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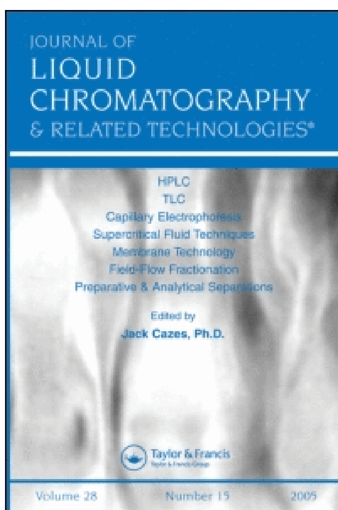

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Validation of HPLC Analysis of Aspartate and Glutamate Neurotransmitters Following <i>o</i>-Phthaldialdehyde-Mercaptoethanol Derivatization

S. Rabouan[a]; J. C. Olivier[b]; H. Guillemin[a]; D. Barthes[a]
[a] Laboratoire de Chimie Analytique, UFR Médecine et Pharmacie, Poitiers, CEDEX, France [b] Laboratoire de Pharmacie Galénique et Biopharmacie, UFR Médecine et Pharmacie, Poitiers, France

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Validation of HPLC Analysis of Aspartate and Glutamate Neurotransmitters Following *o*-Phthaldialdehyde-Mercaptoethanol Derivatization

S. Rabouan,[1,*] J. C. Olivier,[2] H. Guillemin,[1] and D. Barthes[1]

[1]Laboratoire de Chimie Analytique and
[2]Laboratoire de Pharmacie Galénique et Biopharmacie,
UFR Médecine et Pharmacie, Poitiers, France

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ABSTRACT

A conventional HPLC method was developed for simultaneous determination of aspartate (Asp) and glutamate (Glu) in rat brain microdialysate samples after derivatization with *o*-phthaldialdehyde (OPA)-2-mercaptoethanol (ME) reagent. Separation of 20-µL samples was performed on Hypersil® C18 column (5 µm, 150 × 3.2 mm) with gradient elution by a mobile phase based on methanol and 0.05 M sodium acetate buffer, pH 7. Optimal spectrofluorometric detection was at 330 nm excitation and 440 nm emission wavelengths. Manual or automated procedures of

*Correspondence: S. Rabouan, Laboratoire de Chimie Analytique, UFR Médecine et Pharmacie, BP 199, 86005 Poitiers, CEDEX, France; E-mail: sylvie.rabouan@univ-poitiers.fr.

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derivatization were investigated. Best results were obtained when auto addition mode was applied and derivatization carried out at $+10°C$. The correlation coefficients were above 0.999 in the 10^{-7}–10^{-5} mol L^{-1} range, and relative standard deviations for repeatability and reproducibility were, respectively, 7.4% and 7.2% for 10^{-6} mol L^{-1} Asp and 3.5% and 4.5% for 10^{-6} mol L^{-1} Glu. Limits of quantification (LOQ) were 5×10^{-7} mol L^{-1} for Asp (precision 3.8%) and 3×10^{-7} mol L^{-1} for Glu (precision 4.7%).

Key Words: Glutamate; Aspartate; HPLC; Validation; *o*-Phthaldialdehyde 2-mercaptoethanol derivatization; Spectrofluorometry.

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INTRODUCTION

Intracerebral microdialysis is the method of choice for monitoring in vivo levels of neurotransmitters (NT), such as the glutamate (Glu), aspartate (Asp), and glycine (Gly) excitatory NT and the inhibitory γ-aminobutyric acid (GABA) NT.[1–7] The limited volumes of microdialysate samples available (usually 10 to 30 μL) and the extremely low basal concentrations of NT (less than 10^{-6} mol L^{-1}) require an adaptation of the usual analytical conditions. Reversed-phase high-performance liquid chromatography (HPLC), with precolumn derivatization for fluorescence or electrochemical detection, is an appropriate method for analyzing amino acids in microdialysate samples.[8–10] To date, the best sensitivity has been achieved with microbore chromatography, a technology that requires extensive reconstruction of the HPLC systems, i.e., a flow pump reducer and a small-volume cell detector.[11] The purpose of our study was to optimize and to validate a simple and rapid conventional fluorometric HPLC method with an automated precolumn derivatization for the simultaneous determination of Asp and Glu. The method was applied to the analysis of rat basal microdialysates.

EXPERIMENTAL

Apparatus

Fluorescence spectra were obtained using a Perkin-Elmer Luminescence Spectrometer LS50B® (Courtaboeuf, France) with excitation and emission slit controls set at 10 nm and equipped with a 10-mm pathlength quartz cuvette.

The chromatographic system consisted of an L-6100® Merck pump (Nogent-sur-Marne, France) and of a Wisp717plus® refrigerated autosampler

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(Millipore, St Quentin, France). Separation was optimised on a Hypersil® C18 column (150 × 3.2 mm I.D., 5 μm, 120 Å pore size) from Merck (Nogent-sur-Marne, France). Fluorometric detection of eluted derivatives was carried out using the above-mentioned spectrometer equipped with a 16 μL flow cell and with a surface area integration software.

Chemicals and Reagents

Aspartate, Glu, Gly, and GABA were supplied by Sigma (St Quentin-Fallavier, France); and *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol (ME) by Fluka (St Quentin-Fallavier, France). Methanol (Merck-Eurolab, Pessac, France) and deionised water (Biosedra, Louviers, France) were of HPLC grade and exempt of fluorescent impurities. Other reagents were all of analytical grade.

Neurotransmitter 2.5 mM stock solutions were prepared in 0.1 M HCl and stored at −18°C. The working OPA/ME reagent solution for derivatization was prepared daily, stored protected from light and air in plugged vials, and used within 8 hours. In a 10 mL volumetric flask, 20 mg OPA were dissolved in 2 mL methanol, and volume was completed with 0.5 M borate buffer (pH 9.5). Then 23 μL ME were added and the obtained OPA/ME working reagent was thoroughly mixed.

Chromatographic Conditions and Precolumn Derivatization

Solvent A was methanol: 0.05 M sodium acetate buffer, pH 7 (15 : 85) and solvent B was methanol. The gradient programme was: 100% A for 7 min; then 14 min linear to 70% A and 30% B; finally 4 min linear to 30% A and 70% B.

Standard solutions for calibration were prepared in the 10^{-7}–10^{-5} mol L^{-1} range concentration by diluting stock solutions with a 0.01 M borate buffer, pH 9, in order to obtain a final basic pH necessary for the OPA/ME derivatization reaction. Three procedures of derivatization were compared. In Procedure 1, carried out at room temperature (RT), 15 μL of the OPA/ME working reagent were manually added to 30 μL of sample to be derivatized and vortexed for 15 s. After 2 min reaction time, 20 μL of the mixture were injected for analysis. Procedures 2 and 3 consisted of an automated addition of the OPA/ME working reagent using a Wisp717plus® autosampler, by "auto-transfer mode" or by "auto-addition mode," respectively. In Procedure 2, carried out at RT or +10°C, 15 μL of the OPA/ME working reagent (from a transfer vial) were added to 30 μL sample (in a "sample" vial) and mixed by aspiration/forcing back; after 2 min, 20 μL of the

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mixture were injected. In Procedure 3, carried out at +10°C, 10 µL of the OPA/ME working from a transfer vial and 20 µL of sample from a sample vial were successively withdrawn and allowed to mix by diffusion in the loop for 2 min; then, 30 µL of the mixture were injected.

Assay Validation[12,13]

Specificity

For specificity assessment, chromatograms of derivatized NT standards were compared to chromatograms of OPA/ME working reagent, of derivatized artificial CSF solution and of derivatized basal microdialysate sample.

Linearity

Linearity was determined over three days by assessing the correlation between AUC and concentrations at 10^{-7}, 5×10^{-7}, 10^{-6}, 5×10^{-6}, 10^{-5} mol L^{-1} ($n=15$) by least-square regression. The minimum acceptable correlation coefficient to establish linearity was set at 0.990.

Precision

Precision was investigated by studying intra-day variation (repeatability, $n=3$) and inter-day variation (reproducibility, $n=3$). Repeatability was defined as the variability of the method when performed with the smallest variations possible, and variance of repeatability (${s_r}^2$) was calculated as the mean of each day's variance. Reproducibility was defined as the greatest variations possible (different days, technicians, etc.), and variance of reproducibility was defined as the sum of repeatability variance (${s_r}^2$) and inter-group variance (${s_g}^2$) [Eq. (1)].

$$ {s_r}^2 = \frac{\sum_{j=1}^{3} {s_j}^2}{3}; \quad {s_g}^2 = \frac{\sum_{j=1}^{3} (m_j - \bar{m})^2}{2} - \frac{{s_r}^2}{n} \tag{1} $$

where ${s_j}^2$ = variance per day (j), $\bar{m}$ = mean of all calculated concentrations during three days, and n = number of assays per day.

The corresponding relative standard deviations were calculated as follows:

$CV_r = s_r \times 100/\bar{m}$ for repeatability, and
$CV_R = s_R \times 100/\bar{m}$ for reproducibility.

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Accuracy

Aspartate (10^{-6} mol L^{-1}) and Glu (4×10^{-6} mol L^{-1}) samples were prepared in artificial CSF, derivatized and analysed. Determined concentrations C_D were compared with their theoretical values C_T. Recovery (%) was obtained by $C_D \times 100/C_T$.

Limits of Detection and Quantification

The limits of detection (LOD) was defined as the concentration giving a signal-to-noise ratio of 3. The limits of quantification (LOQ) was defined as the lowest concentration providing a relative standard deviation less than or equal to 5%.

Microdialysate Sample Collection

This work was done in accordance with the Principles of Laboratory Animal Care (NIH Publication #86-23, revised 1985). Microdialysis samples were collected from dorsal hippocampus (mm, relative to bregma; P, 5.6: L, 4.8: V, 4.7 from dura mater) of male Sprague–Dawley rats (from 280 to 300 g) from Déprés Breeding Laboratories (St Doulchard, France) as previously described.[15] The CMA/12 microdialysis probe (3 mm membrane length; obtained from Phymep, Paris, France) was perfused with artificial CSF [145 mM NaCl, 0.6 mM KCl, 1.0 mM $MgCl_2$, 1.2 mM $CaCl_2$, and 0.2 mM ascorbic acid in 2 mM potassium phosphate buffer, pH 7.4][14] at a 3 μL min^{-1} flow rate.

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RESULTS AND DISCUSSION

Optimization of the Derivatization Procedure

Excitation and Emission Wavelengths

For NT conversion to derivatives according to the chemical reaction shown in Fig. 1, OPA should be in moderate excess relative to the substances in the mixture in order to avoid extensive formation of secondary products.[16] At room temperature, the derivatization reaction leads to unstable isoindole derivatives, which necessitated limiting reaction time to 2 min.[17] ***o*-Phthaldialdehyde** in excess had no native fluorescence in the conditions of analysis and did not interfere in chromatograms. Excitation spectra of Asp, Gly, and GABA derivatives (10^{-3} mol L^{-1}) obtained for an emission wavelength of 440 nm, showed absorption in a wide range of

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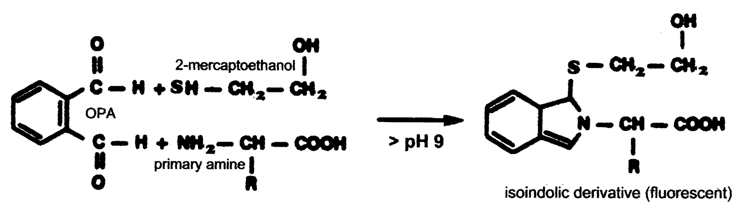

Figure 1. Scheme of the chemical reaction of derivatization of primary amine with OPA and ME leading to isoindolic fluorescent derivatives.

wavelengths (270–380 nm), while the Glu excitation spectrum was in the 330 to 350 nm range. With an excitation wavelength of 330 nm, the common wavelengths for maximum emission were found to be between 435 and 445 nm. Therefore, the excitation and emission wavelengths were chosen to be 330 nm and 440 nm, respectively.

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Signal Response and Linearity

At room temperature, the manual Procedure 1 gave higher slope values (i.e., signal response) than the auto-transfer mode (Procedure 2) (Table 1). This was attributed to the chemical instability of the derivatives, as a degradation peak appeared on chromatograms from samples prepared according to the latter procedure (not shown). With both procedures, correlation coefficients and *y*-values at intercept were satisfactory. An analysis of basal microdialysate, performed at this stage of development of the HPLC method, showed that GABA and Gly peaks were unsatisfactorily resolved from unidentified peak (not shown). Therefore, further procedure optimization was carried out on Asp and Glu only. Procedure 2, performed at +10°C, gave better signal responses than at RT, but responses were, nevertheless, below those obtained with Procedure 1 (Table 1). Furthermore, correlation coefficients were unsatisfactory. With Procedure 3 (carried out at +10°C), no degradation peak appeared on chromatograms, and signal responses were similar to those obtained with Procedure 1. Correlation coefficients were satisfactory. Accordingly, the validation procedure for Asp and Glu determinations was pursued using Procedure 3 derivatization.

Validation of the High Performance Liquid Chromatography Method

Specifity

Figure 2 shows superimposed chromatograms of a standard NT solution and of a basal microdialysate sample. Retention times (±SD %, $n = 6$) were 2.5 min

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Table 1. Linearity responses of NT derivatives using various precolumn derivatization procedures. Standard concentrations ranged from 10^{-7} to 10^{-5} M ($n = 15$).

		At room temperature		At +10°C	
	Neuro-transmitters	Procedure 1 (manual)	Procedure 2 (auto-transfer)	Procedure 2 (auto-transfer)	Procedure 3 (auto-addition)
Slope values	Asp	2.731	1.827	2.32	2.530
	Glu	2.597	1.866	2.15	2.706
	Gly	2.872	1.557	ND	ND
	GABA	2.717	1.511	ND	ND
y-Values for $x = 0$	Asp	0.308	−0.462	−0.01	−0.019
	Glu	−0.021	−0.294	−0.32	−0.094
	Gly	1.385	−1.715	ND	ND
	GABA	0.450	−0.916	ND	ND
Correlation coefficients	Asp	0.993	0.998	0.980	0.997
	Glu	0.997	0.999	0.985	0.999
	Gly	0.975	0.923	ND	ND
	GABA	0.993	0.990	ND	ND

Note: ND, not determined.

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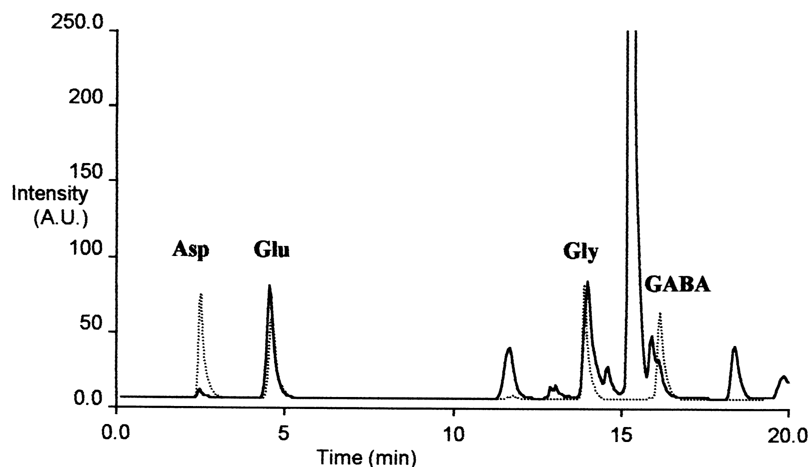

Figure 2. Superimposed chromatograms of a basal microdialysate sample (continuous line) and of a typical standard solution (10^{-6} mol L^{-1}) of Glu, Asp, Gly, and GABA (dotted line).

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(±0.7%), 4.6 min (±0.9%), 14.1 min (±0.3%), and 16.5 min (±0%) for Asp, Glu, Gly, and GABA, respectively. Peaks for Asp and Glu were specific, but the peaks of Gly and GABA were not satisfactorily resolved from unidentified peaks, invalidating the method for the quantification of these two NT.

Accuracy

Measurements of Asp and Glu accuracy were performed at 10^{-6} and 4×10^{-6} mol L^{-1}. The method was accurate, with means for recoveries in the 97.5–100.8% range and relative standard deviations of 6% ($n = 12$).

Precision

Table 2 summarizes the results of precision ($n = 5$) for Asp and Glu (10^{-6} mol L^{-1}). For both compounds, precision was satisfactory. It was significantly better for Glu than for Asp.

Limits of Detection and of Quantification

Limits of detection were determined to be 0.12×10^{-6} and 0.18×10^{-6} mol L^{-1} for Asp and Glu, respectively. As shown in Table 3, LOQ

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Table 2. Precision of Asp and Glu for concentration determination of 10^{-6} M standards ($n = 5$), using the auto-addition precolumn derivatization Procedure 3 (at $+10°C$).

Neurotransmitters	Mean ($mol\,L^{-1}$)	Intra-day (%RSD)	Inter-day (%RSD)
Asp	0.945×10^{-6}	7.4	7.2
Glu	0.972×10^{-6}	3.5	4.5

were $0.5 \times 10^{-6}\,mol\,L^{-1}$ and $0.3 \times 10^{-6}\,mol\,L^{-1}$ for Asp and for Glu, respectively.

Microdialysate Sample Analysis

Results of Asp and Glu determination in microdialysate samples from rat dorsal hippocampus are presented in Table 4. Aspartate concentrations were below the LOQ, precluding interpretation. The estimated concentration was in the same range as previously published work, on microdialysate samples from hippocampus.[11,18] As the Asp basal value was close to the LOQ, a concentration step before derivatization (for example by vacuum-drying the microdialysate sample) should allow determination of basal Asp in microdialysates. Glutamate could be determined, accurately, in microdialysates, as concentration values were 5 to 14 times the LOQ. Basal Glu concentration was in the range generally observed in the hippocampus.[11,19]

Table 3. Limits of quantification, determinations for Asp and Glu ($n = 5$), using the auto-addition precolumn derivatization Procedure 3 (at $+10°C$).

	Asp		Glu	
Theoretical concentration ($\times 10^{-6}\,mol\,L^{-1}$)	0.300	0.500	0.300	0.500
Mean determined concentration ($\times 10^{-6}\,mol\,L^{-1}$)	0.383	0.450	0.330	0.479
Standard deviation	0.027	0.168	0.016	0.081
Relative standard deviation	7.0%	3.8%	4.7%	1.7%
Mean recovery	128%	90%	110%	96%
LOQ (RSD) ($\times 10^{-6}\,mol\,L^{-1}$)	0.5 (3.8%)		0.3 (4.7%)	

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Table 4. Determination of basal concentrations of Glu and Asp in microdialysate samples from rat dorsal hippocampus ($n = 3$).

Neurotransmitters	Concentrations ($\times 10^{-6}$ mol L^{-1}), mean ± SD
Asp	0.19 ± 0.05 < LOQ
Glu	3.77 ± 2.23

CONCLUSION

A chromatographic method was developed for simultaneous determination of Asp and Glu in rat brain microdialysates, using conventional HPLC equipment and automated precolumn OPA/ME derivatization. Limits of quantification were determined to be 0.5×10^{-6} mol L^{-1} for Asp (i.e., 10 pmol injected) and 0.3×10^{-6} mol L^{-1} for Glu (i.e., 6 pmol injected). The method allowed determination of Glu in microdialysate samples obtained from dorsal hippocampus, but was not sensitive enough for Asp determination.

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