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Journal of Chromatography B, 754 (2001) 369–376

JOURNAL OF
CHROMATOGRAPHY B

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Improved method for the measurement of large neutral amino acids in biological matrices

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Received 3 October 2000; received in revised form 1 December 2000; accepted 14 December 2000

Abstract

A high-performance liquid chromatographic method for measuring neutral amino acids in rat sera, brain tissues, and perfusates was developed by using *o*-phthalaldehyde sulfite as a pre-column derivatization reagent. With the present method, it was possible to separate the neutral amino acids within a single run in 25 min, while the acidic amino acids were eluted near or at the solvent front. The recovery was above 88.8% with a relative standard deviation (RSD) below 4.2%. The within- and between-day assay reproducibility for the determination of rat serum amino acids showed RSDs below 1.35 and 7.61%, respectively. In the present study, the neutral amino acids were assayed with high sensitivity, accuracy and good reproducibility in a relatively short time and on a small sample size. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, LC; Amino acids, neutral

1. Introduction

Tryptophan and tyrosine hydroxylase are rate-limiting enzymes for the synthesis of dopamine (DA) and serotonin (5-HT), respectively [1]. As a result, the role of the amino acid precursors of these enzyme systems has received considerable attention [2,3]. It appears that fluctuations in the availability of free tyrosine or tryptophan may be reflected in DA and 5-HT synthesis, respectively, in both tissue and body fluids of experimental animals [2,4,5] and humans [6–8]. A relationship between precursor availability and neurotransmitter synthesis occurs because the hydroxylase may not be fully saturated at all times by tissue concentrations of the precursor [2]. Thus, control of precursor availability may have therapeutic potential where synthesis and function are abnormal [6,9,10].

As a group, the serum large neutral amino acids (LNAAs; tyrosine, valine, isoleucine, leucine, phenylalanine and tryptophan) compete with each other through the same transport system at the blood–brain barrier [11]. As a result, the brain concentration is dependent on the overall concentration of the serum LNAAs. The importance of a serum precursor concentration or the serum ratio (precursor concentration/ΣLNAAs) has become increasingly recognized as a predictor of tissue concentrations. Thus, it is important to elucidate the relationship of the serum to brain precursor concentration [2,4,5,12,13,15].

For the identification and quantification of amino

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PII: S0378-4347(00)00629-0

acids, liquid chromatography (LC) with pre- or post-column derivatization coupled with fluorescence or electrochemical detection has been utilized for its high sensitivity and specificity. Traditionally, amino acids have been separated by ion-exchange LC with post-column derivatization [16]. These methods require dedicated systems. Alternatively, amino acids can be analyzed by reversed-phase high-performance liquid chromatography (HPLC) with pre-column derivatization. Various reagents are available for this purpose, e.g., *o*-phthalaldehyde (OPA) [17], dansylchloride [18], 6-*N*-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate [19], fluoresceinisothiocyanate [20]. Of these reagents, OPA has been widely used [21]. The reaction of OPA with amino acids in the presence of a thiol reducing agent to produce fluorescent products is highly suited to reversed-phase HPLC with fluorescence detection [22]. Additionally, the electrochemical detection of the OPA/β-alkylthiol derivatives of amino acids is relatively unaffected by changes in the derivative structure compared to relative fluorescence of those derivatives [23,24]. Furthermore, many of the published methods that include OPA derivatization of the amino acids require gradient analysis, fluorescence for detection, and elution time of 15 to 60 min [25,26], without the adequate separation of all amino acids [17,19,27–29].

The main objective of this study was to improve the analysis of free neutral amino acids in rat serum and brain tissues using a pre-column derivatization with OPA-sulfite (OPA-S), followed by an isocratic reversed-phase separation and electrochemical detection. The method described below for the neutral amino acids, has many of the advantages of OPA chemistry with improved reagent and derivative stability, economy, and the versatility for use with small sample sizes across a broad range of physiological concentrations.

2. Experimental

2.1. Animal preparation

Male Sprague–Dawley rats 200–250 g (Zivic–Miller Lab., Zelienople, PA, USA) were housed in plastic cages and maintained under an AAALAC accredited facility. All procedures were in strict accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by a local committee.

Throughout each study, animals were deprived of food for 15 h prior to each experiment. For baseline controls animals were decapitated, trunk blood collected and brain tissues were immediately dissected on wet ice and stored at −80°C until analysis. For the kinetic study, animals were administered tyrosine methyl ester·HCl [200 mg/kg equivalent free L-tyrosine in saline: intraperitoneally (i.p.)]. At specified time intervals, the animals were sacrificed and samples collected as previously described.

Microdialysate sample collection was previously described [30]. Briefly, stainless steel guide cannulae were surgically implanted on the brain surface above the medial pre-frontal cortex (MPFC; coordinates relative to bregma, posterior 3.2 mm and from the midline ±0.8 mm) as described by Paxinos and Watson [31]. Microdialysis probes were slowly inserted into the cannulae, 24 h post surgery, and cemented with quick self-curing acrylic resin (Miles, Elkhart, IN, USA). Rats were used only once. The probes were perfused with Dulbecco phosphate buffered saline containing: (in m*M*) 137 NaCl, 2.7 KCl, 0.5 $MgCl_2$, 1.5 KH_2PO_4, 8.1 Na_2HPO_4, 1.2 $CaCl_2$, and 5 glucose (pH 7.4), via polyethylene tubing connected to a Harvard infusion pump at a rate of 1.5 μl/min. Each experiment was started 24 h after probe insertion to include perfusion media pumping and dialysate collection. Dialysates were collected and immediately analyzed or stored at −80°C until day of analysis.

2.2. Amino acid analysis

The chromatography system consisted of a liquid pump-10ADVP, an auto injector-10AD, and an electrochemical detector-ECD-6A (Shimadzu, Columbia, MD, USA). The electrochemical detector utilized a glassy carbon electrode (0.75 V vs. Ag/AgCl). The mobile phase consisted of 0.133 *M* Na_2HPO_4, 0.15 m*M* Na_2EDTA, and 25% methanol adjusted to pH 6.8 with *o*-phosphoric acid. Solutions were filtered through a 0.2-μm nylon-66 membrane filter. A reverse phase column (Allsphere ODS-2 3 μm, 100×4.6 mm; Alltech, Deerfield, IL, USA) was

utilized with a flow-rate of 0.5 ml/min. The column was thermostated at 36°C. Data collection, integration, and calibration was accomplished using a chromatography data acquisition system.

The derivatizing reagent (OPA-S) was prepared by adding the following in order: 10 mg of OPA (Molecular Probes, Eugene, OR, USA), 30 mg of sodium sulfite, 0.25 of ml water, 0.25 ml of methanol and vortex-mixed for approx. 45 s. This is followed by the addition of 4.5 ml of sodium borate buffer (0.4 *M* boric acid adjusted to pH 10.4 with 6 *M* NaOH) [32]. The derivatizing reagent was prepared once weekly and stored at room temperature in an amber bottle.

Individual amino acid stock solutions were prepared by dissolving 10 mg each into 10 ml of diluent (distilled water–methanol, 75:50, v/v). Tyrosine (only) was added to 9.5 ml of diluent, followed by 0.5 ml 30% NaOH. Stock solutions prepared in amber vials and refrigerated were stable for several months. Dilutions of the 1 mg/ml stock amino acids were used to make working standards in μg/ml. Concentration curves were designed to reflect the endogenous concentration range of the biological samples of interest. An internal standard (norvaline) was incorporated with the extracting diluent for quantitation (see individual sample procedures for details).

Standard reactions were prepared as follows: to 2-ml glass vials were added 10 μl of sample (standard solution, biological sample, or blank) and 10 μl of OPA-S. These were allowed to react for a minimum of 5 min. Afterwards, samples were diluted to 100 μl with HPLC mobile phase. Routinely, a 10 μl volume was injected on column.

2.3. Sample preparation

Serum samples were prepared as follows: to a series of 1.5-ml Eppendorf tubes was added 50 μl of sample (serum, standards, or blank) followed by 450 μl of ice cold ethanol [33] containing 10 μg/ml of norvaline. Afterwards, samples were vortex-mixed for 30 s and centrifuged for 45 min at 7300 *g*. Supernatants were derivatized as described in standard reactions (Fig. 1A and B).

Tissue samples were prepared as follows: frozen brain tissues (~ 8 mg) were placed in 1.5-ml centrifuge tubes containing 400 μl of ice-cold 0.1 *M* $HClO_4$ containing 1.0 μg/ml norvaline. The samples were sonicated for 6 to 8 s with a micro-ultrasonic cell disrupter (Kontes Scientific Glassware/Inst., Vineland, NJ, USA). Subsequently, samples were centrifuged for 4 min at 16 000 *g* at 4°C. The supernatants, standards (0.156 to 2.5 μg/ml), and blanks were derivatized as previously described except that an additional 10 μl borate buffer was added to each tube to buffer perchloric acid (Fig. 1C).

Microdialysate samples were directly reacted with the reagent. A 10-μl sample of standard, sample and blanks was added to an autosampler microvials (Alltech), followed by 10 μl of norvaline (1 μg/ml), 10 μl of OPA-S, reacted for 5 min, and diluted to 100 μl with HPLC mobile phase (Fig. 1D).

2.4. Statistical analysis

The accuracy and precision of the HPLC methods were estimated by regression analysis according to the method of inverse regression [34].

3. Results

The reaction of amino acids with OPA-S reagent generates electroactive derivatives [32] that were easily separated by the present conditions. Because acidic amino acids elute near the solvent front, followed by baseline resolution of the neutral amino acids, the method is ideally suited for rapid automated analysis of the latter. The total analysis was approximately 25 min. As demonstrated in Fig. 1A, baseline resolution is achieved for most LNAAs with minor overlap between leucine and phenylalanine.

To assess the reaction time to product formation, valine (10 μg/ml) was selected because of its low molecular mass and early elution time. Reaction end-point was determined by the addition of HPLC mobile phase (see Section 2.2) to the reaction vial followed by an immediate on column (manual) injection. Reaction end-points were determined from 5 to 60 s and 2 to 5, 15, 30 and 45 min. The results showed a 65% product formation at 45 s. The time course to maximum product formation was calculated at 1 min. Additionally, the reaction product was

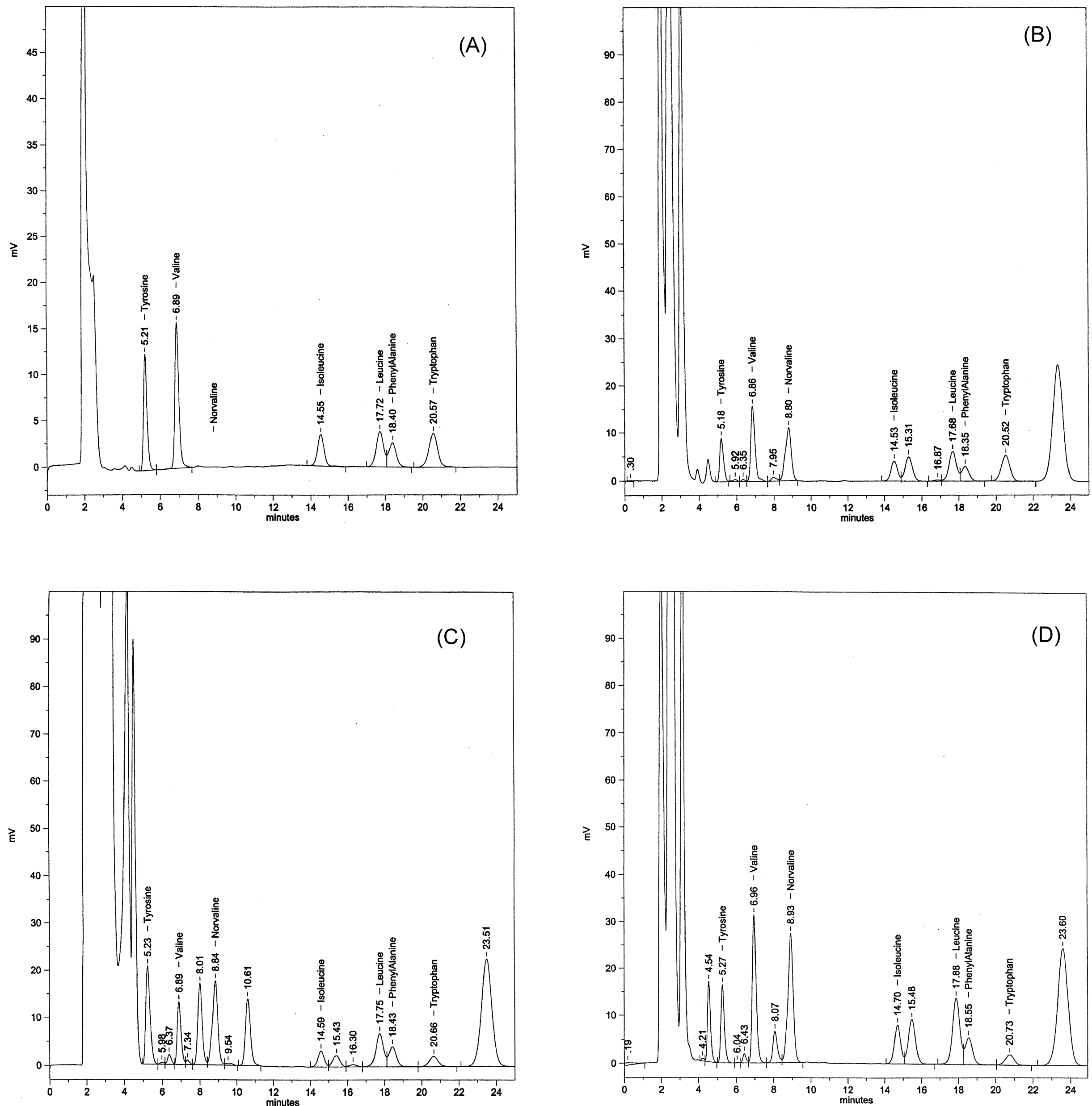

Fig. 1. (A) Elution profile of an OPA-derivatized neutral amino acid standard (1.25 ng each) mix. Column: Allsphere ODS II 3 μm (100×4.6 mm); flow-rate: 0.5 ml/min; column temperature 36°C; In the *y*-axis 1 mV=1 nA. (B) Chromatogram of rat serum neutral amino acids under identical experimental conditions as in (A). Detailed methods described in the text. (C) Chromatogram of neutral amino acids in brain tissue (medial pre-frontal cortex). Sample is a representation of a dosed animal with 200 mg/kg tyrosine at 15 min post i.p. Detailed methods described in the text. (D) Chromatogram of neutral amino acids in dialysate content from the awake rat following 1.5 h baseline stabilization. Detailed methods described in the text. On column injection 20 μl.

followed from 2 to 45 min. In that time range no changes were noted in the peak response, concluding that the reaction was complete within 1 min.

The reagent stability was evaluated as follows: daily, OPA-S was prepared and stored in amber vials. On the eighth day each reagent vial was tested utilizing a solution of valine (2 μg/ml). Each OPA-S reagent was reacted with valine and analyzed as previously described. The results showed no remarkable differences in peak response (relative standard deviation<6.3%). Additionally, the reaction products were checked for stability. A series of standard LNAA mixes, 0.625 to 2.5 μg/ml, were derivatized in triplicate on day one and analyzed for 3 consecutive days. The overall correlation of the study (r) was>0.999 (Table 1).

The ratio of reagent (OPA-S) to amine, reaction time, and derivative stability was extrapolated and or optimized from published data [32]. The optimum ratio of OPA-S to total amine concentration was evaluated utilizing a mix of LNAAs in a concentration range of 0.08 to 10 μg/ml. Optimal reactant's ratio for the maximum amine concentration (60 μg/ml) reaction was 1:33 (amine: OPA-S) in the reaction mix. In that range, the results of the analysis showed a linear relationship (r>0.999) with similar peak responses for each LNAA. Lastly, increased amounts of OPA-S (see Section 2.2) in the reaction mix did not result in an increase in product formation.

To test the reliability of the method for the routine measurements of individual amino acids in serum, intra-assay variability was assessed using pooled sera. On 2 separate days, a pooled rat sera fortified with a mix of LNAAs (1 mg/ml) was diluted with naive pooled sera to achieve exogenous added quantities of 25, 50, and 75 μg/ml LNAAs. Samples were individually extracted, derivatized, chromatographed, and analyzed utilizing the internal standard technique. The results (2 days×2 samples each concentration) showed a relative standard deviation (RSD) in the range of 0.64 to 7.28% and an excellent correlation between the measured endogenous (basal concentrations) and the extrapolated (y-intercept point) amino acid concentrations, $F(1,4)=695.26$, $P<0.001$ (Table 2).

The analytical precision of the method was assessed by the variability of the peak area [(SD/mean)·100] for each amino acid. A standard mix (5 μg/ml) was reacted in triplicate and analyzed within- and between-day. The precision within-day was in the range of 0.98–1.35% ($n=3$) and precision between-days 2.59–7.61% ($n=6$).

The method was applied in vivo to assess the serum concentration of the free LNAAs. Food-deprived animals were sacrificed and blood samples analyzed for the LNAA (see Section 2.2). The results of the study are tabulated in Table 3. Between animals, the RSD for the amino acids was in the range of 9.66 to 16.56%. The calculated ratios for each neutral amino acid (Table 3) compared favorably to similar published studies [14].

Additionally, a group of animals were dosed with tyrosine (256 mg/kg tyrosine methylester·HCl in saline; i.p.). The time course of tyrosine was followed in serum and brain tissues (Fig. 2). Based on the time intervals selected, the highest concentrations in serum tyrosine were observed at 15 min post-injection. In the brain, free tyrosine peaked at 60 min

Table 1
Linear regression analysis of standard large neutral amino acids

Amino acid	Concentration range (ng o/c)[a]	Slope (·10^3)			
		n[b]	Mean[c]	S.E.M.[c]	r[c]
Tyrosine	0.625–2.5	9	1.83	0.078	>0.999
Valine	0.625–2.5	9	2.75	0.158	>0.999
Isoleucine	0.625–2.5	9	1.91	0.087	>0.999
Leucine	0.625–2.5	9	1.74	0.098	>0.999
Phenylalanine	0.625–2.5	9	1.62	0.055	>0.999
Tryptophan	0.625–2.5	9	1.89	0.051	>0.999

[a] Concentration of each amino acid injected on column (o/c; 10 μl).

[b] Standard curves for the individual amino acids were prepared in triplicate on day one and analyzed daily for 3 consecutive days.

[c] Mean is the average of replicates and days ($n=9$); S.E.M.=standard error of the mean; r=correlation coefficient.

Table 2
Amino acid levels of pooled rat sera fortified with varied concentrations of LNAAs

NAA	Nominal[a] (μg/ml)	Found[b] (μg/ml)	Net[c] (μg/ml)	Difference[d] (%)	Accuracy[e] RSD (%)	y-Intercept	Slope[f]	Confidence limit[g] (95%)
Tyr		14.85±0.63			4.20	15.92±1.81		
	25.0	44.58±0.81	29.73	18.92	1.82			
	50.0	66.90±0.93	52.02	4.10	1.39			
	75.0	95.00±0.60	70.14	−6.48	0.63		1.05±0.04	0.89–1.22
Val		22.19±0.93			4.19	22.35±1.36		
	25.0	47.44±0.25	25.25	1.00	0.53			
	50.0	68.46±0.63	46.27	−7.46	0.92			
	75.0	95.05±1.37	72.86	−2.85	1.44		0.96±0.03	0.83–1.01
Ileu		12.80±0.09			0.70	13.52±1.22		
	25.0	38.72±0.45	25.93	3.72	1.16			
	50.0	59.63±0.51	46.84	−6.32	0.86			
	75.0	84.44±0.46	71.64	−4.48	0.55		0.94±0.03	0.82–0.98
Leu		16.15±0.15			0.93	16.43±0.87		
	25.0	39.94±1.73	23.78	−4.88	4.33			
	50.0	60.57±0.44	44.42	−11.16	0.73			
	75.0	84.56±0.37	68.41	−8.79	0.44		0.90±0.02	0.82–0.98
Phe		11.05±0.36			3.30	11.56±1.08		
	25.0	36.50±0.52	25.46	1.84	1.43			
	50.0	57.69±1.13	46.65	−6.70	1.96			
	75.0	82.71±0.47	71.67	−4.44	0.57		0.95±0.02	0.85–1.04
Try		15.65±0.57			3.64	16.06±1.07		
	25.0	41.15±0.85	25.51	2.04	2.07			
	50.0	62.62±1.31	46.98	−6.04	2.09			
	75.0	88.11±2.94	72.47	−3.37	3.34		0.96±0.02	0.86–1.05

[a] Nominal is the exogenous NAA mix added to the sample.
[b] Amino acid concentration found in the sample (mean±SD; n=4).
[c] Net=adjusted concentration (found−baseline); baseline=pooled sera only.
[d] Difference (%) is {[(net−nominal)/added LNAA]·100}.
[e] Relative standard deviation (RSD) is the (standard deviation/sample mean)·100.
[f] Slope is the independent (nominal) vs. dependent (found) variable; the intercept point (y=0) reflects the endogenous level.
[g] Confidence limit (95%) of the slope.

post-injection. In both serum and brain, free tyrosine concentration peaked at an approximate fourfold increase from baseline.

4. Discussion

The present method provides the detection of the

Table 3
Serum amino acid concentrations in Sprague–Dawley rats (n=9)

	Tyrosine	Valine	Isoleucine	Leucine	Phenylalanine	Tryptophan
Range[a]	14.58–20.50	20.46–38.02	11.95–19.49	12.09–21.36	9.81–15.25	12.78–22.25
Mean	17.90	29.31	15.54	17.51	12.06	17.01
SD	1.73	4.03	1.87	2.39	1.65	2.27
Ratio[b]±SD	0.18±0.02	0.29±0.05	0.15±0.01	0.16±0.01	0.12±0.02	0.17±0.02

[a] Sample concentrations are expressed in μg/ml. Animals were deprived of food 15 h prior to serum collection.
[b] Ratio=[serum concentration of an amino acid (AA)/Σ(total−AA)]; total=Tyr+Val+Leu+Ile+Phe+Trp.

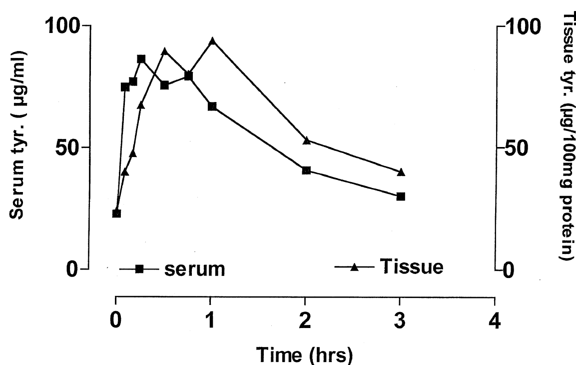

Fig. 2. Time course of tyrosine concentration in serum and brain (medial pre-frontal cortex) after i.p. administration of 200 mg/kg tyrosine to the rats. Each symbol represents a single animal. The solid line is a point-to-point fit.

neutral amino acids in 10-μl sample aliquot in a linear concentration range of 0.125 to 50 μg/ml amino acid concentration in serum, tissue and brain perfusates. The method fulfills the essential requirements of specificity, resolution, sensitivity, reproducibility, accuracy and speed of analysis for a reliable chromatographic procedure.

The specificity is dependent upon the selection of reagent, reaction, and derivative stability. A common problem encountered with the use of OPA-alkylamines is the poor stability (3–5 min lifetime) of the isoindole reaction products. The preparation of OPA–2-mercaptoethanol (reagent) has been shown to have a short half-life (24–72 h) with by-products usually present in the reagent assay [30]. The OPA-S derivatization procedure obviates reagent stability problems, sample to product degradation, and the stench associated with mercaptothiol reagents [32]. The present procedure demonstrates that reactions of primary amines with OPA-S generate isoindole derivatives stable for a period of 72 h. This is an improvement over another method [32] for which OPA-S derivatives were stable for 60 min with subsequent degradation over a 4 h period (50% loss) and disappearance by 24 h. In the present method, the improved stability of the indole sulfite derivatives is the result of the addition of mobile phase to the reaction media which lowers the final pH to ~7.1, auto-sampler temperature control (4°C) and the HPLC mobile phase pH of 6.8. As previously demonstrated, OPA-S derivatives are susceptible to acid hydrolysis at pH below 6.0 [24,32].

The limit of detection (LOD) of the method (at a signal-to-noise ratio of three) for the lowest amino acid response (tryptophan) was 0.0125 μg/ml, while at a signal-to-noise ratio of 10, the lowest limit for reliable quantification (LOQ) was 0.039 μg/ml, corresponding to 220 pg injected (n=6). The sensitivity of the method was well within the LOQ to estimate amino acid concentrations injected on column over a range of 0.156 μg/ml (dialysate) to 10 μg/ml (serum and tissue).

The neutral amino acids from biological matrices or standards were separated utilizing isocratic solvent conditions. An advantage of the OPA-S reaction is that it generates an hydrophilic isoindole derivative. The acidic amino acids (i.e., aspartate, glutamate and GABA) elute near or at the solvent front of the analysis, much earlier than the neutral amino acids. The elution time of the neutral amino acids was sensitive to the concentration of the organic solvent (methanol). Optimally, a 25% methanol resolves all the neutrals with marginal overlap between leucine and phenylalanine. Although the combination of solvent gradient elution with electrochemical detection would reduce assay time, this option is not practical due to baseline shift during the solvent run (particularly at the high level of sensitivity). Because of the large amount of proteins and peptides, the serum and tissue samples were deproteinized with ethanol [35]. Organic solvents are more efficient than acids in the isolation of neutral amino acids [25]. In a direct comparison of sulfosalysilic acid vs. ethanol, the recovery of the neutral amino acids was consistently greater with ethanol [35]. In addition to efficiency of extraction, organic solvents avoid peptide degradation and thus contamination with additional amino acids by acidic hydrolysis of peptides [27].

The accuracy of the method was assessed by linearity of response, replicate analysis of pooled sera, LNAA fortified pooled sera, and a single kinetic analysis utilizing tyrosine for the challenge. The linearity of response was examined over a range from 0.625 to 2.50 μg/ml. The overall correlation coefficient (r) was >0.999 (Table 1). Pooled sera fortified with 25, 50 and 75 μg/ml were analyzed and the results showed an RSD in the range of 0.53 to 4.20%. The recovery of individual amino acids was in the range of 88.8 to 118.92% (Table 2). Basal concentrations of each amino acid (Table 3) were

compared and found to be in the range of similar studies [14,33,36]. A more manageable expression of the changes in serum neutral amino acid concentrations that lead to alterations in brain amino acid concentrations is the ratio of amino acid to the sum of the other competing neutral amino acids (see Table 3). In the present study, the ratios of individual amino acids were compared and found to be in the range of previous published studies [3,14,36,37]. Tyrosine was utilized to assess the appearance and distribution in the serum and tyrosine uptake in brain tissues (Fig. 2). The data are in good agreement with a similar published method [38]. An increase of 390% from the serum and tissue baseline (30 min post i.p.) compared favorably to that study (350%).

In conclusion, using a modification of mobile phase, pH and an hydrophilic derivative, the present method can be used for simple, sensitive, accurate, reproducible, and selective determination of neutral amino acids in biological matrices, using very small volumes of sample and performed in relative short time. In addition, it should be noted that norvaline was utilized as an internal standard. Although our initial evaluation showed no interferences or coelution with norvaline, other internal standards are available (e.g., norleucine, α-aminobutyric acid, etc.) for substitution when other biological matrices are used with the present method.

Acknowledgements

This research was supported by a Veterans Affairs MERIT Grant.

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