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SIMPLE AUTOMATED GAS CHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS AND ITS APPLICATION TO BRAIN TISSUE AND URINE

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SUMMARY

The separation of 21 free amino acids as their isobutyl N(O,S)-pentafluorobenzoyl derivatives by capillary gas chromatography with electron-capture detection is described. This method has been successfully applied to routine analysis of most of these amino acids in brain tissue and urine. The amino acids are converted to their isobutyl N(O,S)-pentafluorobenzoyl derivatives by acid-catalysed esterification followed by aqueous extractive pentafluorobenzoylation. The 21 amino acids can be separated on a single capillary column in 21 min. The high sensitivity permits analysis in minute quantities of sample and the high stability permits the use of an automatic sample injector at ambient temperatures. Thus, over 50 samples can be readily analyzed in a single day.

INTRODUCTION

The prime physiological role of amino acids is protein synthesis. However, in the central nervous system some also function as neurotransmitters or neuromodulators [1—5]. The excitatory effects of glutamate and aspartate [6, 7], the inhibitory effects of glycine, γ-aminobutyric acid and taurine [8—10], and the precursor control of tryptophan, tyrosine and phenylalanine in the synthesis of classical monoaminergic neurotransmitters [11, 12] have recently been reviewed.

Amino acids have been separated since the early 1950s by automated systems using ion-exchange chromatography followed by post column derivatization [13, 14]. The automatic amino acid analyser is an expensive single-purpose instrument that offers good selectivity but often low sensitivity and long chromatographic times. More recently, the use of pre-column derivatization

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with reversed-phase high-performance liquid chromatography (HPLC) has gained wide acceptance [15—27]. Generally, HPLC offers shorter analysis time and higher detector sensitivity compared to the ion-exchange technique, but the fluorescent derivatives, for example those formed by reaction with dansyl chloride or *o*-phthaldialdehyde, are relatively unstable, thus prohibiting automation [28].

The development of gas chromatographic (GC) analyses of amino acids to produce more rapid and sensitive methods and stable derivatives suitable for automation has been the objective of much research. Numerous approaches for the preparation of volatile derivatives suitable for routine quantitative analysis of amino acids have been reviewed [29—33]. The perfluoroacyl alkyl esters, including the N-trifluoroacyl (TFA), *n*-butyl esters [29, 34, 35] and N-heptafluorobutyryl isobutyl esters [36—40], oxazolidinones [41, 42], isobutyloxycarbonyl methyl esters [43] and trimethylsilyl [44] derivatives have been studied most extensively.

Although various analytical methods have been described in the literature, only a few were developed for routine use. Many of the GC methods developed involve lengthy procedures and have not found routine use in analysis of biological samples. We report here a simple, rapid and sensitive method for the derivatization, separation and quantitation of free amino acids in biological samples which is suitable for automation.

EXPERIMENTAL

Reagents and materials

The L-amino acid standards and α-methyl-*p*-tyrosine (internal standard) were purchased from Sigma (St. Louis, MO, U.S.A.) and were of the purest grade available. Pentafluorobenzoyl chloride was obtained from Aldrich (Milwaukee, WI, U.S.A.). All other solvents and reagents were of analytical grade and supplied by Fischer Scientific (Mississauga, Canada). The isobutanol was distilled over calcium hydride and stored over a Linde 4-Å molecular sieve. All amino acid standards were prepared in methanol—6 *M* hydrochloric acid (9:1) and stored at —20°C.

Gas chromatograph and mass spectrometer

GC analysis was carried out with a Hewlett-Packard 5880A gas chromatograph equipped with 7672A automatic sampler and 5880A terminal. All data were analysed by an HP 86 microcomputer. Mass spectra were recorded on 7070E VG Analytical Organic spectrometer.

Extraction and derivatization

Brains from male Sprague—Dawley rats were homogenized in 10 vols. (10 ml/g of tissue) of an ice-cold mixture of methanol—6 *M* aqueous hydrochloric acid (9:1). After centrifugation at 10 000 *g* for 15 min, a 10-μl aliquot of the supernatant was employed for analysis. Human urine samples (24 h) were collected in plastic containers containing 2 ml of 2% EDTA. Aliquots of urine (200 μl) samples were added to 200 μl of an ice-cold mixture of methanol—6 *M* aqueous hydrochloric acid (9:1) and centrifuged. An aliquot (20.0 μl) was

retained for analysis. α-Methyl-*p*-tyrosine (400 ng) was added as internal standard to all samples. The supernatant was dried under a stream of nitrogen and redissolved in a solution of one drop of concentrated hydrochloric acid in 1 ml distilled isobutanol. The reaction tube was flushed with nitrogen prior to capping. The mixture was heated at 100°C for 30 min. After cooling, the isobutanol—hydrochloric acid was evaporated under a stream of nitrogen at 50°C. A 1-ml volume of a solution, containing 5 μl of pentafluorobenzoyl chloride per ml of chloroform, and 1 ml of a saturated aqueous sodium bicarbonate solution were added to the residue. The samples were shaken for 20 min, and the aqueous phase was aspirated off. The organic layer was washed with distilled water (1 ml) before being evaporated to dryness under a stream of nitrogen. The residue was redissolved in 500 μl decane. A 1-μl aliquot of the decane phase was injected by an automatic sampler onto a gas chromatograph equipped with a 15-m narrow-bore SE-54 capillary column and a ^{63}Ni electron-capture detector.

Calibration

For the construction of standard curves, known amounts of authentic samples of the L-amino acids corresponding to 10—1600 ng plus a fixed amount of the internal standard α-methyl-*p*-tyrosine (400 ng) were placed in tubes and carried through in parallel with each set of samples. Curves were constructed by measuring the peak-area ratios of the amino acids to the internal standard recorded by the GC integrator and plotting against the quantities of the amino acids. These data were entered in an HP 86 microcomputer and the amounts present in the biological samples computed using the VisiCalc PLUS program.

Gas chromatographic and mass spectrometric conditions

A Hewlett-Packard 5880A gas chromatograph equipped with a 15-m narrow-bore SE-54 (1% vinyl, 5% phenylmethyl polysiloxane) capillary column (Hewlett-Packard), a ^{63}Ni electron-capture detector, an HP 7672A automatic sampler and an HP 5880A GC terminal was employed. Helium was used as carrier gas with a flow-rate of 2 ml/min. A mixture of argon—methane (95:5) at a flow-rate of 36 ml/min was used as make-up gas. The injection port temperature was maintained at 200°C. The following oven temperature was programmed: initial temperature of 140°C for 0.5 min, increasing at a rate of 5°C/min to 180°C, held for 0.1 min, and increasing at 8°C/min to 310°C where it was maintained for 3 min.

Structures of the derivatized amino acids were confirmed on the mass spectrometer using the following conditions: ion source temperature, 250°C; interface temperature, 290°C; column pressure, 1.035 bar; accelerating voltage, 6 kV; ionization voltage, 70 eV; scan-speed, 0.5 s/decade; dwell time, 0.5 s.

RESULTS AND DISCUSSION

During the past twenty years there have been extensive developments in the separation and analysis of amino acids by GC. Amino acids, being multifunctional, usually require derivatization of reactive groups to obtain suitable

volatile compounds with good peak shape. A large number of different derivatives and techniques for the preparation of derivatives have been employed and extensively reviewed [30—33]. Separation and detection of the amino acid derivatives was usually carried out with conventional GC packed columns and flame-ionization detection, but in more recent years capillary columns and electron-capture detection have been introduced to obtain improved resolution and sensitivity [45—48].

Although various analytical methods have been described in the literature, only a few have found routine use in the analysis of biological samples. Some of the methods involve tedious ion-exchange clean-up procedures or use of two columns for separation. We report here a simple, rapid and sensitive method for the routine separation and quantitation of amino acids which is suitable for complex biological samples such as brain tissue homogenates and plasma and urine samples. The procedures of Hušek et al. [42] and Makita et al. [43] are also rapid with regard to derivatization times, but their applications to analysis of brain tissue have not been published to our knowledge. The procedure of Makita et al. [43], employing flame-ionization detection, provides lower sensitivity than the method described here and requires the use of dual columns in the GC analysis.

For the development of a good analytical procedure suitable for routine use, several factors have to be considered: cost (both in terms of time and expenditure), choice of simple reagents, simplicity of the procedure, availability of laboratory glassware and equipment, possibility of automation, stability and chromatographic properties of derivatives. This method of analysis of amino acids we have developed in our laboratories would seem to meet these criteria.

Any proteins in biological samples interfere in chromatographic analysis and have to be removed. Of all the procedures reported [33], protein precipitation is considered to be the method of choice. The most common protein precipitants are picric acid [49], sulfosalicyclic acid [50], trichloroacetic acid [51] and perchloric acid [51]. With these reagents subsequent elimination of the deproteinizing agent from the sample is essential. Other milder reagents, including ethanol [53], acetone [54], acetonitrile [26] and hydrochloric acid [55], are also being used. However, we found that 10 ml of ice-cold methanol—6 *M* hydrochloric acid (9:1) effectively deproteinized 1 g of brain tissue and an aliquot of the supernatant can be readily brought to dryness for esterification.

The advantages of using isobutanol—3 *M* hydrochloric acid as an esterification reagent for amino acids are well known [36, 39, 40, 47]. Conventionally, anhydrous isobutanol—3 *M* hydrochloric acid is prepared by bubbling the hydrogen chloride gas into the alcohol and then titrating to the desired normality. This reagent should be freshly prepared. The carboxylic acid moiety, however, can be effectively esterified by adding a solution of one drop of concentrated hydrochloric acid per ml of distilled isobutanol into the amino acids prior to heating. The equilibrium of the esterification reaction is expected to shift favourably to the right owing to the large excess (1 ml) of the reactant isobutanol relative to the trace of water present in a drop of concentrated hydrochloric acid. The simplicity of this procedure relative to the difficulties of preparing anhydrous isobutanol—3 *M* hydrochloric acid make this an attractive alternative effective reagent.

As with many other procedures utilizing acid-catalyzed esterification, the amides glutamine and asparagine are converted under the acidic conditions to their dicarboxylic acid counterparts, glutamate and aspartate. Therefore the results for these amino acids are expressed as the summation of glutamate and glutamine and of aspartate and asparagine. This problem represents a disadvantage compared to the procedures of Hušek et al. [42] and Makita et al. [43], both of which permit analysis of glutamate, glutamine, aspartate and asparagine separately. However, there is little information available on the application of these two methods to brain tissue.

The α-amino group plus other reactive groups in the side-chain of the amino acids are commonly acylated by trifluoroacetic anhydride [29, 34, 35, 48, 56] or by heptafluorobutyric anhydride [36, 38—40, 47] at elevated temperature. However, Pearce [45] has cautioned that degradation of such amino acid derivatives may occur with excess heating. We chose to prepare pentafluorobenzoyl (PFB) derivatives of the reactive groups because the extractive N(O,S)-pentafluorobenzoylation is an efficient and reproducible aqueous derivatization procedure [57] that requires only mild conditions and the PFB derivatives provide thermal stability and high electron-capture response [58—64].

Extractive pentafluorobenzoylation with pentafluorobenzoyl chloride as presented here combines efficiently the extraction, derivatization and consumption of the derivatizing reagent within a reasonable time suitable for routine analysis of amino acids. Secondary alcohol groups were not derivatized

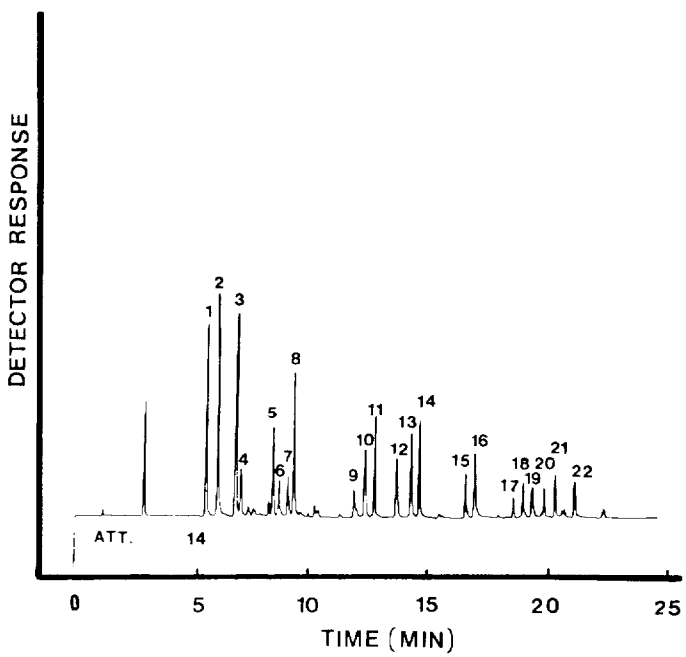


Fig. 1. (A) Typical GC trace of derivatized standards (400 ng) of amino acids carried through the procedure described in the text. Peaks: derivatives of alanine (1), glycine (2), β-alanine (3), valine (4), leucine (5), isoleucine (6), threonine (7), γ-aminobutyric acid (8), hydroxyproline (9), methionine (10), aspartate (11), phenylalanine (12), glutamate (13), serine (14), cysteine (15), α-methyl-*p*-tyrosine (internal standard) (16), citrulline (17), histidine (18), ornithine (19), tryptophan (20), lysine (21) and *p*-tyrosine (22).

by the procedure used. The separation of amino acid standards is demonstrated in Fig. 1.

Calibration linearity for all the amino acids was excellent, with correlation coefficients of $\geqslant 0.99$ obtained routinely. To check the reproducibility of this method, eight 400-ng quantities of each of the amino acids were carried through the entire procedure. Table I shows the relative molar responses and the retention times of each amino acid. It is of interest that even small changes in structure can alter sensitivity dramatically. For example there are decreases when going from leucine to isoleucine, from alanine, β-alanine and glycine to valine or threonine, and from α-methyltyrosine to tyrosine. The reasons for these changes are unclear at present, but it is well known that sensitivity to electron-capture detectors is not always predictable from the structure of the compound being analyzed or even from the number of electrophoric groups added during derivatization [65]. The coefficients of variation were less than 5%, except in the cases of proline and arginine where the coefficients of variation were greater than 10% and thus these two amino acids were not quantitated.

The method allows the determination of amino acids with a high degree of

TABLE I

RETENTION TIMES, RELATIVE MOLAR RESPONSES AND OVERALL REPRODUCIBILITY OF N(O,S)-PENTAFLUOROBENZOYLATED ISOBUTYL ESTER AMINO ACID DERIVATIVES WHEN USING GAS CHROMATOGRAPHY

Amino acid*	Abbreviation	Retention time (min)	RMR**	Coefficient of variation (%)
Alanine	Ala	5.53	1.44	3.3
Glycine	Gly	6.00	1.39	4.1
β-Alanine	β-Ala	7.00	1.46	2.9
Valine	Val	7.22	0.52	2.9
Leucine	Leu	8.31	1.02	2.0
Isoleucine	Ile	8.45	0.45	2.0
Threonine	Thr	8.93	0.45	2.9
γ-Aminobutyric acid	GABA	9.17	1.32	4.1
Hydroxyproline	Hyp	11.78	0.35	2.9
Methionine	Met	12.23	0.82	1.6
Aspartate	Asp	12.64	0.76	4.1
Phenylalanine	Phe	13.57	0.90	2.4
Glutamate	Glu	14.14	1.11	3.7
Serine	Ser	14.51	0.92	2.4
Cysteine	Cys	16.58	0.48	3.7
Citrulline	Cit	18.50	0.31	3.3
Histidine	His	18.90	0.42	3.7
Ornithine	Orn	19.26	0.53	2.0
Tryptophan	Trp	19.81	0.42	3.3
Lysine	Lys	20.25	0.54	4.1
Tyrosine	Tyr	21.08	0.62	3.7
α-Methyl-*p*-tyrosine (internal standard)	α-Me-*p*-Tyr	18.12	1.00	

*The nomenclature of amino acids is according to the IUPAC guidelines.
**Relative molar response (RMR) relative to the internal standard.

TABLE II

FREE AMINO ACID CONCENTRATIONS IN RAT WHOLE BRAIN

Amino acid*	Concentration** (μmol/g wet weight)
Ala	0.64 ± 0.06
Gly	1.74 ± 0.21
β-Ala	0.07 ± 0.01
Val	0.12 ± 0.02
Leu	0.08 ± 0.01
Ile	0.04 ± 0.01
Thr	0.64 ± 0.05
GABA	2.56 ± 0.21
Asp + Asn	3.08 ± 0.24
Glu + Gln	13.79 ± 0.42
Ser	1.55 ± 0.22
Cit	0.04 ± 0.01
His	0.07 ± 0.01
Lys	0.27 ± 0.22
Tyr	0.07 ± 0.01

*The abbreviations are defined in Table I.
**Values represent means ± standard error of the mean from twelve animals.

TABLE III

FREE AMINO ACID LEVELS IN NORMAL HUMAN URINE

Amino acid*	Urine level** (μmol per 24 h)
Ala	239.19 ± 39.64
Gly	1133.50 ± 134.40
Val	59.25 ± 10.74
Leu	43.26 ± 7.63
Ile	24.12 ± 6.06
Thr	212.33 ± 43.16
Hyp	54.95 ± 11.74
Met	71.35 ± 13.95
Asp + Asn	382.02 ± 83.21
Phe	53.53 ± 9.43
Glu + Gln	381.97 ± 53.69
Ser	441.15 ± 63.62
Cys	56.99 ± 7.18
Cit	15.07 ± 2.50
His	1125.20 ± 213.13
Orn	32.14 ± 6.36
Trp	53.60 ± 9.11
Lys	211.30 ± 38.66
Tyr	88.76 ± 11.91

*The abbreviations are defined in Table I.
**Values represent means ± standard error of the mean from ten subjects.

reproducibility. The results obtained in rat brain and in human urine are given in Tables II and III and a typical chromatogram from a rat brain sample is illustrated in Fig. 2.

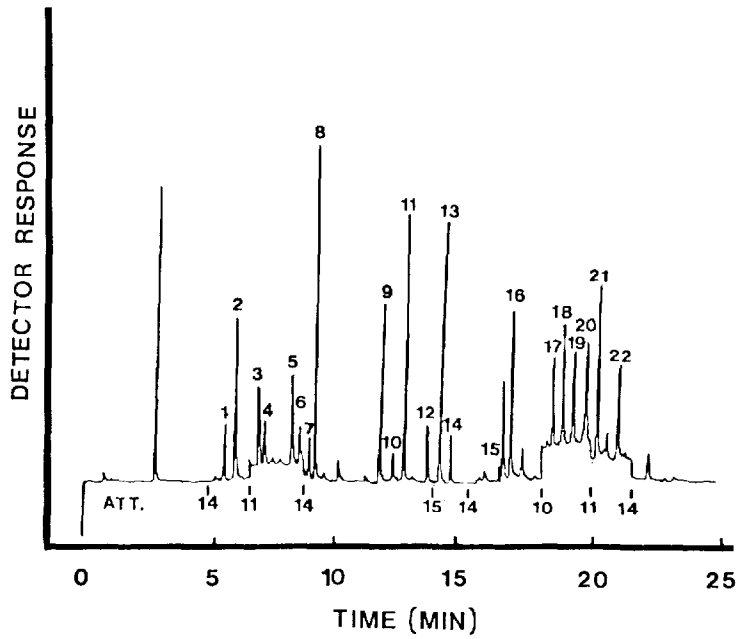


Fig. 2. Typical GC trace of a rat brain extract carried through the extraction and derivatization described in the text. ATT = attenuation. The amino acids are numbered as in Fig. 1.

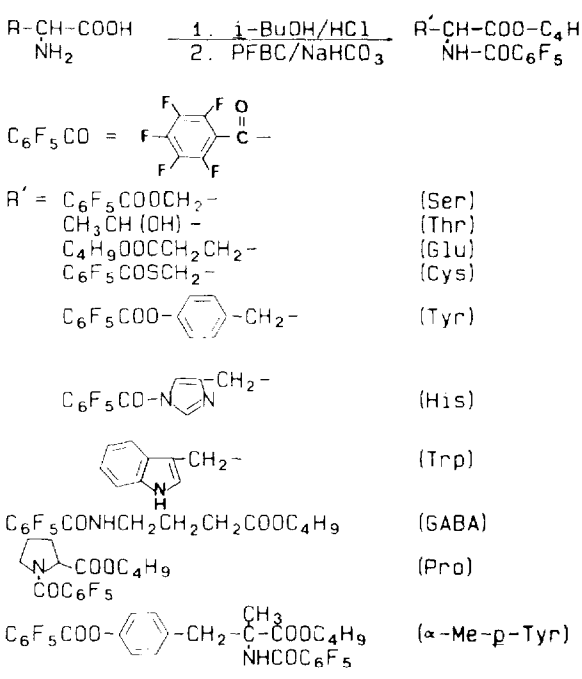


Fig. 3. Structures of derivatives of representative amino acids. The abbreviations are defined in Table I.

In general, the values found by this method are in good agreement with the results reported in the literature [66—69]. However, the procedure appears to be unsuitable for measurement of brain concentrations of hydroxyproline,

methionine, phenylalanine, cysteine, ornithine and tryptophan since levels several times those reported in the literature were obtained. Therefore these amino acids were not included in Table II. The nature of the substances in rat brain which are interfering with analysis of these six amino acids is not known at present.

The stabilities of the derivatives were examined over a period of two weeks at room temperature and four weeks at −20°C. No decomposition was observed with any of the compounds. Thus samples can be injected by the automatic sampler around the clock.

The products corresponding to the peaks attributed to the single amino acids in the chromatogram were characterized by GC—MS after conversion to their pentafluorobenzoyl derivatives. Ten representative isobutyl pentafluorobenzoyl derivatives of amino acids with different reactive substituents in addition to COOH and NH_2 are identified in Fig. 3. The mass spectra of the other twelve derivatized amino acids were also consistent with their structures. All derivatized amino acids contained diagnostic ions of *m/z* M − 101 ($[M - COOC_4H_9]^+$), 195 ($[C_6F_5CO]^+$), 167 ($[C_6F_5]^+$), 57 ($[C_4H_9]^+$) and 41 ($[C_3H_5]^+$). Molecular ions are present except when there is an alcohol (serine and threonine) or a phenol (tyrosine and α-methyl-*p*-tyrosine) function; results are tabulated in Table IV. Other proposed major fragments are illustrated below (Table IV). The identities are given between square brackets and the relative abundances between parentheses.

TABLE IV

MAJOR MASS SPECTRAL FRAGMENTS OF DERIVATIVES OF TEN REPRESENTATIVE AMINO ACIDS

Derivatized amino acid*	Molecular formula	Relative abundance (%)							
		M^{+}**		$[M-101]^{+}$**		*m/z* 195	*m/z* 167	*m/z* 57	*m/z* 41
Pro	$C_{16}H_{16}F_5NO_3$	2	(365)	84	(264)	100	12	5	17
Ser	$C_{21}H_{13}F_{10}NO_5$	—***	(549)	5	(448)	100	16	49	19
Thr	$C_{15}H_{16}F_5NO_4$	—***	(369)	18	(268)	100	22	24	31
Tyr	$C_{27}H_{17}F_{10}NO_5$	—***	(625)	5	(524)	100	13	15	8
α-Me-*p*-Tyr	$C_{28}H_{19}F_{10}NO_5$	—***	(639)	0.1	(538)	100	11	1	2
GABA	$C_{15}H_{16}F_5NO_3$	2	(353)	1	(252)	100	12	24	20
Glu	$C_{20}H_{24}F_5NO_5$	0.2	(453)	11	(352)	100	10	60	35
Cys	$C_{21}H_{13}F_{10}NO_4S$	0.1	(565)	31	(464)	100	43	33	13
His	$C_{24}H_{15}F_{10}N_3O_4$	17	(599)	34	(498)	100	28	35	23
Trp	$C_{22}H_{19}F_5N_2O_3$	11	(454)	3	(353)	9	3	1	1

*The abbreviations are defined in Table I.
**Values in parentheses represent the *m/z* of the fragment.
***Molecular ion not detected.

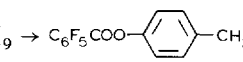
Serine:
$[M - 101]^+ - C_6F_5COOH \rightarrow CH_2{=}C^+{-}NHCOC_6F_5$, *m/z* 236 (52%)
$[M - 101]^+ - C_6F_5CONH_2 \rightarrow {}^+CH{=}CHOCOC_6F_5$, *m/z* 237 (13%)

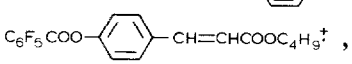
Threonine:
$M^+ - CH_3CHO \rightarrow C_6F_5CONHCH_2COOC_4H_9^+$, *m/z* 325 (9%)
$[325]^+ - COOC_4H_9 \rightarrow CH_2{=}N^+HCOC_6F_5$, *m/z* 224 (13%)
$[325]^+ - (CH_3)_2C{=}CH_2 \rightarrow C_6F_5CONHCH_2COOH^+$, *m/z* 269 (17%)
$[269]^+ - H_2O \rightarrow [C_6F_5(C_3H_2NO_2)]^+$, *m/z* 251 (40%)

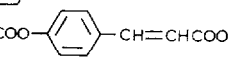
γ-Aminobutyric acid:
$M^+ - OC_4H_9 \rightarrow C_6F_5CONHCH_2CH_2CH_2CO^+$, *m/z* 280 (18%)
$M^{\ddagger} - \dot{C}H_2COOC_4H_9 \rightarrow C_6F_5CONHCH_2CH_2^{\ddagger}$, *m/z* 238 (20%)

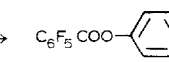
Glutamate:
$M^{\ddagger} - \dot{C}H_2CH_2COOC_4H_9 \rightarrow C_6F_5CONH^+{=}CHCOOC_4H_9$, *m/z* 324 (12%)
$[M - 101]^+ - (CH_3)_2C{=}CH_2 \rightarrow {}^+CH_2CH_2CH(NHCOC_6F_5)COOH$, *m/z* 296 (40%)
$[296]^+ - H_2O \rightarrow [C_6F_5CONH(COC_3H_4)]^+$, *m/z* 278 (38%)
$[324]^+ - C_4H_9OH \rightarrow [C_6F_5CO(C_2HNO)]^+$, *m/z* 250 (16%)

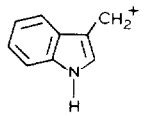
Cysteine:
$M^{\ddagger} - C_6F_5C\dot{O} \rightarrow {}^+SCH_2CH(NHCOC_6F_5)COOC_4H_9$, *m/z* 370 (40%)
$[370]^+ - C_6F_5CONH_2 \rightarrow {}^+SCH{=}CHCOOC_4H_9$, *m/z* 159 (33%)
$[370]^+ - (CH_3)_2C{=}CH_2 \rightarrow {}^+SCH_2CH(NHCOC_6F_5)COOH$, *m/z* 314 (11%)
$[314]^+ - H_2O \rightarrow [C_6F_5CONH(C_3H_2OS)]^+$, *m/z* 296 (23%)

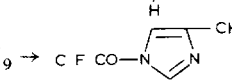
Tyrosine:
$M^{\ddagger} - \dot{C}H(NHCOC_6F_5)COOC_4H_9 \rightarrow$  *m/z* 301 (11%)

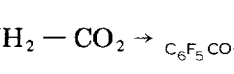
$M^{\ddagger} - C_6F_5CONH_2 \rightarrow$  , *m/z* 414 (17%)

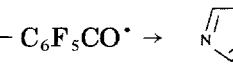
$[414]^{\ddagger} - (CH_3)_2C{=}CH_2 \rightarrow$  *m/z* 358 (26%)

α-Methyl-*p*-tyrosine:
$M^{\ddagger} - CH_3\dot{C}(NHCOC_6F_5)COOC_4H_9 \rightarrow$  , *m/z* 301 (28%)

Tryptophan:
$M^{\ddagger} - \dot{C}H(NHCOC_6F_5)COOC_4H_9 \rightarrow$  , *m/z* 130 (100%)

Histidine:
$M^{\ddagger} - \dot{C}H(NHCOC_6F_5)COOC_4H_9 \rightarrow$  , *m/z* 275 (3%)

$M^{\ddagger} - \begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!C{=}CH_2 - C_6F_5CONH_2 - CO_2 \rightarrow$  , *m/z* 288 (23%)

$M^{\ddagger} - C_6F_5CON{=}CHCOOC_4H_9 - C_6F_5CO^{\cdot} \rightarrow$  , *m/z* 81 (60%)

In summary, a simple, relatively rapid assay employing double derivatization and electron-capture GC has been developed and applied to brain tissue and urine. Disadvantages of the method include the inability to separately analyze glutamate and glutamine and aspartate and asparagine and the failure thus far to separate several of the amino acids from interfering substances in brain tissue. However, the high sensitivity of the derivatives permits analysis to be made on small aliquots of brain homogenates or urine (amino acids in aliquots

equivalent to 10 μl of each are readily measured). The stability of the final derivatives also permits the use of an automatic injector.

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