eluted with

TABLE I

AMINO ACID ANALYSIS OF HUMAN PLASMA AND ERYTHROCYTES

Amino acid	Concentration (mean ± S.D.) (μM)			
	Plasma*	Erythrocytes**		
		Methanol	PCA	SSA
Asp	2 ± 0.3	124 ± 13	153 ± 15	109 ± 14
Glu	31 ± 4	169 ± 25	229 ± 86	182 ± 22
Ser	105 ± 12	97 ± 14	133 ± 8	102 ± 6
Gly	166 ± 21	266 ± 34	388 ± 37	260 ± 23
Asn	62 ± 7	83 ± 11	85 ± 13	71 ± 4
Gln	534 ± 43	269 ± 36	348 ± 31	269 ± 22
Thr	134 ± 18	40 ± 4	—	—
Ala	306 ± 35	291 ± 39	321 ± 11	256 ± 20
His	15 ± 9	16 ± 5	19 ± 1	18 ± 6
Pro	278 ± 53	171 ± 26	177 ± 31	162 ± 24
Arg	79 ± 1	0.6 ± 0.6	9 ± 1	15 ± 12
Tyr	122 ± 23	80 ± 13	41 ± 13	35 ± 14
Val	251 ± 23	184 ± 24	168 ± 8	131 ± 25
Met	23 ± 5	25 ± 4	—	—
Cys	—	—	—	—
Ile	89 ± 7	73 ± 9	72 ± 2	59 ± 9
Leu	179 ± 14	141 ± 18	127 ± 3	101 ± 13
Phe	78 ± 7	73 ± 7	55 ± 6	50 ± 9
Trp	137 ± 18	124 ± 18	270 ± 16	262 ± 37
Lys	117 ± 23	107 ± 10	230 ± 12	211 ± 23

*Four determinations on the same sample of human plasma. Cysteine and cystine cannot be determined by this method.

**Three determinations each by methanol, PCA and SSA extraction of the same sample of packed, unwashed human erythrocytes. Threonine was quantified only in the methanol extractions. Cysteine and cystine could not be determined by this method.

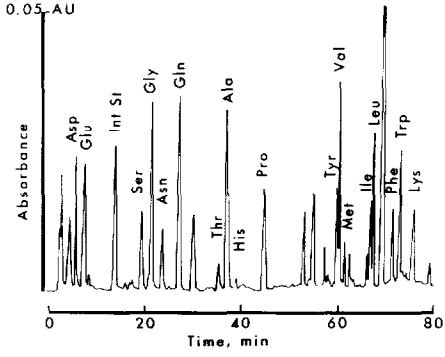

Fig. 3. Chromatographic separation of amino acids in a 50-μl sample of packed, unwashed human erythrocytes extracted with methanol and derivatized with PITC. One fifth of the sample was injected on the column. The internal standard was α-aminoadipate (2 nmol). Detection at 254 nm (0.05 a.u.f.s.).

threonine. This peak was not observed with methanol extraction. Extraction efficiencies for the three methods are comparable (75–95%). The average results of extractions of the same human erythrocyte sample by the three extraction procedures are shown in Table I. Except for threonine, comparable results are produced with each extraction method. These values for amino acid concentration in human erythrocytes correlate well with the literature values [15].

Quantitation of asparagine and glutamine in L5178Y cells treated with asparaginase or depleted media

Our recent report [12] showed that pretreatment of L5178Y cells with *Escherichia coli* asparaginase prevented methotrexate cytotoxicity and decreased the extent of methotrexate polyglutamation. This pretreatment caused intracellular asparagine concentrations to fall from 1–2 μmol/g to less than 0.06 μmol/g (the limit of sensitivity of the Joel 5AH analyzer system). Glutamate and glutamine levels were not decreased.

In recent experiments, we have investigated the effect of incubation of these cells in glutamine-free or asparagine-free media on methotrexate cytotoxicity and polyglutamation. L5178Y cells were extracted with SSA after incubation for 6 h in RPMI 1640 media plus 10% dialysed fetal calf serum deficient in glutamine or asparagine or this media containing 0.1 I.U. *E. coli* asparaginase. The amino acids in the extracts were analyzed by this PITC method and the standard ninhydrin method. Total intracellular methotrexate was determined as described [12].

Table II shows the intracellular concentrations of asparagine, glutamine, aspartic acid and glutamic acid in these cells, and the percentage of the intracellular methotrexate concentration in complete media controls. Table II shows that a decrease in methotrexate retention occurred with either low intracellular asparagine or glutamine. *E. coli* asparaginase and asparagine-free media produced comparable changes in intracellular asparagine concentration. Nevertheless, *E. coli* asparaginase produced a greater decrease in methotrexate accumulation and more protection from methotrexate cytotoxicity (data not

TABLE II

EFFECT OF MEDIA ON INTRACELLULAR FREE AMINO ACIDS AND METHOTREXATE RETENTION IN L5178Y CELLS

Medium*	n**	Concentration*** (μmol/g of cell)				Methotrexate§ (%)
		Asn	Gln	Asp	Glu	
Complete	2	1.18	2.08	1.74	9.85	100
+Asparaginase	6	0.039 ± 0.011	1.30 ± 0.63	1.13 ± 0.47	7.46 ± 2.34	70
—Asn	4	0.042 ± 0.011	2.14 ± 1.00	1.37 ± 0.44	6.09 ± 1.12	85
—Gln	2	1.74	0.063	1.06	1.45	53

*Complete media was RPMI 1640 with 10% dialyzed fetal calf serum. Asparaginase was 0.1 I.U./ml *E. coli* asparaginase in complete media. —Asn and —Gln were complete media devoid of asparagine and glutamine, respectively.

**n is the number of samples analyzed by HPLC.

***Average ± S.D. values at 3 and 6 h incubation in each medium.

§Average percentage of intracellular methotrexate concentration in control media at 6 h from two separate experiments. Complete media 10.1—13.2 nmol/g of protein.

shown) than asparagine-free media. Thus, *E. coli* asparaginase may be exerting effects other than asparagine depletion [16].

The analyses of amino acids in these samples were performed by this present HPLC method and by a Joel 5AH analyzer with ninhydrin detection. The results from eight samples of media using the two methods were nearly identical (linear regression on a total of 40 pairs of values for Asp, Glu, Asn, Gln, Ser and Gly was HPLC = 0.87 · Joel − 0.016, r^2 = 0.98). The cell extracts showed greater variation. Linear regression on a total of 64 pairs for Glu, Asn, Gln, Ser and Gly was HPLC = 0.76 · Joel − 0.28, r^2 = 0.83. Aspartate showed a ratio of HPLC to Joel of only 0.4 ± 0.2 (n = 14). This lower value by HPLC is due to the co-elution of reduced glutathione with aspartate on the Joel analysis [17]. Aspartate is resolved from glutathione with the HPLC method.

DISCUSSION

Precolumn derivatization of samples with PITC and separation of the PTC-amino acids by reversed-phase HPLC permitted quantitative analysis of the amino acids in physiological fluids and cell extracts within 80 min. The derivatization is rapid and quantitative with nearly identical absorbances for all amino acids except threonine, histidine, cysteine and lysine. Lysine produces a double PITC derivative with almost twice the detector response of the internal standard. Threonine and cysteine have lower peak areas but are linear with concentration. Some problems exist in the quantification of histidine and cysteine by this method. Conditions may have to be modified to optimize histidine derivatization and maintain derivative stability. With cysteine a peak was seen at a late retention time with lower absorbance than that of most other amino acids. With cystine a peak was not detected in 80 min. Analysis of cysteine by conversion to the 5-carboxymethyl derivative may circumvent these problems since this compound produced a PTC derivative which was well separated on our HPLC system and had a similar absorbance (Fig. 1) [14, 18].

The derivatization is simple and reproducible. Chromatograms show minimal background or interference due to reagent peaks. Excess reagent is easily removed by evaporation under reduced pressure since the coupling reagent and solvents are volatile. The PTC-amino acids (except histidine) have a linear detector response and high molar absorptivities. Detection limits at 254 nm are of the order of 10 pmol.

Several methods of sample extraction with erythrocytes were compared. Methanol, PCA and SSA produced similar extraction efficiencies of 75—95%. Methanol extraction was simpler and more rapid since removal of salts by anion-exchange chromatography and lyophilization was not required. We have found that the Savant evaporator concentrates the pellet into a smaller area compared to lyophilization, thereby aiding in dissolving the sample in small volumes for the derivatization procedure. In addition, the acid extraction technique did not permit quantitation of threonine since controls contained a peak that coeluted with threonine. We did not test ultrafiltration or short precolumn absorption methods that have been used successfully for other HPLC methods [19].

Concentrations of amino acids in plasma and erythrocytes determined by

these methods were consistent with the literature values obtained by classical amino acid analysis [14, 15]. Furthermore, parallel analyses by the two methods in our laboratory gave comparable results.

The separations achieved by this HPLC method allowed us to quantify extremely small concentrations of asparagine and glutamine in extracts of L5178Y cells and media treated with *E. coli* asparaginase or depleted media. Asparagine was well separated from the high concentrations of glycine in these samples. Unlike standard amino acid analysis in lithium citrate buffers, aspartate was separated from reduced glutathione, and asparagine and glutamine were separated from glutamate in this HPLC system. These experiments showed that depletion of intracellular asparagine or glutamine was associated with decreased accumulation and cytotoxicity of methotrexate.

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