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ANALYSIS OF AMINO ACIDS IN BRAIN AND PLASMA SAMPLES BY SENSITIVE GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Gas chromatography–mass spectrometry–selected-ion monitoring provided a simple and sensitive method for analyzing amino acids in plasma and brain samples. Although the sensitivities of chemical ionization and electron-impact ionization were similar chemical ionization produced higher-mass ions, which might increase the selectivity of the assay. Both chemical and electron-impact ionization distinguished the natural amino acids from the ^{15}N-labelled amino acids. The recovery of amino acids from plasma and brain samples was ca. 75%. The amino acid levels determined by gas chromatography–mass spectrometry were comparable with the amino acid levels determined by high-performance liquid chromatography or amino acid analyzer.

INTRODUCTION

There is growing evidence that amino acids such as γ-aminobutyric acid (GABA), glumatic acid (Glu), aspartic acid (Asp), and glycine (Gly) play an important role as neutrotransmitters in the central nervous system [1–3]. Other amino acids, such as tyrosine and tryptophan, are precursors for the synthesis of many important neurotransmitters [4]. It has also been reported that alteration in brain amino acid levels may be associated with the development of several brain disorders [5–7]. Since there is no radioactive isotope of nitrogen suitable for use in animals (^{13}N is extremely unstable and has a short half-life), ^{15}N-labelled amino acids could be used in studying amino acid metabolism and the role of amino acids in the development of various brain disorders.

The common methods for the analysis of amino acids are high-performance liquid chromatography (HPLC) and amino acid analysis [8–11]. Although these methods are simple and sensitive, they lack confirmatory capability and cannot distinguish natural amino acids from ^{15}N-enriched amino acids [12]. These at-

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tributes are required (1) to study the metabolism of amino acids in plasma and brain samples and (2) to establish possible role of amino acids in the etiology of various brain disorders. Previous studies have shown that gas chromatography–mass spectrometry (GC–MS) can be applied to the trace analysis of amino acids, confirmation of amino acids, and detection of natural and ^{15}N-amino acids [12–20]. This paper describes a GC–MS method for the trace analysis of amino acids in plasma and brain samples.

EXPERIMENTAL

Materials

The GC–MS system used was a Hewlett-Packard Model 5987 with electron-impact (EI) and chemical ionization (CI), and an integral gas chromatograph. Amino acid standards were obtained from Sigma (St. Louis, MO, U.S.A.). Pentafluoropropionic anhydride (PFP) and hexafluoroisopropanol (HFIP) were obtained from Aldrich (Milwaukee, WI, U.S.A.). ^{15}N-Labelled amino acids were obtained from MSD Chemicals (Montreal, Canada). The capillary column used was a DB-5 (30 m × 0.25 mm I.D.) from (J&W Scientific, Rancho Cordova, CA, U.S.A.).

Methods

Preparation of the brain samples. Five rats weighing ca. 150 g were anesthetized with diethyl ether. Each rat was surgically operated, the brain sample was collected and frozen immediately for the analysis of amino acids. At the time of analysis, each sample was homogenized in isotonic saline (2.0 ml/g) and incubated at room temperature for 30 min. After incubation, 15 ml of methanol were added to each sample and the sample was again homogenized. The combined homogenate was incubated at 70 °C for 30 min. After incubation, each sample was centrifuged for 15 min at 1500 *g*. The clear supernatant was collected in another test-tube and dried at 70 °C by flash evaporation. The dried residue was directly derivatized for the analysis of amino acids.

Preparation of the plasma samples. A 5-ml volume of plasma was mixed with 15 ml of methanol. The mixture was incubated at 37 °C for 30 min and at 80 °C for 30 min. After incubation, the mixture was centrifuged for 15 min at 1500 *g*. The clear supernatant was collected and dried at 70 °C under reduced pressure. The dried residue was directly used for derivatization.

Derivatization procedure. The derivatization procedure of Wolfensberger [21] was used in this study. A 500-μl volume of a mixture of PFP and HFIP (2:1), and 5 mg of glass beads were added to the dried tissue or plasma residue. Standard amino acids (1.0 μg) were derivatized by mixing them with the above reagents. The mixture was incubated at 25 °C for 60 min and at 80 °C for 4 h. After incubation, the derivatization reagent was dried under a stream of nitrogen. The dried residue was redissolved in 100 μl of hexane, and 1 μl of the derivatized residue was subjected to GC–MS. ^{15}N-Labelled amino acids were derivatized similarly. The stability of the derivatized samples was determined by derivatizing various amino acids and analysing the concentration at various subsequent times.

GC–MS conditions. GC–MS was preformed under the following conditions: in-

TABLE I

SELECTION OF IONS FOR THE ANALYSIS OF AMINO ACIDS

Amino acid	EI mode		CI mode	
	Ion (m/z)	Abundance (%)	Ion (m/z)	Abundance (%)
GABA	176	100	232	100
			206	80
Tyrosine	253	100	478	100
Glycine	176	100	372	100
Alanine	190	100	238	100
Glutamic acid	202	100	230	100
Glutamine	202	100	230	100
	230	80	426	50
Aspartic acid	384	100	384	100
Ornithine	216	100	–	–
	176	80	–	–
Phenylalanine	298	100	426	100
			294	80
Lysine	230	100	589	100
	176	80	–	–
Leucine	203	100	428	100
	232	70	232	80
	371	60		
Isoleucine	202	100	232	100
	232	70	428	20
	371	60		
Methionine	202	100	446	100
			398	50
Proline	216	100	260	100
Tryptophan	245	100	481	100
Valine	203	100	414	100
	218	75		

let temperature, 180 °C; initial temperature, 60 °C; oven temperature programme, isothermal at 60 °C for 3.0 min, then increasing 5.0 °C/min to 180 °C; run-time, 40 min. For EI, the source temperature was 200 °C, the electron energy voltage was 70 eV, and the source pressure was $4.5 \cdot 10^{-6}$ Torr. For CI, using methane as the reagent gas, the source pressure was $2.0 \cdot 10^{-4}$ Torr. The equipment was programmed to monitor the selected ions shown in Table I.

Quantitative analysis. Known concentrations of derivatized amino acids (1.0–4.0 ng/ml) were analysed to generate a standard curve. The integrated area of the peak at various standard concentrations was determined for each amino acid. A standard curve was drawn by plotting the concentration of standards on the x-axis and the integrated peak area on the y-axis. The concentrations of various amino acids in the brain or plasma samples were determined from the standard curve. Recoveries were determined by adding known amounts of amino acids to

the sample and determining the amounts recovered [^{15}N]GABA was used as the internal standard for the quantitative analysis of amino acids in plasma and brain samples.

RESULTS AND DISCUSSION

Derivatization of amino acids

This study indicated that GC combined with EI- or CI-MS can be used for the trace analysis of amino acids in brain, plasma and other tissues. When the mass spectrometer was programmed to monitor only a few selected ions, the sensitivity of this method was similar to the sensitivity of the HPLC method (Table II). Although underivatized amino acids are not volatile, removal of the acidic or basic character by derivatization makes them fairly volatile [21]. Earlier investigations used acidic esterification as the derivatization procedure for the analysis of amino acids by GC–MS [22–24]. Wolfensberger et al. [25] observed that the esters of α-amino acids formed diketopiperazines, which interfere with the analysis of several amino acids. Also, the acid esters of amino acids produce smaller ions and result in the minor peaks of one amino acid interfering with the diagnostic peaks of others [26]. In the present study, a mixture of PFP and HFIP was used to form the PFP-HFIP amino acid derivatives. These derivatives are stable for three to five days and form diagnostically significant ions in the mass spectrometer (Fig. 1, Table III). Contrary to the observations of this study, previous studies have reported that PFP-HFIP derivatization was unstable and provided multiple derivatives [27,28]. Although the cause of this discrepancy is not known, the concentration and incubation temperature used for derivatization in the studies of Corradetti et al. [28] and in the present study were different.

TABLE II

BRAIN AMINO ACID LEVELS IN RAT BRAIN

Values are mean ± S.D., $n = 3$.

Amino acid	GC–MS (μmol/g)	HPLC* (μmol/g)
GABA	2.17 ± 0.13	1.52 ± 0.11
Alanine	0.53 ±0.01	0.61 ± 0.12
Glycine	0.61 ± 0.13	1.48 ± 0.46
Leucine	0.23 ± 0.07	0.40 ± 0.06
Isoleucine	–	3.69 ± 0.68
Aspartic acid	2.30 ± 1.01	3.70 ± 0.68
Glutamic acid	5.60 ± 1.07	9.38 ± 0.67
Glutamine	1.36 ± 0.21	0.97 ± 0.16
Tyrosine	0.19 (one sample)	0.31 ± 0.10
Lysine	–	3.9 ± 0.62
Tryptophan	0.11 (one sample)	0.32 ± 0.03

*Unpublished data.

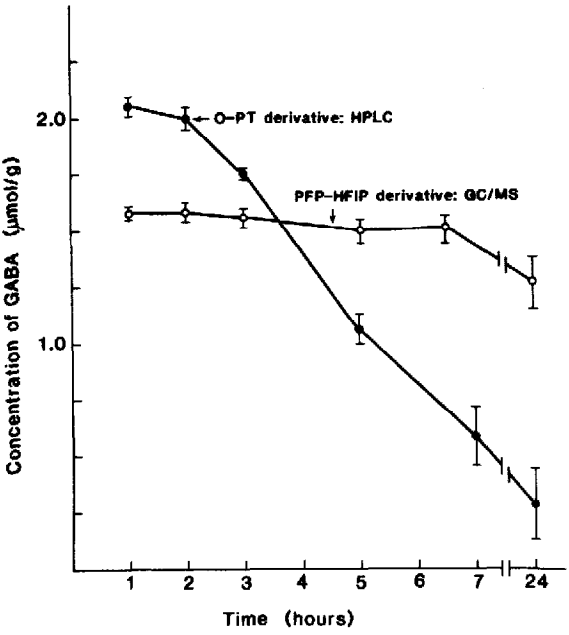

Fig. 1. Relative stabilities of *o*-phthaldialdehyde (O-PT) and PFP-HFIP derivatives of GABA. The concentrations of GABA at various time intervals were determined by using freshly derivatized standards.

Fragmentation of PFP-PFIP derivatives of amino acids

GABA. The molecular ion was not observed in the EI spectrum, whereas the M + 1 ion (*m/z* 400) was observed in the CI spectrum. The major ions produced by EI were *m/z* 176 (CF_3–CF_2–CO–NH–CH_2) and *m/z* 232 [M – ($OCH(CF_3)_2$)]. The major ions produced by CI were *m/z* 232, 206, and 400 (Table III). The ions produced from [^{15}N]GABA were *m/z* 177 and 233 in the EI mode, and *m/z* 233, 207 and 401 in the CI mode. This study indicated that both EI and CI distinguished the natural amino acid from the ^{15}N-labelled amino acid.

Tyrosine and phenylalanine. The major ions produced by the EI fragmentation of tyrosine were *m/z* 253, 293 and 266. The ion at *m/z* 266 was formed by the loss of –OH and –COO–$CH(CF_3)_2$ groups. The mechanism by which ion *m/z* 253 was formed is not known. Although the molecular ion was not detected in EI mode, the M + 1 ion (*m/z* 478) was present in highest abundance in CI mode. The major ion produced by the EI fragmentation of phenylalanine was *m/z* 298, which was formed by the loss of –NH–$COCF_2$–CF_3 moiety. The M + 1 ion (*m/z* 462) was present in highest abundance when phenylalanine was subjected to CI ionization. Another ion formed by CI was *m/z* 294, by the loss of the –O–$CH(CF_3)_2$ group (Table III).

Tryptophan. The major ions produced by the EI fragmentation of tryptophan were *m/z* 245 [loss of –CF_2–CF_3, 2(CF_3) and –CO–CF_2–CF_3], 286, and 480 [loss of –$OCH(CF_3)_2$]. The ions produced by CI fragmentation of tryptophan were *m/z* 481 [loss of –$OCH(CF_3)_2$] and 501 (loss of –CO–CF_2–CF_3). Molecular ions were not observed in either modes (Table III).

TABLE III

MASS FRAGMENTATION PATTERNS OF VARIOUS AMINO ACIDS BY EI AND CI

The base ion is set at 100% and other ions are relative to the base ion. Values in parentheses are percentages abundance.

Amino acid	Mol. mass derivatized	EI ions (*m/z*)	CI ions (*m/z*)
GABA	399	176(100), 232(30)	232(100), 206*(80), 400(25)*
[^{15}] GABA	400	177(10), 233(30)	233(100), 207*(80), 401(25)*
Tyrosine	477	253(100), 293(25), 266(10)	478(100)*, 253(10)
Glycine	371	176(100), 224(25)	372(100)*, 176(25), 204(20)
Alanine	385	190(100), 238(50)	238(100), 386(30)*
Glutamic acid	593	202(100), 230(10), 265(10)	230(100), 380(25)
[^{15}] Glutamic acid	594	203(100), 231(10), 266(100)	231(100)
Glutamine	753	202(100), 230(80)	230(100), 426(50)
Aspartic acid	579	384(100), 216(80)	384(100), 412(80)
[^{15}N] Aspartic acid	580	384(100), 217(80)	384(100), 412(80)
Ornithine	–	216(100), 176(80), 231(10)	–
Phenylalanine	461	298(100), 461(1)**	462(100)*, 294(80)
Lysine	588	230(100), 176(80), 218(20)	589(100)*, 421(25), 230(25)
[^{15}N] Lysine	589	232(100), 178(80), 220(20)	591(100)*, 231*
Leucine	427	203(100), 232(70), 371(60), 352(25)	428(100)*, 232(80)
Isoleucine	427	203(100), 232(70), 371(70), 352(25)	232(100), 428(20)*
Methionine	445	202(100), 203(95), 229(10), 445(1)**	446(100)*, 398(50)
Proline	411	216(100), 264(10), 411(1)**	260(100)
Histidine	–	188(100), 259(25), 216(20)	–
Tryptophan	646	245(100), 286(10), 480(25)	481(100), 501(25)
Valine	413	203(100), 218(75), 371(25)	414(100)*, 442(20), 218(70)

*M + 1 ion.
**Molecular ion.

Proline. The major ions produced by the EI ionization of proline were *m/z* 216 [loss of $-COO-CH(CF_3)_2$ group] and 264. The major ion produced by CI fragmentation of proline was *m/z* 260, by loss of $-CO-CF_2-CF_3$ (Table III).

Glycine, lysine, and ornithine. In EI ionization, these amino acids produced an ion at *m/z* 176, which was recognized as $CF_3-CF_2-CONHCH_2$. Other major ions produced by EI ionization were *m/z* 224 for glycine, *m/z* 230 [formed by the loss of $-COO-CH(CF_3)_2$ and $-(NH-CO-CF_2-CF_3)$ groups] for lysine and *m/z* 216 for ornithine. [N^{15}]Lysine produced ions at *m/z* 591 (M + 1), 232, and 178, which were different from the ions produced by natural lysine (Table III).

Alanine. The major ions produced by EI ionization of alanine were *m/z* 190 [loss of $-COO-CH(CH_3)_2$] and 238 (loss of $-CO-CF_2-CF_3$). CI of alanine produced an ion at *m/z* 238 in highest abundance. The M + 1 ion (*m/z* 386) was present in 30% abundance (Table III).

Glutamic acid and glutamine. EI ionization of glutamic acid and glutamine produced ions at *m/z* 202 [loss of $(CF_3)_2CH-O-$ and $-COO-CH(CF_3)_2$ groups] and 230 [loss of two $(CF_3)_2CH-COO$ groups]. However, the ratio of the abundances

of ions 202 and 230 was 10.0 for glutamic acid and 1.2 for glutamine. CI of glutamic acid and glutamine produced an ion at *m/z* 230 highest abundance. Other major ions produced by glutamic acid and glutamine were at *m/z* 380 and 426, respectively (Table III). The ^{15}N-labelled derivative produced ions at *m/z* 203, 231, and 266, which were different from the ions produced by the natural amino acids.

Aspartic acid. The ion at *m/z* 384 [produced by loss of $-NH-CO-CF_2-CF_3$ and $-COO-CH(CF_3)_2$ groups] was present in highest abundance in the mass spectra produced by EI ionization or by CI of aspartic acid (Table III). Other ion produced by the EI ionization of aspartic acid was at *m/z* 216 (Table III). [^{15}N]-Aspartic acid produced ions at *m/z* 384 and 217.

Leucine and isoleucine. EI ionization of these amino acids produced ions at *m/z* 203, 232, 371, and 352. Ion 203 was produced by loss of $(CH_3)_2CH-CH_2-$ (for leucine) or $CH_3-CH_2-CH(CH_3)-$ (for isoleucine and $(O-CH(CF_3)_2)$. The ion at *m/z* 232 was produced by the loss of the $COO-CH(CF_3)_2$ group, and the ion at *m/z* 371 was produced by the loss of the $CH_3-CH_2CH(CH_3)_2$ or $(CH_3)_2CH-CH_2$ group. CI of leucine produced the ion at *m/z* 428 (M + 1) in highest abundance, whereas isoleucine produced the ion at *m/z* 232 in highest abundance. The abundance of the M + 1 ion produced from CI of isoleucine was ca. 20%.

Methionine. CI of methionine produced the ion at *m/z* 446 (M + 1) in highest abundance. The other major ion produced by CI was at *m/z* 398. Ions at *m/z* 202 and 203 were present in highest abundance when methionine was subjected to EI ionization (Table III).

Selection of ions for monitoring amino acids

The criterion described by Singh et al. [29] was used to select ions for the quantitative analysis of amino acids. For EI analysis, the ion at *m/z* 176 was included to monitor GABA, lysine, glycine, and ornithine (Table I). The ion at *m/z* 190 was included to monitor alanine, and that at *m/z* 202 was included to monitor glutamic acid, glutamine, and methionine (Table I). For CI analysis, M + 1 ions could be monitored to analyse most of the amino acids. Therefore, it is proposed that CI might eliminate the problem of minor peaks of one amino acid interfering with the diagnostic peak of others, which is commonly associated with EI analysis.

EI and CI chromatography

Under the chromatographic conditions used in this study, our capillary column provided excellent separation of different amino acids in the blood or brain samples (Figs. 2 and 3). Individual amino acids were identified by their specific mass ions (Figs. 2 and 3). Sass and Fisher [30] and Singh et al. [31] have observed that CI is at least ten times more sensitive than EI ionization for the quantitative analysis of various drugs. Contrary to their observations, the results of this study indicated similar sensitivities of the CI and EI procedures for the analysis of amino acids. However, CI was more specific than EI because of the high abundances of M + 1 ions produced by CI.

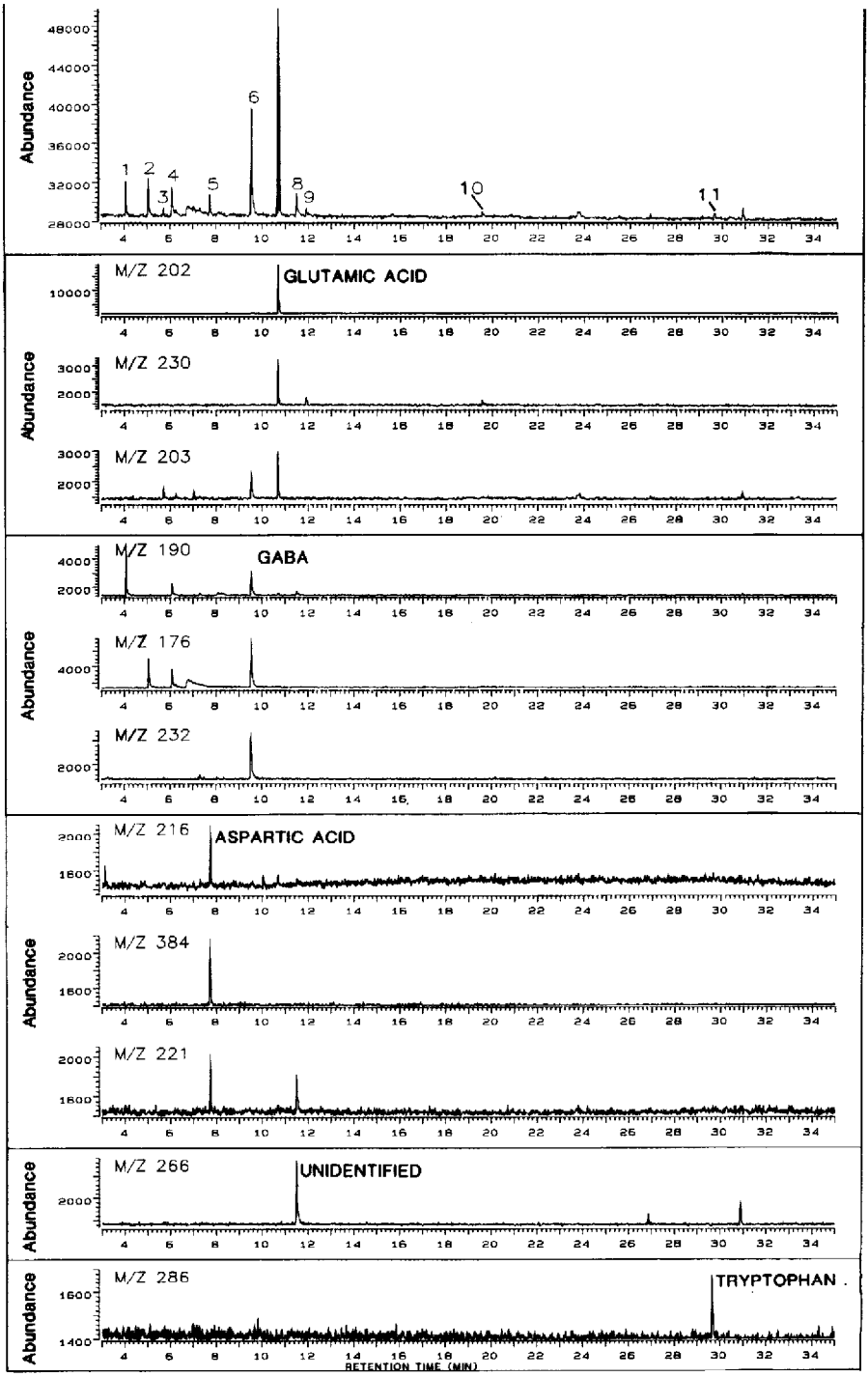

Fig. 2. Chromatographic separation of various amino acids in brain. Peaks: 1 = alanine; 2 = glycine; 3 = leucine; 4 = isoleucine; 5 = aspartic acid; 6 = GABA; 7 = glutamic acid; 8 = glutamine; 9 = unidentified; 10 = tyrosine; 11 = tryptophan. The ion profiles of selected amino acids are also illustrated.

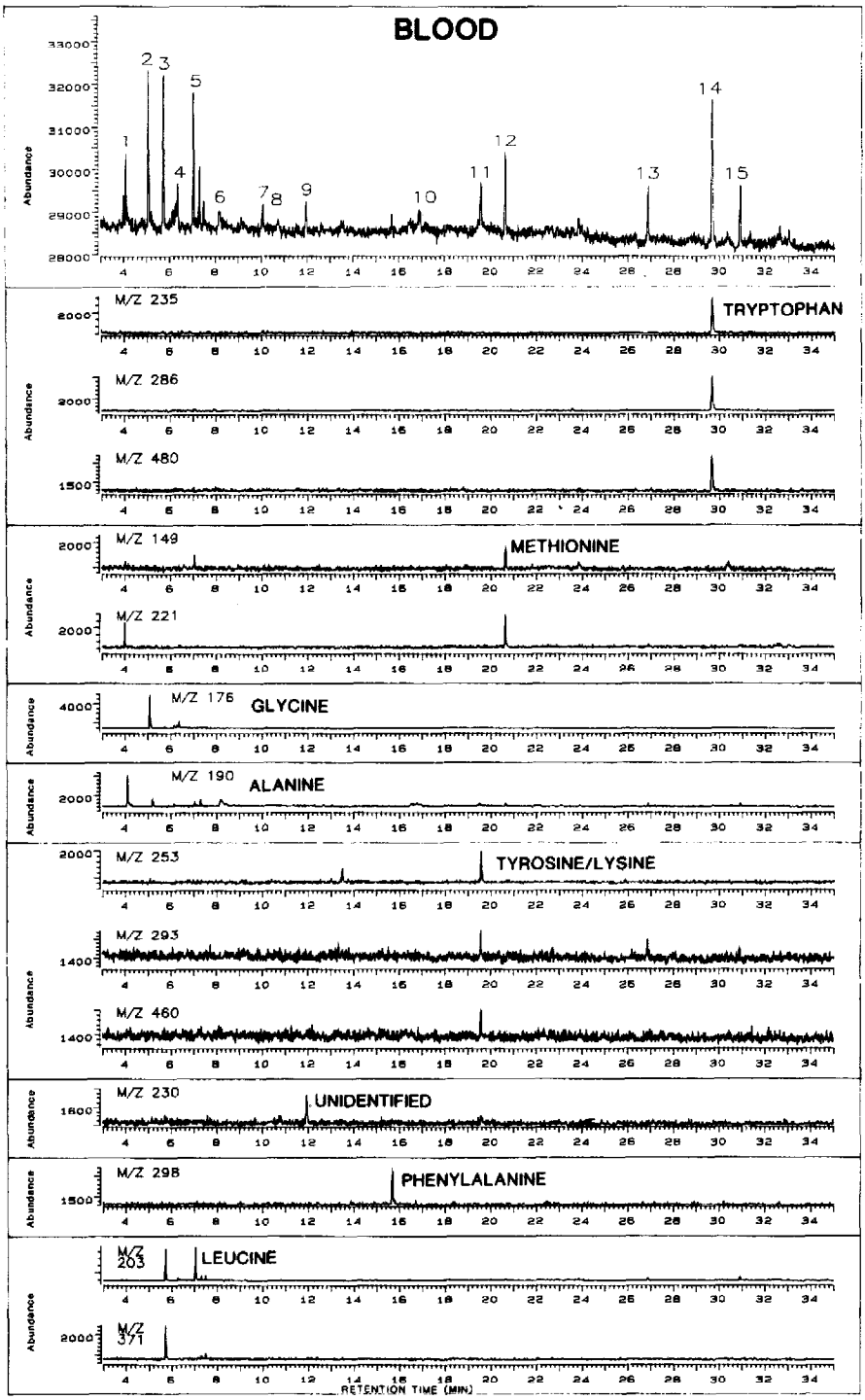

Fig. 3. Chromatographic separation of various amino acids in plasma. Peaks: 1 = alanine; 2 = glycine; 3 = leucine; 4 = isoleucine; 5 = aspartic acid; 6 = unidentified; 7 = glutamic acid; 8 = glutamine; 9 = unidentified; 10 = phenylalanine; 11 = tyrosine/lysine; 12 = methionine; 13 = unidentified; 14 = tryptophan; 15 = unidentified. The ion profiles of selected amino acids are also illustrated.

Quantitative analysis

When [^{15}N] GABA was used as an internal standard for the quantitative analysis of amino acids, the recovery of the extraction procedure was ca. 75%. Other compounds that might be suitable for use as an internal standard are deuterium-labelled amino acids and phenylethylamine [21]. The amounts of amino acids present in the plasma or brain samples were determined from the standard curve. As shown in Tables II and IV, we observed comparable values when various amino acids were determined by GC–MS, HPLC [32] or amino acid analysis [32]. For certain amino acids, such as GABA, alanine, and glycine, the values obtained using GC–MS were different from the values obtained using HPLC or amino acid analysis. This might be because of differences in the extraction and derivatization procedures. Other investigators have reported similar variations in amino acid levels [23–37]. However, repeated use of the same technique produced very little variation in the amino acid levels [37,38].

Previous studies have commonly used *o*-phthalaldehyde amino acid derivatives for the analysis of amino acids by HPLC [10]. Since these derivatives are stable for only 10–30 min, the derivatized samples must be analysed immediately. We have observed a time-dependent decline in the concentrations of the *o*-phthalaldehyde derivatives of GABA and Glu when samples were analyzed at various times after derivatization (Fig. 1). This study has indicated that the PFP-HFIP amino acid derivatives are stable for three to five days (Fig. 1), which makes the GC–MS procedure more reproducible and suitable for the analysis of a large number of samples by using autosamplers.

From the results of this study, it is concluded that (1) GC–MS–selected-ion monitoring provides a simple and sensitive method for measuring amino acids, (2) both CI and EI distinguish natural and ^{15}N-labelled amino acids, (3) the sensitivities of the GC–MS, HPLC and amino acid analysis methods are comparable, and (4) the PFP-HFIP amino acid derivatives are stable for three to five days.

TABLE IV

PLASMA AMINO ACID LEVELS IN RAT PLASMA

Values are mean ± S.D., $n = 4$.

Amino acid	GC–MS (μmol per 100 ml)	Automatic analysis* (μmol per 100 ml)
Alanine	10.29 ± 1.44	9.83 ± 1.75
Glycine	7.16 ± 2.13	3.19 ± 0.93
Leucine	3.70 ± 0.70	2.16 ± 0.63
Isoleucine	3.69 ± 0.68	4.39 ± 0.56
Aspartic acid	2.86 ± 0.32	1.55 ± 0.36
Glutamic acid	2.25 ± 0.42	3.26 ± 0.33
Tyrosine	0.36 ± 0.04	0.54 ± 0.13
Phenylalanine	–	0.59 ± 0.08
Methionine	0.47 ± 0.21	0.50 ± 0.12

*Ref. 30.

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