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Automated determination of neuroactive acidic sulphur-containing amino acids and γ-glutamyl peptides using liquid chromatography with fluorescence and electrochemical detection

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ABSTRACT

A column liquid chromatographic method is presented for the determination of trace levels of acidic sulphur-containing amino acids and γ-glutamyl di- and tripeptides in microdialysates sampled from rat brain *in vivo*. Automated precolumn derivatization was performed with *o*-phthaldialdehyde–β-mercaptoethanol. The derivatives were separated by reversed-phase liquid chromatography with electrochemical and fluorescence detection. The mean relative standard deviation (n = 10) was 1.03 and 4.59% for retention times and peak heights, respectively. The mean correlation coefficient of linearity (r) was 0.9982 in the range 4.5–450 pmol (n = 15), and the lowest detectable amount was 200 fmol for the homocysteinesulphinic acid derivative, (k' = 5.4, at a signal-to-noise ratio of 3). A microcolumn electrochemical detection method, developed for volume-limited samples, produced a fifteen-fold increase in mass sensitivity. Neurochemical applications using microdialysis *in vivo* are presented.

INTRODUCTION

Several acidic sulphur-containing amino acids (ASCAAs, Fig. 1) and γ-glutamyl di- and tripeptides (GGPs, Fig. 2) are known to be constituents of the mammalian brain [1–5]. The physiological role of the GGPs is not clear; however, the diverse roles of glutathione (γ-glutamylcysteinylglycine, GSH, reduced form and

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GSSG, oxidized form) are well documented [6]. Several of the GGPs are potent displacers of glutamic acid (Glu) receptor binding, and may therefore be involved in the regulation of amino acidergic neurotransmission [7]. Their presence in the brain may also be related to the γ-glutamyl cycle [8]. Some of the ASCAAs have an excitatory [9–11] and neurotoxic [12,13] effect similar to, or greater than, that of aspartic acid (Asp) and Glu. Furthermore, some ASCAAs are released *in vitro* by chemical depolarization in a transmitter-like fashion [14].

Hitherto, all studies involving determination of endogenous ASCAAs and their function in the brain have been performed *in vitro*, but no such studies have been conducted *in vivo*. In the past few years, microdialysis has successfully been applied to *in vivo* sampling of compounds involved in normal and pathological neurotransmission [15–18]. The microdialysates represent an averaged diluted reflection of the chemical profile in the extracellular fluid around the dialysis membrane. Because the tissue concentration of the GGPs and the ASCAAs in mammalian brains are in the range 0.5–30 μM [1,19,20], the expected dialysate concentration will be in the low nanomolar range (due to high intra to extracellular concentration ratios and dialysis recovery factors; see ref. 16).

To our knowledge, no analytical method has yet been presented that allows simultaneous determination of all these compounds at the low concentration levels encountered in microdialysates recovered *in vivo* [14,19–22].

The *o*-phthaldialdehyde–β-mercaptoethanol (OPA–βME) reagent has been

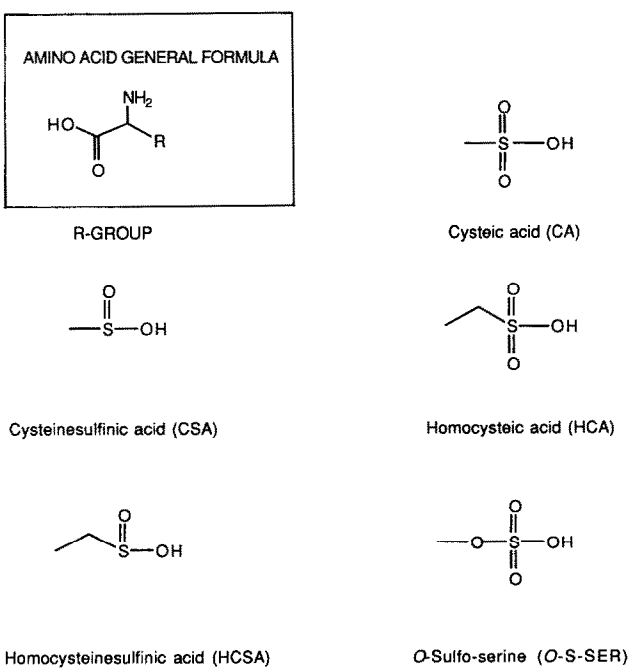

Fig. 1. Structures of acidic sulphur-containing amino acids.

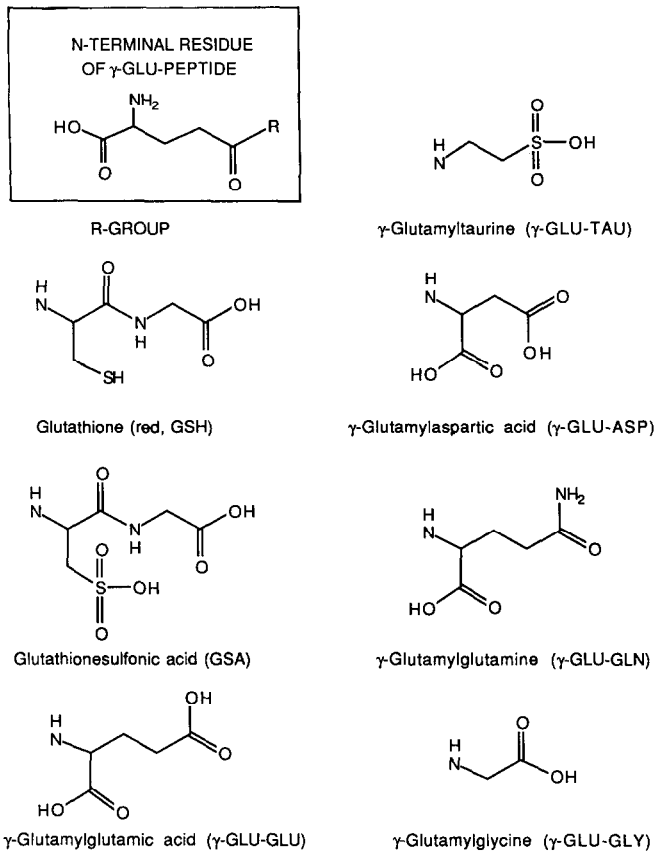

Fig. 2. Structures of γ-glutamyl peptides.

successfully employed for precolumn derivatization of primary amino acids in physiological samples [23,24]. The formed 1-alkylthio-2-alkylisoindole derivatives [25] are amenable to fluorescence and electrochemical detection [26,27]. The mass limit of detection is in the femtomole range [28,29], and the only major drawback is the chemical instability of the derivatives in solution [25,30].

In the present study, automated precolumn derivatization using OPA–βME and reversed-phase liquid chromatography (LC) was utilized to determine ASCAAs and GGPs at low nanomolar levels. Fluorescence and electrochemical detection were both used. The method was applied to samples recovered *in vivo* from rat brain using the microdialysis technique. For volume-limited samples, a microcolumn LC method was developed.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a Varian LC 5500 (Varian Assoc., Sunnyvale, CA, U.S.A.) ternary gradient pump, which was modified by an extra pulse damper installed between the flow-controller and the injector to reduce flow pulsations. Injections were performed by a CMA/200 autosampler (Carnegie Medicine, Stockholm, Sweden), fitted with either a 7.4-μl or a 100-μl loop. The reversed-phase columns employed were a 300 mm × 4.6 mm I.D. Spherisorb ODS-2 (5 μm stationary phase, Phase Separations, Clwyd, U.K.) packed at our laboratory, or two 150 mm × 4.6 mm I.D. with a TSK ODS-80TM (5 μm stationary phase, Tosoh, Tokyo, Japan) coupled in series. A Schoeffel-Kratos FS 980 LC fluorescence detector (Applied Biosystems, Ramsey, NJ, U.S.A.) operating at 330 nm excitation wavelength (bandwidth 5 nm) was equipped with a 418-nm cut-off emission filter. Electrochemical detection was performed with a BAS (Bioanalytical Systems, West Lafayette, IN, U.S.A.) LC4B amperometric detector (filter-setting 0.1 Hz) equipped with a TL 5A thin-layer cell with a glassy carbon working electrode and an Ag/AgCl reference electrode. The potential of the working electrode was maintained at +700 mV unless otherwise stated.

The same LC system with the following modifications was used for the micro-column system. A flow-splitting arrangement was used as previously described [31]. The columns (300 mm × 1.0 mm I.D.) were packed with Sperisorb ODS-2 5 μm (see refs. 32 and 33 for a detailed description). The end-fitting of the column outlet was connected by a short piece of 0.10 mm I.D. stainless-steel tubing into the flow-cell housing. A tandem LC4B amperometric detector with a TL 5A thin-layer cell and dual-series working electrode arrangement (Hg/Au and glassy carbon) was used. The Hg/Au electrode was positioned upstream (+200 mV *vs.* Ag/AgCl) and the glassy carbon electrode downstream (+700 mV *vs.* Ag/AgCl).

All chromatographic data were acquired and processed on an NEC APC 4 personal computer using the Maxima 820 software (Waters, Dynamic Solutions, Milford, MA, U.S.A.) Uncorrected fluorescence spectra were obtained on an Aminco Bowman (Silver Spring, MD, U.S.A.) spectrofluorimeter, and absorbance measurements were obtained on a Hitachi 100-60 (Hitachi, Tokyo, Japan) UV–VIS spectrophotometer.

Mobile phases

All buffers were 1.0 m*M* with respect to Na_2EDTA and were prepared by dissolving salts of pro analysi grade in distilled deionized water. A phosphate buffer (50 m*M*) was prepared from monosodium phosphate. The pH was adjusted to 4.69 (A), 4.67 (B) and 4.45 (C) with 1.0 *M* NaOH (A and B) or 1.0 *M* H_3PO_4 (C). A citrate buffer (50 m*M*) was prepared from trisodium citrate dihydrate, and the pH was adjusted to 4.75 with 5 *M* H_3PO_4 (D). The organic modifiers were prepared by mixing 220 ml of tetrahydrofuran (THF) with 780 ml

of methanol (E) or by mixing 15 ml of THF, 185 ml of acetonitrile and 800 ml of methanol (F). Organic solvents of HPLC grade or spectroscopic grade were purchased from Rathburn Chemicals (Walkerburn, U.K.). All aqueous eluents were filtered through a 0.22-μm Milli-Q system (Millipore, Bedford, MA, U.S.A.) and degassed in an ultrasonic bath prior to use. The mobile phases were made fresh on a daily basis, and continuously sparged with research-grade helium during the analytical work.

Standard solutions

Stock solutions (1 m*M*) were prepared in distilled deionized water and stored at −80°C. These standards were subsequently diluted to working concentrations with water containing 1 m*M* Na_2EDTA. All amino acids and peptides were obtained from Sigma (St. Louis, MO, U.S.A.), except for S-sulpho-L-cysteine, L-homocysteinesulphinic acid (HSCA) and γ-D-glytamyltaurine, which were obtained from Tocris Neuramin (Essex, U.K.), and γ-L-glutamyl-L-valine, γ-L-glutamyl-L-alanine, β-L-aspartyl-L-alanine, α-L-aspartyl-L-alanine, α-L-aspartylglycine and L-cysteinesulphinic acid, which were obtained from Bachem (Bubendorf, Switzerland). The γ-L-glutamylaspartic acid was a generous gift from Dr. Vince Varga at the University of Tampere, Finland.

Reagents

A borate buffer was prepared by dissolving boric acid in distilled deionized water to a final concentration of 0.4 *M*. The pH was adjusted to 11.6 with 1.0 *M* NaOH. The OPA–βME reagent was prepared by adding 100 mg of OPA (recrystallized from *n*-heptane three times) and 100 μl of βME to 1 ml of methanol. This solution was diluted in 8.9 ml of the borate buffer. An iodoacetamide solution (100 m*M*) was prepared in the borate buffer and an N-ethylmaleimide (NEM) solution (50 m*M*) in distilled deionized water. All reagent chemicals were obtained from Sigma.

Derivatization procedures

All derivatizations were performed using an OPA–βME reagent concentration of 75 m*M*, giving at least at 200 times excess over the total primary amine concentration [28]. A pH of 11.6 of the borate buffer was sufficient to yield a required pH value over 9.0 in the derivatization solution in all types of sample studied [26]. The autosampler was programmed to add 20 μl of the OPA–βME reagent (1.5 μl in the microcolumn system) to 80 μl of the sample (6 μl in the microcolumn system). After a 2-min reaction time, the whole volume or aliquots were injected on the column using a partially filled loop technique.

When protective alkylation of the sulphydryl groups was employed with iodoacetamide or NEM, a two-reagent software for the autosampler was used. After the OPA–βME derivatization was completed, either the buffered iodoacetamide solution or the NEM solution was added to the sample and allowed to react for 2 or 5 min, respectively, until injection.

Sample preparation

Male Wistar rats weighing *ca.* 300 g were anesthetized with halothane (5%) and intubated. Anesthesia was maintained with N_2O–O_2 (70:30, v/v) and halothane (< 1%). The animals were placed in a stereotactic frame, and two dialysis probes were positioned bilaterally in the corpus striatum. The dialysis probes (4 mm length of dialysis tubing) were perfused with a Krebs–Ringer bicarbonate buffer at a flow-rate of 1.0 μl/min. Samples were collected for periods of 20 min and stored at −80°C until analysed.

RESULTS AND DISCUSSION

Derivatization

All ASCAAs and GGPs examined, apart from S-sulpho-cysteine and GSSG, reacted quantitatively with OPA–βME. These two compounds are, depending on derivatization conditions, partly or totally reduced by βME to cysteine and GSH, respectively [34,35]. The formation of the derivatives was monitored by measuring the fluorescence response in the derivatization solution (λ_{exc} = 345 nm, λ_{em} = 450 nm). The maximum fluorescence response was obtained for all derivatives within 30–60 s and remained constant for several minutes (Fig. 3). A check of the long-term stability of the derivatives exposed to daylight at room temperature

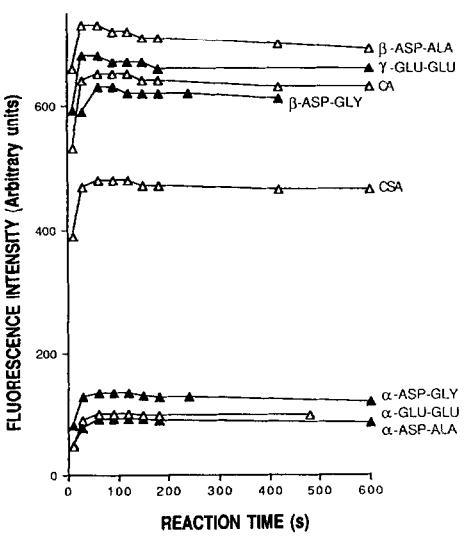

Fig. 3. Influence of reaction time on the relative fluorescence intensity. The derivatization was performed by adding 100 μl of the reagent solution to 400 μl of a standard solution (100 μ*M*) of each amino acid or peptide. The fluorescence response was then measured spectrofluorimetrically at the times indicated. Abbreviations as in Figs. 1 and 2. β-ASP-ALA = β-aspartylalanine; β-ASP-GLY = β-aspartylglycine; α-ASP-GLY = α-aspartylglycine; α-GLU-GLU = α-glutamylglutamic acid; α-ASP-ALA = α-aspartylalanine.

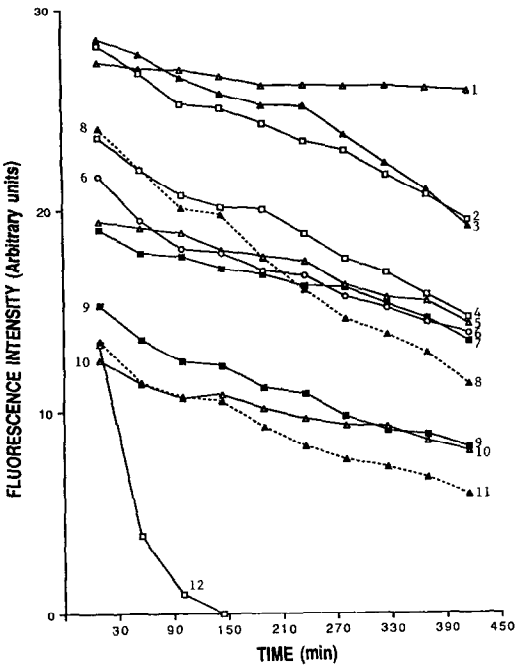

Fig. 4. Degradation profiles for the OPA–βME derivatives. Injections of a derivatized 1 μ*M* mixed standard. Column, Spherisorb ODS-2 (300 mm × 4.6 mm I.D.); flow-rate, 1.0 ml/min. Mobile phase gradient: 0 min, 87% A and 13% E; 3 min, 87% C and 13% E; 25 min, 75% C and 25% E. Curves: 1 = GSH; 2 = HCSA; 3 = γ-Glu-Tau; 4 = HCA; 5 = γ-Glu-Glu; 6 = CA; 7 = γ-Glu-Gln; 8 = GSA; 9 = CSA; 10 = γ-Glu-Asp; 11 = β-aspartylglycine; 12 = O-phosphoserine. For mobile phase conditions and abbreviations, see Experimental and Figs. 1 and 2, respectively.

(Fig. 4) revealed slow degradation ($t_{1/2} > 6$ h) of all derivatives with the exception of O-phosphoserine ($t_{1/2} < 20$ min). Superior stability compared with the 1-alkylthio-2-alkylisoindoles usually formed with OPA–βME and amino acids was obtained with GSH, which reacts with OPA to yield a highly fluorescent tricyclic adduct [36]. For this derivative, no decline in fluorescence was observed during the first 6 h. Notably, the tricyclic OPA–GSH adduct was preferentially formed even in the presence of a large excess of βME. The relatively high stability of the derivatives makes precise timing of the derivatization and the injection steps less critical than for many other amino acids, *e.g.* glycine, ornithine and lysine [28,37].

Thiols are susceptible to catalytic oxidation by metals and metal ions in aqueous media, especially under mild alkaline conditions [38]. This oxidation may result in the formation of sulphinic and sulphonic acids from thiols, *e.g.* cysteic acid and cysteinesulphinic acid may be formed from cysteine oxidation [39], thereby preventing correct determination. Derivatization of standard solutions (5–200 μ*M*) of cysteine, GSH, homocysteine and their disulphides, and of spiked microdialysates recovered from rat brains showed no formation of cysteic acid, cysteinesulphinic acid, homocysteinesulphinic acid, homocysteic acid or glutathione sulphonic acid.

Separation

A common feature of the ASCAAs is the low pK_a of the sulphinate and sulphonate functional groups, which makes them more acidic than most other amino acids. The GGPs have two α-carboxylate functional groups, and are therefore more acidic than their α-glutamyl peptide analogues, owing to the lower pK_a value of the α- than the γ-carboxylate group. If the pH of the mobile phase were adjusted to keep the α-carboxylates of the GGPs and the ASCAAs ionized, while the β- and γ-carboxylate groups of other acidic amino acids (*e.g.* the dicarboxylic amino acids Asp and Glu) were protonated, then this would result in a net charge difference on the derivatives. Fig. 5 shows that, if the mobile phase pH is kept below 4.9, the separation is free of interferences from aspartic acid and other amino acids. As organic modifiers, methanol–acetonitrile and methanol–THF mixtures yielded comparable chromatographic resolution. With a ternary gradient, twelve amino acids/peptides were resolved within 18 min (Fig. 6A and B). A total of 95 reference compounds were studied for possible chromatographic interference (68 amino acids, 4 polyamines, 5 catechol- and indoleamines, 4 α-Asp peptides, 4 β-Asp peptides, 6 γ-Glu peptides and 4 α-Glu peptides; a list can be

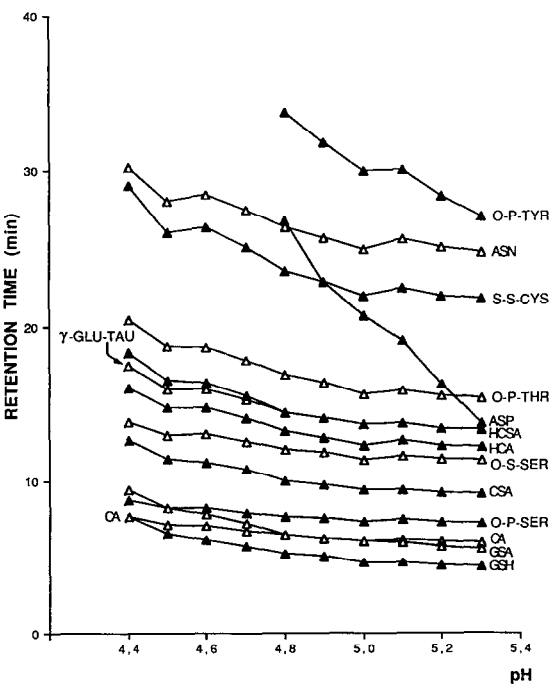

Fig. 5. Retention time *versus* buffer pH. Mobile phase, THF–methanol–50 m*M* phosphate buffer (2.6:10.4:87.0, v/v); flow-rate, 1.0 ml/min; column, Spherisorb ODS-2 (300 mm × 4.6 mm I.D.). O-P-TYR = O-phosphotyrosine; ASN = aspargine; S-S-CYS = S-sulphocysteine; O-P-THR = O-phosphothreonine; ASP = aspartic acid; O-P-SER = O-phosphoserine. Other abbreviations as in Figs. 1 and 2.

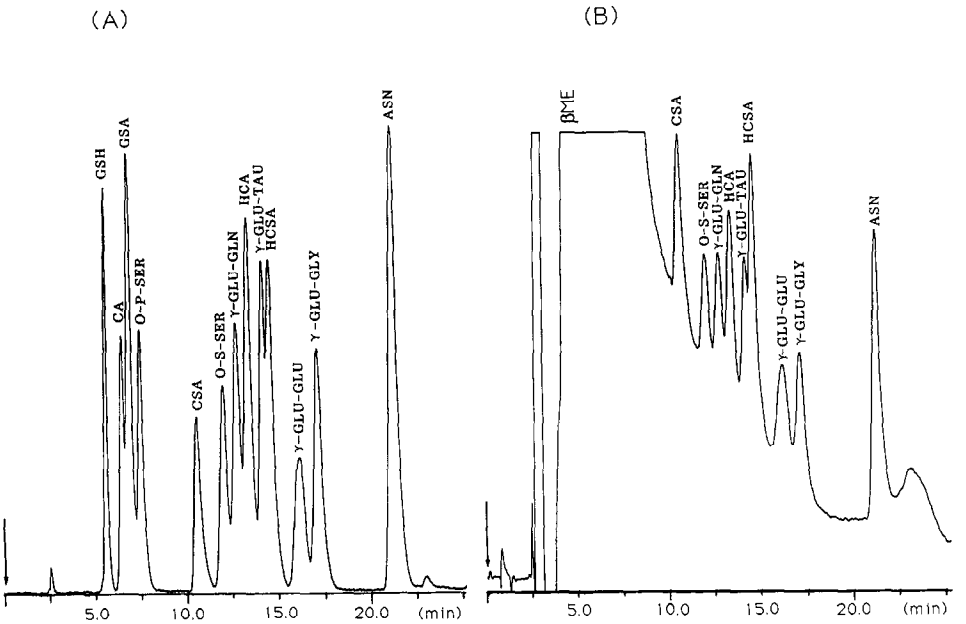

Fig. 6. Separation of a mixture of standards (40 pmol of each compound injected). Column, Spherisorb ODS-2 (300 mm × 4.6 mm I.D.); flow-rate, 1.0 ml/min. Mobile phase gradient; 0 min, 87% A and 13% E; 3 min, 87% C and 13% E; 25 min 75% C and 25% E. O-P-SER = O-phosphoserine; ASN = aspargine. For mobile phase conditions and abbreviations, see Experimental and Figs. 1 and 2, respectively. (A) Fluorescence detection; (B) electrochemical detection.

obtained upon request). Of these reference compounds, coelution was observed only for γ-glutamylaspartic acid with γ-glutamylglutamine and β-aspartylglycine with O-sulpho-serine. These peptides were resolved using a reversed-phase column with different selectivity and other mobile phase conditions (Fig. 7). Under these separation conditions, however, γ-glutamyltaurine and γ-glutamylglycine coeluted.

Detection

Uncorrected fluorescence spectra were registered in a solvent similar to the mobile phase for the derivatives of all compounds listed in Table I. Excitation maxima at 320 nm and emission maxima at 450 nm were found for all derivatives.

Generally, when peptides react with the OPA–βME reagent, derivatives are obtained that exhibit very low fluorescence emission intensities [27,40,41]. The peptides that have been investigated and reported in the literature have, to our knowledge, all been α-peptides, *i.e.* the α-carboxylate group of the N-terminal amino acid has participated in the carboxamide linkage. However, for dicarboxylic amino acids, the γ-carboxylate (*e.g.* Glu) and the β-carboxylate group (*e.g.* Asp) can participate in peptide bonding. In the present study, the fluorescence emission intensities of these β-aspartyl and γ-glutamyl peptide derivatives were

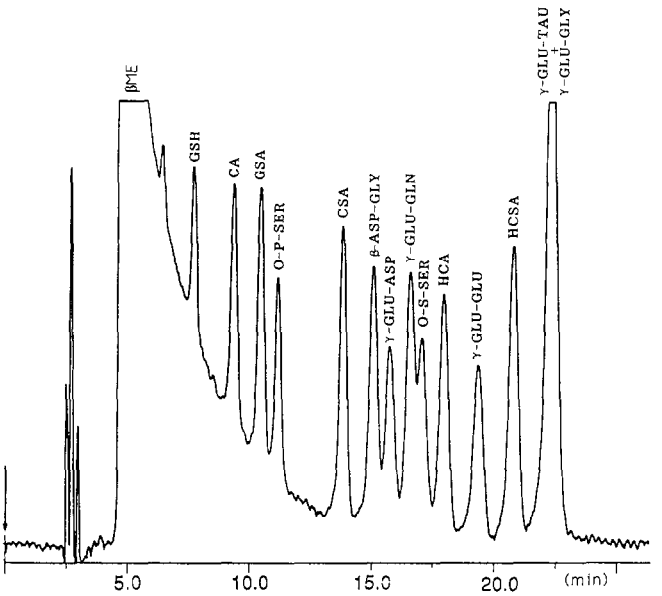

Fig. 7. Separation of a mixture of standards (20 pmol of each compounds injected). Mobile phase, D–F (19:81, v/v). Two ODS-80TM columns (150 mm × 4.6 mm I.D.) coupled in series. Flow-rate, 1.2 ml/min. Electrochemical detection. For mobile phase conditions and abbreviations, see Experimental and Figs. 1 and 2, respectively.

TABLE I

SPECTROSCOPIC PROPERTIES OF SOME OPA–βME DERIVATIVES

The derivatives were generated by adding 100 μl of the reagent solution to 100 μl of an amino acid or peptide solution (100 μ*M*). After reaction for 2 min, a 100-μl aliquot was dissolved in 1.0 ml of eluent (A–C, 15:85. v/v). The fluorescence response and absorbance were measured after 2 min.

Amine	RFI[a]	Absorbance λ_{320nm}/AU[b]
Alanine	1.00	0.0445
Cysteinesulphinic acid	0.98	
Cysteic acid	1.14	
Homocysteinesulphinic acid	1.13	
β-Aspartylalanine	1.14	0.0496
α-Aspartylalanine	0.14	0.0498
β-Aspartylglycine	1.18	0.0482
α-Aspartylglycine	0.24	0.0461
γ-Glutamylglutamic acid	1.40	0.0385
α-Glytamylglutamic acid	0.41	0.0365
γ-Glutamylaspartic acid	1.18	
γ-Glutamylglutamine	1.42	
γ-Glytamyltaurine	1.44	

[a] Relative fluorescence intensity normalized to the alanine derivative.

[b] Absorbance units.

observed to be several times higher than those of their α-analogues and similar to those for single α-amino acids (Table I). The absorbance at 320 nm (Table I) clearly indicates that quenching due to a α-positioned carboxamide linkage is the cause of the lower fluorescence emission intensities. This is consisent with earlier results [40,41].

Hydrodynamic voltammograms show that the half-wave potentials ($E_{1/2}$) for the OPA–βME derivatives of ASCAAs and GGPs are in the range 0.50–0.55 V (see Fig. 8). The working potential was set to 700 mV, where the oxidation of the derivatives is close to being diffusion-limited. At this working potential, background currents were generally less than 1 nA, and noise levels were *ca.* 5 pA. When acetonitrile (HPLC grade)–methanol mixtures were employed, high background currents, of the order of 30–40 nA were observed. However, the background currents dropped to less than 1 nA when HPLC-grade acetonitrile was replaced by spectroscopic grade acetonitrile or THF.

With electrochemical detection, the electroactivity of βME limits the trace-level determinations of the most polar amino acid derivatives. Despite the facts that the half-wave potential of the derivatives is *ca.* 0.35 V lower than for β-mercaptoethanol (Fig. 8) and slow heterogenous electron-transfer kinetics has been reported for thiol oxidation on glassy carbon surfaces [42,43], the oxidation of βME is still pronounced at the working potential of +0.7 V. Thus, owing to the relatively high concentration of βME emanating from unconsumed reagent, a large interfering peak is produced at the beginning of the chromatogram. If elec-

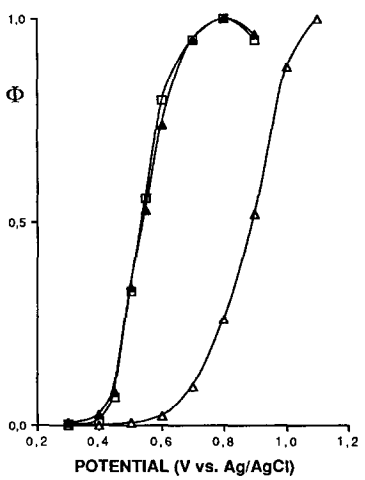

Fig. 8. Hydrodynamic voltammogram of β-mercaptoethanol (△) and OPA–βME derivatives of γ-glutamylglycine (□) and cysteinesulphinic acid (▲). Mobile phase, THF–methanol–0.05 *M* sodium phosphate buffer (pH 4.60) (1 m*M* Na_2EDTA) (3.0:12:85, v/v); column, Spherisorb ODS-2 (300 mm × 4.6 mm I.D.); flow-rate, 1.2 ml/min. Φ is defined as the ratio of the peak current to the limiting current (for βME a diffusion-limited response was not obtained, and Φ was calculated as the ratio of the peak current to the current response at +1.25 V).

trochemical detection is to be an attractive alternative to fluorescence detection, then the excess βME must be eliminated or minimized. The alkyl halide iodoacetamide has been used in conjunction with the OPA chemistry to eliminate excess thiol after derivatization [44]. We found that the use of either iodoacetamide or NEM (an alternative SH-blocking agent) was unsatisfactory owing to interfering peaks formed from side-reactions in the samples. The addition of a primary amine (30 commercially available amines tested, none further purified) was unsuccessful because of interfering peaks. Electrochemical detection was, however, still possible for most of the ASCAAs and GGPs (see Fig. 6B).

The mass limit of detection (at a signal-to-noise ratio of 3) for the homocysteinesulphinic acid derivative ($k' = 5.4$) under gradient separation conditions was 200 fmol and 425 fmol using fluorescence and electrochemical detection, respectively. Improved detection limits were obtained (factor of 2 for the HCSA derivative) for electrochemical detection under isocratic separation conditions.

Precision and linearity

The repeatability of retention times, as shown in Table II, was 1.03% (mean R.S.D., $n = 10$) and of peak heights 4.92% and 4.25% for electrochemical and fluorescence detection, respectively (mean R.S.D., $n = 10$). The mean correlation coefficients (r) of linearity (4.5–450 pmol, $n = 15$) were 0.9975 (range 0.9959–

TABLE II

RELATIVE STANDARD DEVIATION OF RETENTION TIMES AND PEAK HEIGHTS (CONVENTIONAL SYSTEM)

Amino acid peptide	Retention time (mean, $n = 10$) (min)	R.S.D. (%)	Peak height (22.5 pmol, $n = 10$) (R.S.D., %)	
			ED[a]	FLD[b]
Glutathione	5.34	0.55	4.91	2.80
Cysteic acid	6.27	0.64	3.04	2.72
Glutathionesulphonic acid	6.64	0.76	3.11	3.09
O-Phosphoserine	7.27	0.82	6.05	6.46
Cysteinesulphinic acid	9.98	1.44	6.40	4.26
O-Sulphoserine	11.31	1.52	6.39	3.41
γ-Glutamylglutamine	12.24	1.53	5.96	4.50
Homocysteic acid	12.74	1.24	4.73	4.21
γ-Glutamyltaurine	13.61	1.17	6.17	4.78
Homocysteinesulphinic acid	14.06	1.09	4.34	4.22
γ-Glutamylglutamic acid	16.18	0.80	5.24	4.92
γ-Glutamylglycine	16.90	0.81	2.75	5.64

[a] Electrochemical detection.

[b] Fluorescence detection.

0.9992) for electrochemical and 0.9988 (range 0.9966–0.9999) for fluorescence detection.

Applications

The method is currently used in microdialysis studies of the possible role of these compounds in neurotransmission and neuropathology. An average recovery of 100.9% (range 97.2–104.8%, $n = 6$) was obtained for ten ASCAAs and GGPs added to microdialysates. The low detection limits of the method allowed monitoring of basal levels in the range 2.5–20 n*M* of the various amino acids and peptides, *e.g.* 7.10 ± 2.13 n*M* (mean ± S.D., $n = 6$) was determined for γ-glutamyltaurine in microdialysates from striatum. Furthermore significant changes, 2.8-fold and 1.7-fold ($p < 0.01$, Dunnett *t*-test, $n = 6$), were observed for cysteinesulphinic acid and γ-glutamyltaurine, respectively, during perfusion with 10 m*M* dihydrokainic acid (Fig. 9), an uptake blocker of acidic amino acids [45]. For the first time, an increase was also demonstrated of these neuroactive ASCAAs and GGPs in the extracellular space during global cerebral ischemia [46].

Microcolumn LC

For sample-limited situations a microcolumn method was developed. The column (300 mm × 1.0 mm I.D.) was packed with the same reversed-phase material as in the conventional system; thus, the separations could be performed under the same chromatographic conditions. The benefits of using microcolumns in LC

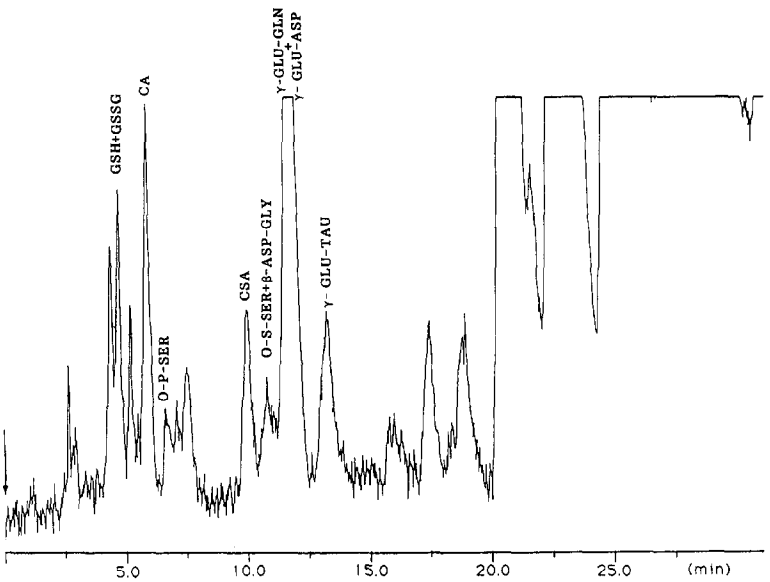

Fig. 9. Chromatogram of a microdialysate sampled during administration of 10 m*M* dihydrokainic acid via the dialysis probe. Experimental conditions and abbreviations are the same as in Fig. 6A.

have been discussed in detail elsewhere [47]. With concentration-sensitive detectors and the same amount of solute injected on the column, a decrease in column I.D. results in less dispersion and therefore a higher solute concentration at the peak maxima. If extra-column band-broadening is insignificant, a larger response is obtained provided that the concentration sensitivity of the detector is preserved during miniaturization of the detector flow-cell. Thin-layer electrochemical detectors are compatible with 1 mm I.D. columns without any major modifications [48]. The lower volumetric flow-rates result in decreased noise levels [48] and therefore higher signal-to-noise ratios.

The large amount of βME from unconsumed reagent was partly eliminated by use of a Hg/Au amalgam electrode positioned upstream in the detector flow-cell. The function of this electrode is similar to the working electrode in a dual Hg/Au electrode arrangement for detection of thiols as previously described [43]. Thiols are eliminated via their catalytic oxidation of the mercury surface at 200 mV according to:

$$2RSH + Hg^0 \rightarrow Hg(SR)_2 + 2H^+ + 2e^-$$

The 1-alkylthio-2-alkylisoindoles are detected downstream at a glassy carbon working electrode operating at 700 mV. No currents were measured at the Hg/Au electrode, which works as a thiol scavenger. At a reagent concentration of 20 m*M*, which is required for a total primary amine concentration of *ca.* 100 μM, the βME peak is reduced by 75% (5 μl injection volume). With this detector arrangement, background currents were less than 0.5 nA and noise levels *ca.* 0.46 pA. The Hg/Au electrode has a limited lifetime because of its scavenger function, but normally the electrode was active for at least two weeks. However, reactivation of the electrode is easily performed by applying a new mercury film on the gold electrode surface.

The mass limit of detection (signal-to-noise ratio = 3) under isocratic separation conditions was 13 fmol for the homocysteinesulphinic derivative (k' = 5.2). This is an improvement by a factor of 15 over the conventional system (isocratic separation). The concentration limit of detection was 1.3 n*M*.

The repeatability of retention times and peak heights was 1.14 and 4.79% (mean R.S.D., n = 8), respectively, using the microcolumn method (Table III). The mean correlation coefficient of the linearity (r) was 0.9979 (range 0.9930–0.9996, 0.5–50 pmol, n = 12).

The separation of a standard mixture of ASCAAs is shown in Fig. 10 A and B.

CONCLUSION

Simultaneous determination of GGPs and ASCAAs at low nanomolar concentrations has been demonstrated. These compounds represent the major number of acidic non-protein amino acids and acidic peptides known to exist in the mammalian central nervous system. The fluorescence emission intensities of the

TABLE III

RELATIVE STANDARD DEVIATION OF RETENTION TIMES AND PEAK HEIGHTS (MICRO-COLUMN SYSTEM)

Amino acids	Retention time (mean, n = 8) (min)	R.S.D. (%)	Peak height (500 fmol, n = 8) (R.S.D., %)
Cysteic acid	7.26	1.16	4.04
O-Phosphoserine	8.36	1.11	3.62
Cysteinesulphinic acid	11.19	1.15	3.48
O-Sulphoserine	12.89	0.91	4.98
Homocysteic acid	14.52	1.45	7.85
Homocysteinesulphinic acid	16.34	1.06	4.76

β-aspartyl and γ-glutamyl derivatives are several times higher than those of their α-analogues, and similar to those of single α-amino acids. This makes the OPA–βME reagent, in combination with fluorescence detection, highly attractive for trace-level determination of these peptides. Furthermore, the OPA–βME reaction in this application is not restricted by exact timing of derivatization and

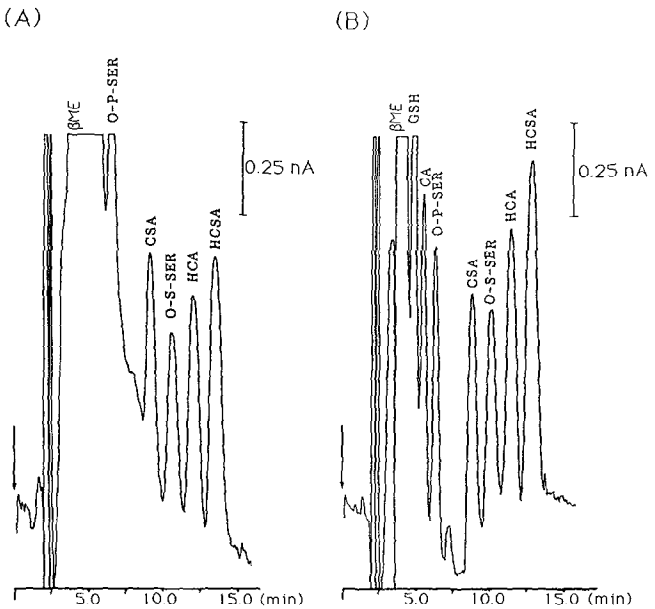

Fig. 10. Separation of a mixture of standards (4.8 pmol of each compound injected). Mobile phase, B–E (12:88, v/v); column Spherisorb ODS-2 (300 mm × 1.0 mm I.D.); flow-rate, 65.2 μl/min; injection volume, 5.8 μl. For mobile phase conditions and abbreviations, see Experimental and Figs. 1 and 2, respectively. (A) Electrochemical detection; single glassy carbon working electrode (700 mV, 5 nAFS); (B) electrochemical detection: dual series electrode Hg/Au and glassy carbon (200 mV/700 mV, 5 nAFS).

injection steps, because the derivatives are relatively stable. The conventional LC method can be used on samples recovered from the rat brain *in vivo* using the microdialysis technique if temporal resolution requirements are low or if multiprobe implantations are possible. The minimal sampling time using a single dialysis probe and a 2 μl/min perfusion rate is therefore *ca.* 40 min (20 min for dual probe implantations). As a means of increasing the temporal resolution, the micro LC method can be used. The high sensitivity thereby achieved allows sampling to be performed during only 3 min. The lack of a commercialy available fluorescence detector suitable for microcolumns restricted the detection to electrochemistry in the microcolumn method. For further improvements, *i.e.* determination at sub-nanomolar concentrations, the use of laser-induced fluorescence detection is attractive. The fluorescence spectral characteristics of the derivatives of GGPs and ASCAAs make them suitable for excitation with the 325-nm line of a HeCd laser or the UV multi-line of an argon-ion laser. Such work is in progress.

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