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USE OF STABLE ISOTOPES AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY IN THE STUDY OF DIFFERENT POOLS OF NEUROTRANSMITTER AMINO ACIDS IN BRAIN SLICES

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SUMMARY

A method was developed for simultaneous determination of endogenous and newly synthesized neurotransmitter amino acids (4-aminobutyric acid, glutamate and aspartate) and glutamine in brain *in vitro*. Brain slices were incubated in artificial cerebrospinal fluid in the presence of ^{13}C-labeled precursors (glucose, pyruvate or acetate). After the incubation, the slices were homogenized in cold 80% ethanol and the supernatants were evaporated to dryness. The resultant residues were derivatized with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide and analyzed by capillary gas chromatography–mass spectrometry in the electron-impact mode. N(O)-*tert.*-Butyldimethylsilyl derivatives of the naturally occurring amino acids, their ^{13}C-enriched counterparts and deuterated internal standards were detected as their $[M-57]^+$ fragments using selected-ion monitoring. The method was shown applicable to studying compartmentation of neurotransmitter amino acids.

INTRODUCTION

The balance between the activity of inhibitory amino acid neurotransmitters [4-aminobutyric acid (GABA)] and excitatory amino acid neurotransmitters [glutamate (Glu) and aspartate (Asp)] may play a crucial role in the pathogenesis and potential treatment of epilepsy[1,2]. These amino acids have several other roles unrelated to neurotransmission; they are involved in energy metabolism, protein metabolism and ammonia metabolism. Total tissue concentrations of neurotransmitters amino acids, therefore, may not be indicators of active neurochemical processes and the importance of compartmentation must be recognized[3–5]. Functional neuronal activity and the accompanying dynamic changes may be better represented by turnovers or concentrations of newly synthesized neurotransmitters[6–9]. Glutamine (Gln) is metabolically and functionally closely related to these amino acids[10–13] and also needs to be considered when studying their dynamic processes. In fact, the interrelationship between these amino acids is sometimes referred to as: the "glutamine cycle", "glutamate–glutamine cycle" or "GABA–glutamine–glutamate cycle".

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Labeled precursors have been used from the outset to study the dynamic metabolic characteristics and compartmentation of these amino acids[14,15]. These initial studies utilized radioactive precursors exclusively. More recently, with the advancements in gas chromatography–mass spectrometry (GC–MS) and derivatization techniques, stable-labeled precursors have also been frequently utilized[9,16,17]. In addition to avoiding the use of radioactivity, stable isotopes in conjunction with GC–MS offer certain advantages. The latter technique allows simultaneous and specific quantitation of labeled and unlabeled species. Furthermore, it provides more specific information on labeling, *e.g.* it allows quantitation of the number of carbons which are labeled, which is important due to recycling of the label. Lastly, it can provide quantitative information on which part of a molecule was labeled, *e.g.* amino *versus* amide nitrogen or which part of the carbon skeleton.

Recently, we have reported a GC–MS method for determination of these amino acids and their stable-isotopic enrichment following the formation of *n*-butyl ester pentafluoropropionyl amide derivatives[18]. While this method offered certain advantages over the existing methods, it required prior separation of dicarboxylic from neutral amino acids because the acid-catalyzed esterification caused deamidation and, therefore, converted Gln to Glu. As a result, two separate GC–MS analyses were needed for each sample. The usefulness of formation of N(O)-*tert.*-butyldimethylsilyl derivatives of physiological compounds, including amino acids, is becoming recognized[19,20]. However, only recently has a method been reported for the simultaneous analysis of intact Glu and Gln and their ^{15}N isotopic enrichment in plasma following the formation of *tert.*-butyldimethylsilyl derivatives[21].

In vitro brain slice preparations are commonly used because they provide intact neural circuitry and allow considerable control of cellular environment without interferences from external influences[22]. Hippocampal slices are possibly the most extensively studied. The hippocampus exhibits high sensitivity *in vitro* as well as *in vivo* to epileptiform seizures[23].

In this study, three different stable-labeled precursors were used: [$^{13}C_6$]D-glucose, [2,3-$^{13}C_2$]sodium pyruvate and [1,2-$^{13}C_2$]sodium acetate. Each of these substrates introduces a $^{13}C_2$ fragment via acetyl coenzyme A into the tricarboxylic acid (TCA) cycle and subsequently the amino acids following the transamination of α-keto acids. Terms "basal" and "new" are used here to denote concentrations of naturally occurring and $^{13}C_2$-enriched amino acids, respectively.

EXPERIMENTAL

Chemicals

Natural isotopes of L-amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). [$^{13}C_6$]D-Glucose (98.9 atom% ^{13}C), [2,3-$^{13}C_2$]sodium pyruvate (99.4 atom% ^{13}C), [1,4-$^{13}C_2$]GABA (99 atom% ^{13}C), [2,2,3,3,4,4-$^{2}H_6$]GABA (99 atom% ^{2}H), [2,3,3,4,4-$^{2}H_5$]L-Glu (99.2 atom% ^{2}H), [2,3,3,4,4-$^{2}H_5$]L-Gln (98.7 atom% ^{2}H) and [α-$^{15}N_1$]L-Gln (99 atom%^{15}N) were obtained from MSD Isotopes (St. Louis, MO, U.S.A.) and [1,2-$^{13}C_2$]sodium acetate (99 atom% ^{13}C) from Cambridge Isotope Labs. (Woburn, MA, U.S.A.).

N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) and derivatization-grade acetonitrile were from Regis (Morton Grove, IL, U.S.A.). Deion-

ized water was obtained using the Milli-Q reagent-grade water system (Millipore, Bedford, MA, U.S.A.). Organic solvents were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Other chemicals were of the best commercially available grade.

Hippocampal slices

Male CR:NGP(S) mice (25–30 g, about six weeks old; Harlan Sprague Dawley, Frederick, MD, U.S.A.) were killed by decapitation and the brains quickly excised and rapidly cooled in ice-cold oxygenated artificial cerebrospinal fluid (ACSF). Both hippocampi were removed and 500-μm transverse slices were cut perpendicular to the hippocampal long axis with a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY, U.S.A.). Two pools, consisting of seven or eight center-cut slices each, were obtained by assigning alternating slices from both hippocampi. Each pool of slices was then allowed to recover for 1 h after transfer to individual incubation chambers containing 15 ml of ACSF maintained at 34.5°C in a shaking incubator set at 48 cycles per min. The normal ACSF was (in m*M*): NaCl 125, KCl 3, KH_2PO_4 1.4, $MgSO_4$ 1.3, $NaHCO_3$ 25, $CaCl_2$ 1.2, and D-glucose 10 at pH 7.4. The chambers consisted of two nesting 30-ml polypropylene beakers with the bottom of inner beaker replaced with nylon mesh. A polyethylene tube to deliver O_2–CO_2 (95:5) was taped to the side of the inner beaker approximately midway into the ACSF. The inner chamber, containing the slices, was then capped with Parafilm. This arrangement allowed rapid replacement of media or any pharmacologic manipulations with minimal disruption of the slices since the outer beakers containing the experimental compounds could be rapidly exchanged. The equilibration period was followed by a 30-min experimental incubation period with different stable-labeled precursors. The slices were collected for analyses by transfer to nylon mesh using a modified Pasteur pipette. This allowed rapid transfer of the slices with a chilled spatula into a cold 15-ml polypropylene test tube (dry ice–ethanol). The tubes were stored at −20°C until analysis. After the slices were collected, the osmolarity and pH of the ACSF remaining in each incubation chamber were checked to assure constant experimental conditions.

Analysis of amino acids

Frozen hippocampal slices were homogenized in 0.8 ml of cold 80% aqueous ethanol using a Polytron homogenizer (Brinkmann Instruments). After centrifugation (10 000 *g*, 20 min, 4°C), the supernatant was transferred to clean tubes and stored in the refrigerator. For the analysis, 150 μl of the supernatant were combined with 20 μl of 1 *M* ammonium formate (pH 4.5) and 50 μl of the aqueous internal standard solution. For the standard curve, 100 μl of 80% ethanol was combined with 20 μl of 1 *M* ammonium formate (pH 4.5), standards solution (GABA, Asp, Glu and Gln) and 50 μl of the internal standard solution. The samples were evaporated at room temperature using a Savant SpeedVac (Savant Instruments, Farmingdale, NY, U.S.A.). The residue was derivatized with 200 μl of acetonitrile–MTBSTFA (3:1, v/v) for 30 min at 80°C. The derivatives were analyzed in an electron-impact mode using a Model 5987A gas chromatograph–mass spectrometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with a Model 5880 gas chromatograph and 1000E data system. A 10 m × 0.18 mm, 0.4-μm cross-linked and bonded 5% phenylmethyl DB-5 fused-

silica capillary column (J & W Scientific, Folsom, CA, U.S.A.) was inserted directly into the source. Helium was used as a carrier gas with a back pressure of 15 p.s.i. The injector was set for 1:15 split ratio. The oven temperature profile was as follows: hold 0.5 min at 50°C, program 20°C/min to 180°C, program 18°C/min to 270°C and hold. Total run time was 12 min. Injector, transfer line and source temperatures were 250, 260 and 200°C, respectively. The filament current was 300 μA and the electron energy 70 eV. Prominent and characteristic $[M-57]^+$ ions were monitored for GABA (*m/z* 274), Asp (*m/z* 418), Glu (*m/z* 432) and Gln (*m/z* 431) and their M+1 and M+2 isotopes and the deuterated internal standards with a dwell time of 25 ms. [$^2H_5$]Glu was used as the internal standard for both Glu and Asp. Peak areas were used for quantitation. Concentrations of the endogenous amino acids were determined based on the corresponding standard curves. Stable-isotopic enrichment was calculated according to Dunstan[24] whereby first-order corrections (for natural abundance) and second-order corrections (for the incorporation of an isotope) are made. This is important under the experimental conditions where some recycling of the label takes place. In our case, relative abundances of (M+2)-labeled molecule ($^{13}C_2$) are corrected for the contributions from the natural abundance of the M+2 isotope plus a contribution from M+1 isotope of the (M+1)-labeled molecule ($^{13}C_1$). Concentrations of the $^{13}C_2$-labeled (newly synthesized) amino acids were based on the concentration of the endogenous amino acids and the corresponding corrected relative abundances.

Standard curve and hippocampal slice samples were spiked with [$\alpha^{15}H_1$]L-Gln to determine if deamidation of Gln to Glu was occurring during the analysis. Under the experimental conditions, the deamidation was not detectable.

Protein determinations

Protein concentrations were determined in homogenates of hippocampal slices using the BCA Protein Assay (Pierce, Rockford, IL, U.S.A.) and protein standard solution (containing 5 g of human albumin and 3 g of human globulin) from Sigma.

RESULTS AND DISCUSSION

The use of stable isotopes and GC–MS allows determinations of basal and newly synthesized amino acids. This approach may be useful to study the dynamics of different pools of amino acids.

Representative selected-ion chromatograms for GABA, Asp, Glu and Gln and their M+2 isotopes are shown in Fig. 1. These chromatograms are a result of an injection of 1% of the derivatized sample. Each hippocampal slice sample contained about 1 mg of protein and about 0.2 mg of protein was used for each analysis. The analysis is flexible in that several parameters (sample size used, volume of injection, split ratio of the injector and the volume of the derivatization reagent) can be altered to suit specific needs.

Representative standard curve data are summarized in Table I. Linear least-squares regression analysis showed good linearity and a reasonable *y*-intercept for all amino acids. Good accuracy and precision were shown for endogenous amino acids (Table II).

A good correspondence between theoretical and measured relative abundances

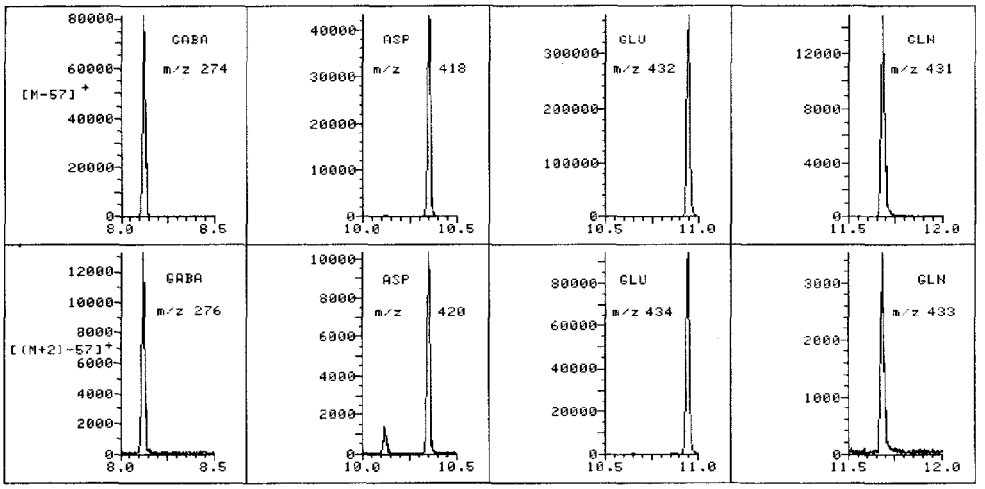

Fig. 1. Selected-ion chromatograms of amino acids in hippocampal slices (corresponding to 0.195 mg protein) incubated 30 min with 0.1 m*M* [$^{13}C_2$]pyruvate. The [M − 57]$^+$ fragments (top tracings) and their M + 2 isotopes (bottom tracings) are shown for GABA, Asp, Glu and Gln. The sample contained (per 100 mg of protein): 1.01 μmol GABA, 1.45 μmol Asp, 7.02 μmol Glu, 0.57 μmol Gln, 64.9 nmol [$^{13}C_2$]GABA, 82.5 nmol [$^{13}C_2$]Asp, 540.6 nmol [$^{13}C_2$]Glu and 25.6 nmol [$^{13}C_2$]Gln.

of M + 2 isotopes is evident in Table III. Validation data for the determination of isotopically enriched amino acids are presented for GABA (Table IV). A good agreement between added and measured [$^{13}C_2$]GABA was found in the range of values found experimentally.

The idea of "metabolic compartmentation" of Glu and related amino acids dates back some thirty years and, subsequently, on the basis of kinetic data, it has been proposed that the mammalian brain contains at least two pools of Glu (for review see refs. 13 and 25). Glucose and pyruvate are preferential precursors of the large pool thought to be neuronal and a site of synthesis of neurotransmitter Glu, GABA and Asp. On the other hand, acetate preferentially labels the small pool which is thought to be glial and a site of Gln synthesis. Consistent with this, the ratio of

TABLE I

STANDARD CURVE DATA FOR UNLABELED AMINO ACIDS

Ethanolic solution was spiked with varying amounts of unlabeled reference amino acids and constant amounts of the internal standards (I.S.) and these samples were used to generate standard curves. Linear least-squares analysis was used to define standard curves. $n = 6$.

Amino acid	*Range (ng per sample)*	*Correlation (r^2)*	*Slope*	*Intercept*	*I.S.*	*Amount of I.S. (ng)*
GABA	100– 600	0.9998	0.00214	0.0036	[$^2H_6$]GABA	500
Asp	100– 600	0.9954	0.000488	−0.001	[$^2H_5$]Glu	1500
Glu	400–2400	0.9991	0.000746	−0.0076	[$^2H_5$]Glu	1500
Gln	100– 600	0.9997	0.003992	0.0188	[$^2H_5$]Gln	250

TABLE II

ACCURACY AND PRECISION DATA FOR UNLABELED AMINO ACIDS

Ethanolic solutions were spiked with known amounts of unlabeled reference amino acids and constant amount of the internal standards, and these samples were treated as unknowns. Concentrations of amino acids were determined based on the standard curves generated as described in Table I.

Amino acid	*Added (ng)*	*Measured (mean ± S.D., n = 8) (ng)*
GABA	150.0	151.5 ± 1.1
	550.0	549.5 ± 3.9
Asp	150.0	148.2 ± 2.8
	550.0	552.0 ± 18.5
Glu	600.0	598.0 ± 19.6
	2200.0	2198.4 ± 32.3
Glu	150.0	149.8 ± 4.1
	550.0	546.4 ± 7.9

TABLE III

RELATIVE ABUNDANCES OF M+2 ISOTOPES

Theoretical values were calculated according to Dunstan[24]. Experimental values were determined from (a) standard curve samples containing unlabeled reference amino acids and (b) hippocampal slices incubated in the absence of labeled precursors.

Amino acid	*Relative abundance (%)*		
	Theoretical	*Experimental (mean ± S.D., n = 6)*	
		a	*b*
GABA	9.52	9.58 ± 0.09	9.56 ± 0.12
Asp	16.32	16.52 ± 0.33	16.60 ± 0.11
Glu	16.65	16.69 ± 0.17	16.72 ± 0.27
Gln	16.45	16.76 ± 0.38	17.02 ± 0.46

TABLE IV

ACCURACY AND PRECISION DATA FOR LABELED GABA

Ethanolic solutions were spiked with unlabeled reference amino acids (including 250 ng of GABA), constant amount of the internal standards and known amounts of [$^{13}C_2$]GABA. The amounts of [$^{13}C_2$]GABA present in these samples were calculated as described in the Experimental section.

Added (ng)	*Measured (mean ± S.D., n = 8) (ng)*
5.0	5.1 ± 0.29
10.0	9.7 ± 0.26
20.0	19.2 ± 0.45

TABLE V

EFFECT OF PRECURSOR ON NEWLY SYNTHESIZED Glu/Gln

Hippocampal slices were incubated for 30 min in the presence of a labeled precursor. The ACSF contained 9 m*M* D-glucose + 1 m*M* [$^{13}C_6$]D-glucose, 10 m*M* D-glucose + 0.1 m*M* [$^{13}C_2$]pyruvate or 10 m*M* D-glucose + 0.5 m*M* [$^{13}C_2$]acetate.

Precursor	*Glu/Gln ratio (mean ± S.D., n = 6)*
[$^{13}C_6$]Glucose	19.1 ± 3.4
[$^{13}C_2$]Pyruvate	23.1 ± 4.3
[$^{13}C_2$]Acetate	2.6 ± 0.3

TABLE VI

EFFECT OF POTASSIUM ON BASAL AND NEW AMINO ACIDS

Concentrations of basal and new amino acids are reported in μmol per 100 mg of protein and nmol per 100 mg of protein, respectively. The ACSF contained 10 m*M* D-glucose + 0.5 m*M* [$^{13}C_2$]acetate and the incubation time was 30 min. Each value represents a mean (± S.D.) for six samples.

K^+ (*mM*)	*GABA*		*Asp*		*Glu*		*Gln*	
	Basal	*New*	*Basal*	*New*	*Basal*	*New*	*Basal*	*New*
4.4	1.17	39.6	1.32	30.4	6.67	285.0	0.37	109.0
	(0.06)	(6.9)	(0.26)	(9.2)	(0.36)	(35.5)	(0.04)	(10.3)
51.4	1.36[a]	11.2[a]	0.20[a]	4.6[a]	6.64	369.7[a]	1.16[a]	64.2[a]
	(0.12)	(2.9)	(0.06)	(2.9)	(0.42)	(44.4)	(0.10)	(9.8)

[a] Significantly different from matched CONTROL (4.4 m*M* K^+) by paired two-tailed Student's *t*-test, $p < 0.05$.

newly synthesized Glu/Gln was about 20 when labeled glucose or pyruvate was used and about 2 for labeled acetate (Table V). In addition, consistent with the presence of different pools, changes in the cellular environment can have different effects on basal and new amino acids. This can be seen in Table VI with high potassium, a commonly used depolarizing agent. For example, high potassium increased basal and decreased new GABA and did not affect basal but increased new Glu.

In summary, a relatively simple GC–MS method has been described for the simultaneous quantitation of neurotransmitter amino acids and their isotopically enriched counterparts following incubation of brain slices in the presence of stable-labeled precursors. The analysis requires no extraction, uses very small amounts of tissue and allows simultaneous determination of Glu and Gln. It can be easily modified for quantitation of other amino acids, as we have done for alanine, glycine and taurine (data not shown). The method is suitable for studying different pools of neurotransmitter amino acids and their dynamics.

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