Amino Acids (2007) 32: 203–212
DOI 10.1007/s00726-006-0370-6
Printed in The Netherlands

Amino Acids

Quantification of free amino acids and dipeptides using isotope dilution liquid chromatography and electrospray ionization tandem mass spectrometry

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Received January 20, 2006
Accepted May 3, 2006
Published online October 10, 2006;

Summary. Our aim was to develop a liquid chromatography and electrospray ionization tandem mass spectrometry ($LCMS^2$) method to measure free amino acid (FAA) and dipeptide (DP) concentrations in biological fluids. We synthesized chloroformate derivatives of FAA and DP, identified the major precursor ions and used $LCMS^2$ to obtain the most intense product ions. Using serial dilutions of unlabeled and labeled standards ($[^2H_3]$-L-Dopa, homoarginine, homophenylalanine, $[^{15}N]$-Glutamine and $[^2H_3]$-methionine), we observed linear relationships in MS response that we used to calculate the amounts of FAA and DP in biological samples. This method is sensitive with a limit of detection (LOD) for most of the FAAs and DPs tested in the 0.05–1 pmol range and is linear over 3–5 orders of magnitude when many metabolites were measured simultaneously. Reproducibility and between run or daily variations were <10% for most FAAs and DPs. We applied this method to human samples and quantitatively measured 21 FAAs and 2 DPs in 200 µl CSF, 31 FAAs and 6 DPs in 100 µl plasma, and 23 FAAs and 5 DPs in 200 µl urine. These data demonstrate the potential for using $LCMS^2$ to discover changes in FAA and DP metabolic pathways that occur during disease pathogenesis.

Keywords: Amino acids – Dipeptides – Liquid chromatography – Tandem mass spectrometry – Cerebrospinal fluid – Plasma – Urine

Abbreviations: $LCMS^2$, Liquid chromatography and electrospray ionization tandem mass spectrometry; SRM, selected reaction monitoring; FAA, free amino acid; DP, dipeptide; GABA, γ-aminobutyric acid; βABA, β-aminobutyric acid; ABA, α-aminobutyric acid; DABA, 2,4-diaminobutyric acid; Dopa, 3,4-dihydroxyphenyl-L-alanine

Introduction

Amino acids, the building blocks of proteins, are molecules with both ammonium and carboxylate groups separated by –CHR where R is a unique group specific for different amino acids (Liu et al., 2002; Tipton et al., 1999). FAAs and DPs also serve as signaling molecules (Greengard, 2001) and have antioxidant and buffering properties (Kang et al., 2002; Babizhayev et al., 1994; Boldyrev, 2001; Gallant et al., 2000; Liu et al., 2003). These FAA and DP functions make their metabolism an important consideration in the pathophysiology of many diseases including those of the brain (Thomas, 1995; Zauner and Bullock, 1995). Thus, measurement of FAAs and DPs in physiological fluids may provide fundamental information about diseased conditions.

There are 20 major coded amino acids in humans. The primary source of these amino acids for endogenous protein synthesis is from the diet. However, mammals cannot synthesize 8–10 important amino acids and must obtain these essential amino acids from their diet. Proteolysis in the gastrointestinal tract generates FAAs absorbed by the jejunum and released into the blood for general circulation. The liver and other tissues take up FAA from the plasma pool for the synthesis of plasma and intracellular proteins. Alternatively, FAAs are used for other physiologic functions. For instance, transamination and deamination reactions interconvert amino acids to secondary metabolites or for energy generation (Bixel et al., 2001).

Of the primary amino acids, several excitatory and inhibitory FAAs have been widely studied in the CNS (Baker et al., 1993; Bullock, 1994; Hattori and Wasterlain, 1990; Headley and Grillner, 1990; Elekes et al., 1986). The major excitatory amino acid is glutamate while the major inhibitory amino acid is glycine. Biosynthetic pathways produce other amino acids or derivatives that are also signaling molecules or have physiological properties. For example, tyrosine is converted to L-Dopa and to dopamine by the consecutive activities of tyrosine hydrolase and aromatic amino acid decarboxylase (Milner and

Wurtman, 1986). DPs are also important in brain function. Various DPs such as carnosine, anserine and taurine are derived from the diet or are synthesized by the brain and muscle cells (Bakardjiev and Bauer, 2000; Hoffmann et al., 1996).

The aim of these studies was to develop and use $LCMS^2$ to measure concentrations of FAA and DPs. Our data show that 75 different FAAs and DPs can be simultaneously quantified using $LCMS^2$. This approach is sensitive and specific and should be useful in elucidating changes in FAA and DP biochemical pathways that occur in diseases.

Materials and methods

Materials

Ammonium formate, ultrapure HPLC grade water and methanol were bought from VWR (West Chester, PA). The EZ-Faast amino acid analyses kit containing sorbent extraction tips, extraction solutions, amino acid standards, derivatization reagents and C18 liquid chromatography column, was bought from Phenomenex (Torrance, CA). Bovine serum albumin and FAA standards including the internal standard ($[^2H_3]$-L-Dopa, $[^{15}N]$-glutamine) not provided in the EZ-Faast kit were bought from Sigma (St Louis, MO). Protein dye reagent concentrate was from BioRad Laboratories (Hercules, CA).

Recruitment and diagnosis

Study participants who gave informed consent were recruited prospectively from the North Los Angeles area for our IRB-approved research. These subjects had no classifiable brain disorder after complete neurological and psychiatric evaluation. Comorbid conditions such as hypertension were recorded but were not a reason for exclusion.

Sample collection

We collected CSF by lumbar punctures performed by a neurologist between 1 and 5 pm, either in the lateral or sitting positions, using a 22 gauge Quincke type needle, between either the L3/4 or L2/3 positions. CSF was drip collected in three consecutive fractions (~7 ml each), centrifuged at $3000 \times g$ to pellet cells, fractionated into 1.0 ml aliquots and stored at $-80\,^{\circ}C$.

Whole blood (~10 ml) was collected by venipuncture into anticoagulant (K_3 EDTA vacutainers, Becton Dickinson) and centrifuged at $3000 \times g$ to separate plasma from blood cells. Plasma fractions (0.5 ml) were stored at $-80\,^{\circ}C$.

Approximately 30 ml of midstream urine was collected during the same visit as CSF and plasma collections. Urine samples were centrifuged at $3000 \times g$ to remove particulates, fractionated into 5 ml aliquots and stored at $-80\,^{\circ}C$ until needed for analysis.

Extraction and derivatization of FAA

The procedure consisted of a solid phase extraction step, followed by a derivatization procedure using the EZ-Faast amino acid analyses kit from Phenomenex. Briefly, internal standards were added to plasma (100 µl), CSF (200 µl) or urine (200 µl) and FAAs and DPs were extracted using Sorbent Tips and reagents from the EZ-Faast kit following the instructions of the kit's manufacturer (Phenomenex). Extracted FAAs and DPs were converted to chloroformates (Husek, 1998) and analyzed by LC tandem MS as described below.

$LCMS^2$ of amino acids

Precursor ions of FAAs and DPs were obtained using a full scan MS infusion experiment. Tandem MS was used to obtain the most intense product ion from each FAA and DP. Selected reaction monitoring (SRM) parameters (collision energy and tube lens voltage) were optimized for each FAA and DP. FAAs (10 µl) were then separated using a C18 Luna HPLC column (2×250 mm, maintained at $45\,^{\circ}C$) on an HP1100 liquid chromatography system (Agilent, Palo Alto, CA). FAAs were eluted from the column using a linear methanol gradient starting with water:methanol (32/68 v/v) containing 10 mM ammonium formate at 0.2 ml/min over 10 min. The total run time including column equilibration was 35.5 min. The HPLC eluent was directly interphased to the electrospray ionization (ESI) source of a triple quadrupole mass spectrometer (TSQ Quantum from Thermo Electron, San Jose, CA) operated at a spray voltage of 4500 V, sheath Gas pressure of 30 units, auxiliary gas pressure of 0, capillary temperature of $300\,^{\circ}C$ and collision pressure of 1.5 units. Positive ions were acquired in the profile mode with 7 different scan events using SRM after collision-induced dissociation (10 V) of protonated or ammonium precursor ions. All SRM transition peak intensities were integrated, processed and mole quantities determined using ICIS and Xcalibur software, respectively (Thermo Electron). Mole quantities were determined from standard curves obtained using known amounts of FAA and DP (0–100 pmol) standards and 100 pmol internal standards ($[^2H_3]$-L-Dopa, $[^2H_3]$-methionine, $[^{15}N]$-glutamine, homoarginine, homophenylalanine). The ratio of FAA or DP peak area to IS peak area was obtained and a linear equation relating mole amounts of FAA to this ratio was obtained. This linear equation was then used to calculate the mole quantities of each FAA or DP in CSF, plasma and urine.

Data analyses

Statview (Abacus Concepts, Berkeley, CA) was used to calculate mean concentrations and LOD values, determine linear equations and correlation (r^2) of standard curves of FAAs and DPs.

Results

Detection of FAAs and DPs by $LCMS^2$

Before levels of FAAs and DPs could be measured in samples, it was necessary to develop a sensitive and specific assay to determine the mole values of these metabolites. We used LC-positive ion-electrospray ionization tandem MS with SRM for these studies. Chloroformate derivatives were obtained for each FAA and DP and precursor ions were identified. MS^2 was performed to obtain the most intense product ions. A precursor ion-product ion transition optimization was performed for each metabolite (Table 1). As shown in Table 1, each FAA and DP displayed a distinct precursor ion-product ion transition that made it possible to selectively detect each of them in our samples. For FAAs with the same parent ion m/z such as 1-methyl-histidine and 3-methyl-histidine, resolution by LC or detection of different optimized product masses allowed accurate quantification of these amino acids.

Table 1. Precursor ions, product ions, collision energy and parameters used for MS optimization

Amino acids and dipeptides	Parent ion (m/z)	Product ion (m/z)	Collision energy (V)	Tube lens offset (V)
Ethanolamine	148.00	106.00	47	68
Pyroglutamic acid	172.00	130.00	10	165
Serine	234.00	174.00	10	101
Glutamine	275.00	172.00	18	103
Arginine	303.00	200.00	24	98
Citrulline	304.00	244.00	10	98
Homophenylalanine	308.00	117.10	42	111
Homoarginine	317.00	170.00	28	107
Phosphothreonine	325.00	117.00	60	65
Anserine	369.00	212.00	37	103
Acetylcholine	148.08	87.07	18	61
Glycine	204.00	118.00	47	63
Asparagine	243.00	201.00	22	102
Threonine	248.00	160.00	18	133
4-Hydroxyproline	260.00	172.00	18	88
3-Methyl-histidine	298.00	210.00	26	100
1-Methyl-histidine	298.00	256.00	27	100
Glycine-proline	301.00	158.00	20	110
β-Alanine	218.00	116.00	22	73
Alanine	218.00	130.00	21	73
4-Aminobutyric acid	232.00	130.00	17	103
α-Aminobutyric acid	232.00	144.00	10	103
β-Aminobutyric acid	232.00	172.00	10	103
Histamine	284.00	138.00	25	89
2,4-Diaminobutyric acid	333.00	273.00	10	103
Ornithine	347.00	201.00	22	97
Proline-hydroxyproline	357.00	156.00	21	127
Proline	244.00	156.00	15	100
Methionine	278.00	190.00	27	90
[$^2H_3$]-Methionine	281.00	193.00	10	75
Aspartic acid	304.00	216.00	21	108
Phosphothreonine	325.20	117.00	57	60
Lysine	361.00	170.00	25	119
Histidine	370.00	196.00	28	93
Lysine-alanine	432.00	170.00	36	120
Carnosine	441.00	284.00	26	92
Homoserine	230.00	188.00	10	140
Valine	246.00	156.00	18	97
Thioproline	262.00	174.00	17	109
Ethionine	292.00	204.00	10	85
Glutamic acid	318.00	172.00	27	91
α-Aminoadipic acid	332.00	244.00	19	107
Tryptophan	333.00	230.00	20	75
γ-Glutamyl-ε-lysine	532.00	414.00	17	91
γ-Glutamyl-ε-lysine	532.30	84.08	53	121
Isoleucine	260.00	130.00	26	81
Leucine	260.00	172.00	18	81
Allo-isoleucine/ norleucine	260.00	200.00	17	86
Phenylalanine	294.00	106.00	10	52
Cysteine	336.00	190.00	37	71
Aminopimelic acid	346.00	156.00	27	85
Adrenaline	424.00	252.00	21	148
Noradrenaline	445.48	107.02	53	95
4-Aminobenzoic acid	266.00	224.00	21	89
Homophenylalanine	308.00	117.00	38	102
Homocysteine	350.00	204.00	18	92
Tyrosine	396.00	294.00	10	74
Dopamine	412.00	266.00	19	82
Cystathionine	479.00	230.00	21	131
Cystine	497.00	248.00	19	103
Homocystine	525.00	262.00	21	97
3-Nitro-L-tyrosine	534.23	94.01	53	94
Urea	117.00	72.00	17	57
Sarcosine	218.15	88.08	22	82
Phosphoserine	317.21	84.10	42	94
[^{15}N]-Glutamic acid	319.17	85.06	37	101
Noradrenaline	445.24	152.03	38	85
Phosphotyrosine	498.00	412.00	26	99
L-Phenylalanine	294.18	120.06	37	89
Melatonin	326.00	94.00	41	65
Serotonin	349.16	160.04	38	93
3-Hydroxytyramine	429.25	137.11	38	75
3-Chloro-L-tyrosine	447.24	170.01	37	102
L-Dopa	515.29	178.02	42	98
[$^2H_3$]-L-Dopa	518.29	180.02	47	119
2,4,5-Trihydroxy-DL-phenylalanine	600.32	149.93	42	107

We converted FAA and DP standards to chloroformate derivatives. Upon infusion into a mass spectrometer, we identified the major protonated or ammonium precursor ions and used tandem MS to determine 2–4 major product ions. The most intense product ions were used for SRM studies by optimizing the precursor-product ion transitions with respect to the collision energy and the tube lens voltage

Quantification of FAA and DPs

Serial dilutions of authentic FAA and DP standards were made with a fixed amount (100 pmol) of five IS. An example of the total ion current (TIC) of authentic FAAs or DPs and extracted SRM of 5 internal standards is shown in Fig. 1A–F. Freshly made stock solutions of FAAs and DPs yielded one major ion while after storage, some standards gave multiple peaks, likely because of oxidation or degradation of the amino acids. The relative retention time of all standards deviated $<2.5\%$ relative to the expected retention time or when compared to internal standards for experiments using the same solvent composition and LC gradient. The relationship between the concentrations of each FAA, DP and IS ([$^2H_3$]-L-Dopa, homoarginine, homophenylalanine) were linear for most FAAs and DPs, while standard curves using [^{15}N]-Glutamine and [$^2H_3$]-methionine were linear only for their respective unlabeled amino acids. For most standard curves using [$^2H_3$]-L-Dopa as an IS, the correlation of mole quantities to peak area ratios was linear ($R^2 > 0.85$) (Fig. 1G,

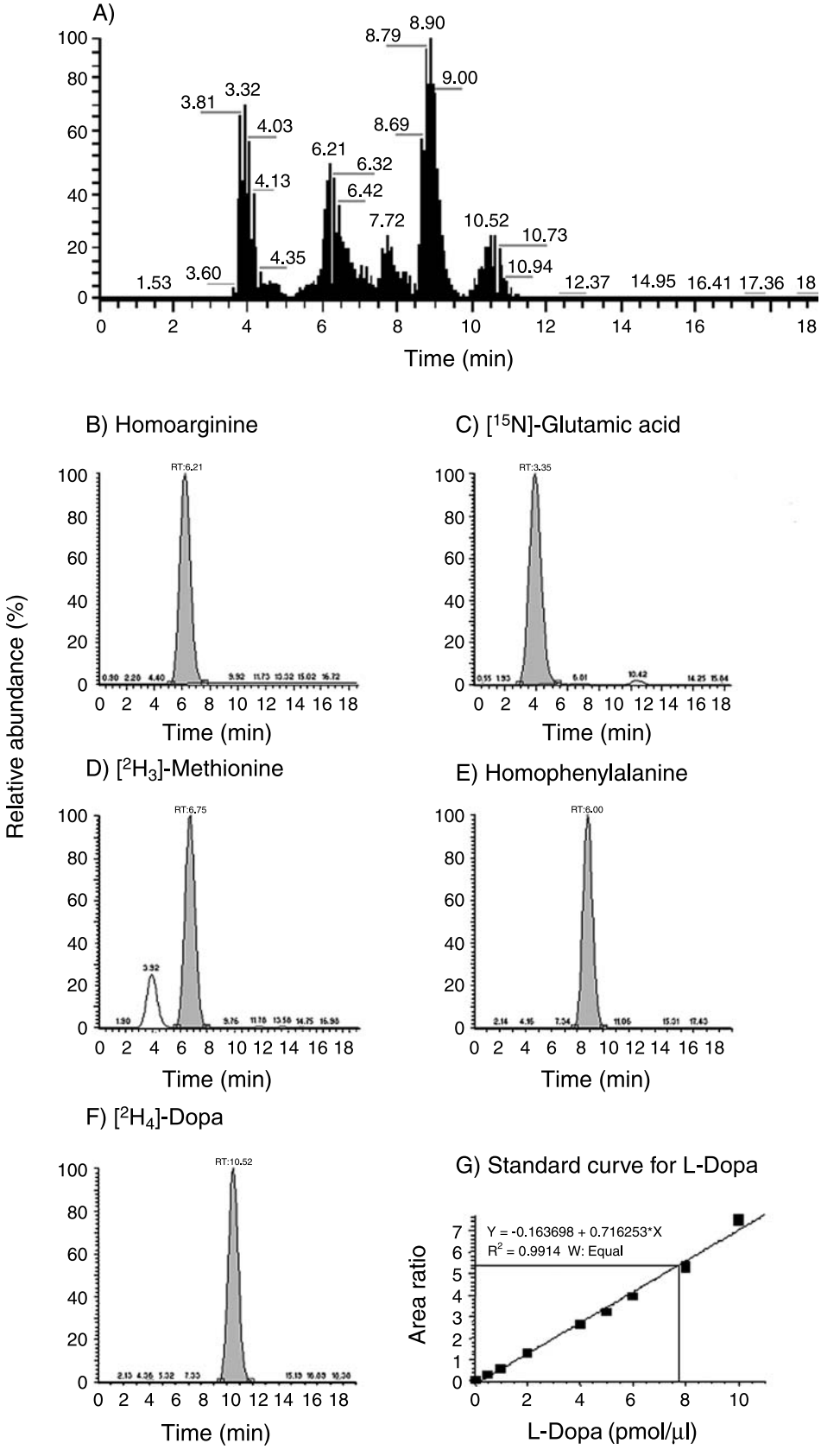

Fig. 1. LCMS^2 of FAA and DP standards. We used LCMS^2 to obtain the TIC of FAA and DP standards (**A**), the extracted SRM chromatograph of five internal standards (**B**–**F**) and the standard curve for L-Dopa using [$^2H_4$]-Dopa as an internal standard (**G**). Ten microliter of standard mixture containing 5 pmol/µl or serially diluted L-Dopa was injected in these studies. Multiple injections on the same day or repeated several days apart gave similar chromatograph and ion spectra

Table 2. Quantification of amino acids and dipeptides using [$^2H_3$]-Dopa as an internal standard

Amino acids and dipeptides	Correlation (r^2)	Deviation (% mean)	LOD (pmol)
Ethanolamine	0.39	0.81	ND
Pyroglutamic acid	0.88	15.74	0.5
Serine	0.94	2.21	0.05*
Glutamine	0.93	3.84	0.05*
Arginine	0.92	4.21	0.2
Citrulline	0.93	3.99	0.05
Homophenylalanine	IS	IS	IS
Homoarginine	IS	IS	IS
Anserine	0.97	2.16	0.2
Acetylcholine	0.82	17.22	0.6
Glycine	0.92	5.28	0.05
Asparagine	0.73	7.68	0.2
Threonine	0.95	4.03	0.4
4-Hydroxyproline	0.85	7.80	0.5***
3-Methyl-histidine	0.96	1.88	0.05*
1-Methyl-histidine	0.96	2.16	0.05*
Glycine-proline	0.89	8.21	0.05***
β-Alanine	0.22	66.40	ND
Alanine	0.93	2.05	0.5
4-Aminobutyric acid	0.71	13.50	0.5
α-Aminobutyric acid	0.72	22.12	1
β-Aminobutyric acid	0.65	26.94	0.5
Histamine	0.75	9.91	0.5
2,4-Diaminobutyric acid	0.85	12.06	0.4
Ornithine	0.82	9.087	0.5
Proline-hydroxyproline	0.94	2.26	0.05***
Proline	0.96	2.76	0.2
Methionine	0.66	32.44	ND
[$^2H_3$]-Methionine	IS	IS	IS
Aspartic acid	0.95	3.08	0.2
Lysine	0.85	11.73	2
Histidine	0.88	0.65	0.5**
Lysine-alanine	0.96	1.72	0.05**
Carnosine	0.93	5.95	0.8
Homoserine	0.88	14.32	0.05
Valine	0.95	1.28	0.05
Thioproline	0.96	1.24	0.05
Ethionine	0.96	0.83	0.05
Glutamic acid	0.94	1.44	0.05**
α-Aminoadipic acid	0.94	0.74	0.05**
Tryptophan	0.96	1.96	0.05**
γ-Glutamyl-ε-lysine	0.93	1.06	0.1
Isoleucine	0.94	0.55	0.05*
Leucine	0.94	0.73	0.05***
Norleucine	0.93	2.29	0.4
Phenylalanine	0.94	0.33	0.05*
Cysteine	0.93	4.87	0.05*
Aminopimelic acid	0.94	1.58	0.05*
Adrenaline	0.95	2.27	0.05*
Noradrenaline	0.94	5.12	0.1
4-Aminobenzoic acid	0.96	3.30	0.5
Homophenylalanine	IS	IS	IS
Homocysteine	0.97	2.22	0.5
Tyrosine	0.96	0.77	0.1*
Dopamine	0.96	2.14	0.1*
Cystathionine	0.94	0.84	0.05*
Cystine	0.95	1.81	0.05
Homocystine	0.91	1.91	0.05**
3-Nitro-L-tyrosine	0.89	5.60	0.1
Sarcosine	0.74	15.63	1
[^{15}N]-Glutamic acid	IS	IS	IS
Phosphotyrosine	0.92	4.30	0.05*
L-Phenylalanine	0.94	0.89	0.05***
Melatonin	0.86	5.95	0.05
Serotonin	0.93	4.25	0.05***
3-Hydroxytyramine	0.91	3.19	0.05***
3-Chloro-L-tyrosine	0.97	0.74	0.05***
L-Dopa	0.97	2.96	0.2
[$^2H_3$]-L-Dopa	IS	IS	IS
2,4,5-Trihydroxy-DL-phenylalanine	0.87	6.66	0.2

Serial dilutions (0–100 pmol) of amino acids and dipeptides and 100 pmol internal standards were prepared and subjected to LCMS^2. Peak areas were obtained and the ratios of peak areas of each FAA or DP compared to that of internal standards. A plot of the amount of each FAA or DP against the peak area ratio (peak area FAA or DP/[$^2H_3$]-L-Dopa) was obtained and the linear correlation obtained for four separate experiments. The mean deviation was then calculated. For limit of detection (LOD) studies, compounds were diluted (0–1000 fmol) and tandem MS obtained. Signal to noise ratio (S/N) was obtained for each FAA and DP. The concentration that gave peak intensity that was >2 fold above the noise (signal of compound divided by signal of background) was recorded as the LOD for that particular compound

IS Internal standard; *ND* not determined

* S/N ratio >5; ** S/N ratio >10; *** S/N ratio >25

Table 2). Injection of multiple standards yielded a percentage difference <10% between expected and calculated concentrations for most FAAs and DPs. The limit of detection (LOD) obtained by serial dilution of each standard FAA and DP was in the low pmol level when multiple samples were monitored. This method is sensitive with a LOD for most FAAs and DPs in the 0.05–1 pmol range (Table 2). The dynamic range (0.01–100 pmol) is 3–5 orders of magnitude for the different metabolites.

FAA and DP concentrations in CSF, plasma and urine

After developing a sensitive method for FAA and DP detection, we applied it to human samples (CSF, plasma and urine). FAAs and DPs in CSF reflect their brain metabolism. Levels of FAAs and DPs in plasma indicate uptake, removal or release en route to the brain and other organs, while the levels in urine represent the removal pathway for FAA metabolites. Using LCMS^2 and the isotope dilution strategy, the TIC (Fig. 2A) and several important FAAs were identified in human CSF (Fig. 2). Similarly, examples of several FAAs and DPs detected in

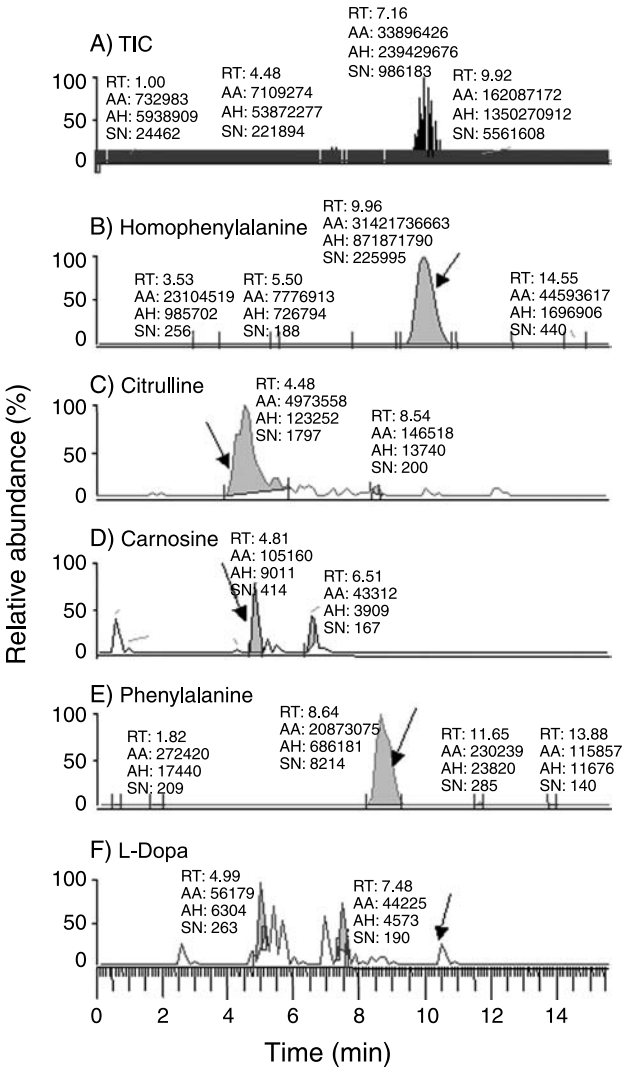

Fig. 2. Detection of important FAAs in CSF using LCMS^2. TIC of amino acids and DP in CSF (**A**) obtained by LCMS^2 in the SRM mode. Spectra of homophenaylalanine internal standard (**B**), citrulline (**C**), carnosine (**D**), phenylalanine (**E**), and L-Dopa (**F**). The arrows indicate peaks corresponding to the retention times of authentic FAA standards

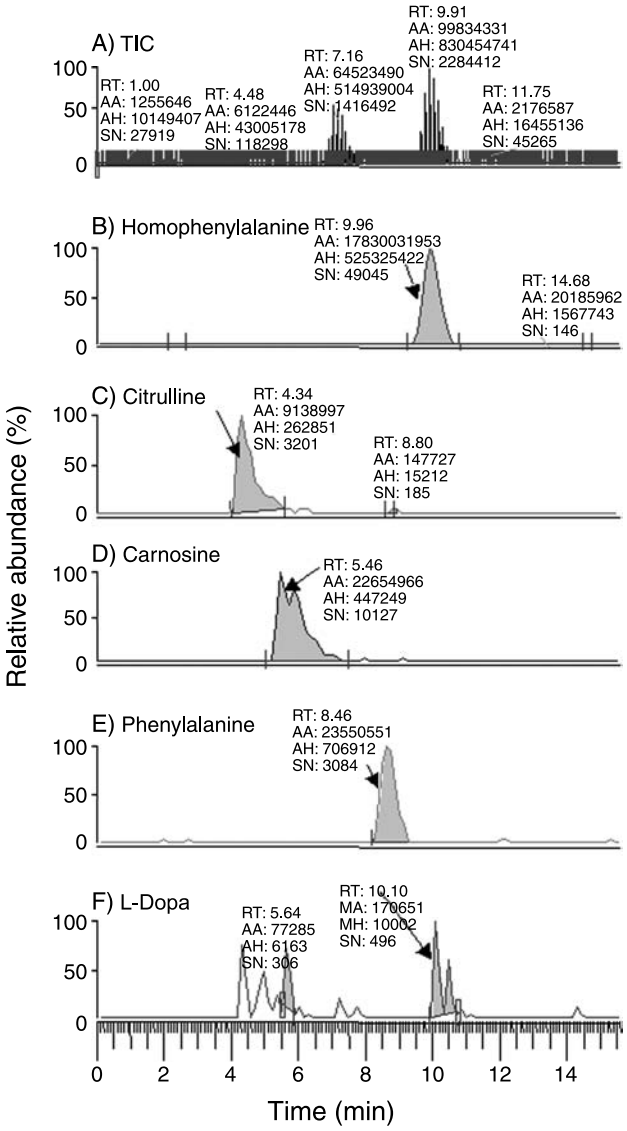

Fig. 3. Detection of important FAAs in plasma. TIC of amino acids and DP in plasma (**A**) obtained by LCMS^2 in the SRM mode. Spectra of homophenaylalanine internal standard (**B**), citrulline (**C**), carnosine (**D**), phenylalanine (**E**), and L-Dopa (**F**). The arrows indicate peaks corresponding to the retention times of authentic FAA standards

plasma and urine are shown in Figs. 3 and 4, respectively. We quantified 21 FAAs and 2 DPs in CSF, 31 FAAs and 6 DPs in plasma and 23 FAAs and 5 DPs in urine (Table 3). Under conditions where a particular FAA or DP was not resolved or not detected in a specific body fluid, increasing the amount of fluid used or changing the LC conditions improved detection. For example, although we did not clearly integrate and measure dopamine in CSF, peaks with $S/N > 2$ can be obtained by doubling the amount of CSF used in our studies. Therefore, more FAA and DPs can be detected by increasing our sample load for CSF, plasma or urine. Alternatively, sensitivity can be improved for a specific metabolite by increasing the scan time or decreasing the number of metabolites measured per scan. The values of many of the FAA measured by LCMS^2 were similar to those measured by ionic exchange chromatography (Molina et al., 1998) or were in the range of published values (http://www.labcorp.com/datasets/labcorp/html/appendix_group/appendix/section/amino.htm#amino). Any discrepancies in concentrations may be because of differences in the methods for quantification or in the ages of the study participants. Regardless, these data show that quantitative levels of many FAAs and DPs can be obtained using differentially labeled stable isotope standards.

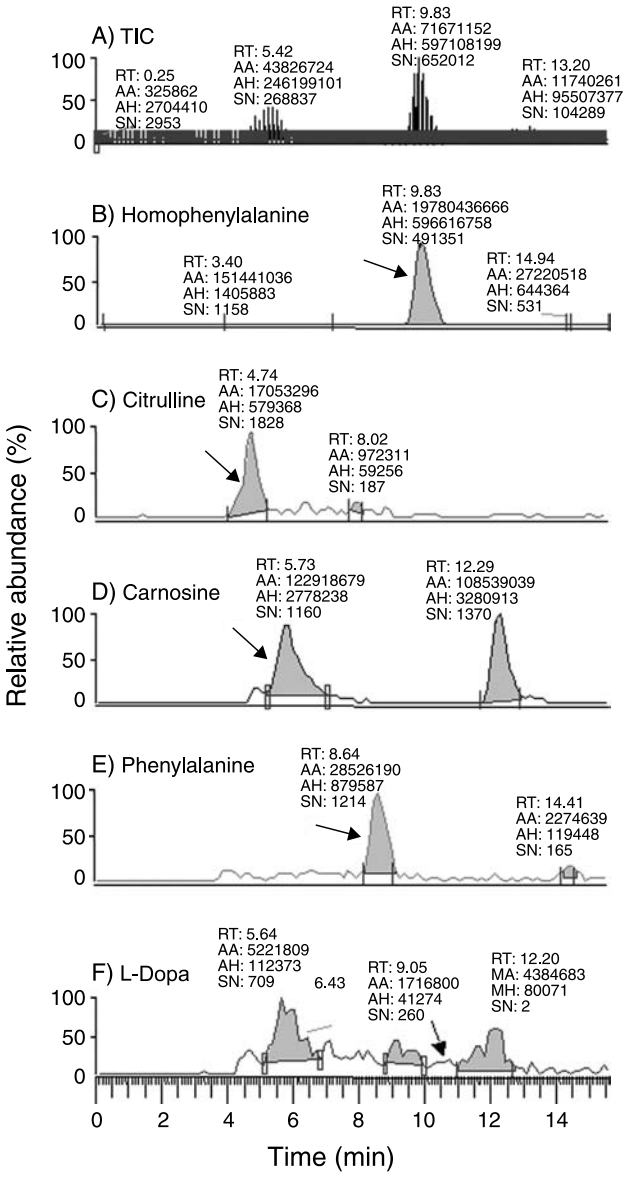

Fig. 4. Detection of important FAAs in urine. TIC of amino acids and DP in urine (**A**) obtained by $LCMS^2$ in the SRM mode. Spectra of homophenaylalanine internal standard (**B**), citrulline (**C**), carnosine (**D**), phenylalanine (**E**), and L-Dopa (**F**). The arrows indicate peaks corresponding to the retention times of authentic FAA standards

Comparison of FAA and DPs in body fluids

To aid in interpreting the data presented here, we will divide and discuss our data based on functional or biochemical properties that include:

1) Histidine (imidazole)-containing FAAs or DPs that have antioxidant properties.
2) Aromatic-containing FAAs that are neurotransmitters.
3) FAAs and DPs associated with urea metabolism/detoxification and NO formation.
4) Glutamate- and proline-derived FAAs and DPs.
5) Aspartate-, serine- and pyruvate-derived FAAs.

The major histidine-containing FAAs and DPs are histidine, 1-methyl-histidine, 3-methyl-histidine, carnosine and anserine (Table 3). For most of these compounds, the concentration was highest in urine > plasma > CSF.

Tryptophan, phenylalanine, tyrosine, L-Dopa and dopamine were the major aromatic-containing FAA measured in CSF, plasma or urine (Table 3). Concentrations of these FAAs were highest in the plasma > urine > CSF.

Arginine, citrulline and ornithine were the major FAAs associated with urea metabolism and NO synthesis detected in CSF, plasma or urine. The concentration gradient was urine > plasma > CSF except arginine that was highest in plasma (Table 3).

Several FAAs derived from glutamate metabolism were detected in CSF, plasma and urine. Aspartate-derived FAAs (asparagine, threonine, isoleucine and lysine), serine derived FAAs (cysteine, glycine) and pyruvate-derived FAAs (valine, leucine) were detected in CSF, plasma or urine. Lysine concentration was highest of the aspartate-derived FAAs in CSF (Table 3).

Together, these data show that many FAAs and DPs can be measured in body fluids and the concentration gradient between body fluids is different for amino acid biochemical groups.

Discussion

Several approaches have been used to measure FAA concentrations (Durkin et al., 1988; Husek, 1991; Petritis et al., 2000; Piraud et al., 2005a; Schwarz et al., 2005; Cellar et al., 2005). $LCMS^2$ offers several advantages over TLC, microfluidic devices and capillary electrophoresis or HPLC of derivatized samples (Casetta et al., 2000; Piraud et al., 2003). First, a combination of LC and MS increases resolution and thus improves the detection of a specific FAA or DP. Second, $LCMS^2$ is sensitive over a wide dynamic range. Third, $LCMS^2$ is reproducible. Mole quantities of FAAs and DPs can be accurately determined using internal standards to reduce sample loss during extraction (Piraud et al., 2005b). With careful optimization, quantitative data can be obtained for many different FAAs and DPs using a single internal standard. Finally, many different FAAs and DPs can be simultaneously detected using $LCMS^2$. The present study shows the use of $LCMS^2$ to determine the concentrations of many FAAs and DPs that vary in concentration over many thousand folds in the same sample. The sensitivity, specificity and dynamic range of this ap-

Table 3. FAA and DP concentrations in control CSF, plasma and urine

Amino acids	CSF (nmol/dl)	Plasma (nmol/dl)	Urine (nmol/dl)
1) Histidine-containing FAAs and DPs			
Histidine	1854.17 ± 639.10	13049.86 ± 1687.42	37263.90 ± 11541.33
1-Methyl-histidine	402.35 ± 139.98	1647.64 ± 356.72	38638.63 ± 9239.50
3-Methyl-histidine	347.45 ± 71.41	739.81 ± 153.56	27817.89 ± 6739.06
Carnosine	ND	654.23 ± 100.61	18694.81 ± 9996.17
Anserine	ND	26.63 ± 7.97	41893.77 ± 23311.63
2) Aromatic-containing FAAs			
Tryptophan	160.22 ± 36.05	2916.74 ± 709.06	1661.36 ± 346.69
Phenylalanine	678.94 ± 79.15	7707.78 ± 996.12	1763.99 ± 317.26
Tyrosine	2553.68 ± 270.19	16382.48 ± 2310.10	13205.55 ± 3295.70
Dopa	ND	513.01 ± 121.61	ND
Dopamine	33.67 ± 5.03	2219.31 ± 433.48	ND
3) FAAs involved in the urea cycle or in NO synthesis			
Arginine	2183.74 ± 273.39	9831.56 ± 625.94	2447.29 ± 479.65
Citrulline	2884.71 ± 292.21	10043.02 ± 418.00	19848.25 ± 5056.55
Ornithine	ND	5391.24 ± 2442.10	6639.78 ± 1758.87
4) Glutamate- and proline-derived FAA and DPs			
Glutamic acid	ND	2716.21 ± 1032.96	ND
Pyroglutamine	12.87 ± 1.04	32.25 ± 4.98	298.64 ± 118.88
γ-Glutamyl-ε-lysine	ND	13.29 ± 2.65	ND
GABA	ND	193.01 ± 59.96	ND
βABA	ND	292.56 ± 51.90	ND
ABA	ND	2157.76 ± 700.68	ND
DABA	ND	9848.23 ± 1281.46	71953.51 ± 17976.57
Glutamine	13909.06 ± 1144.36	22332.97 ± 3527.70	1645.66 ± 553.01
Proline-hydroxyproline	6.95 ± 1.21	906.71 ± 138.32	759.49 ± 119.19
4-Hydroxyproline	29.80 ± 3.00	568.28 ± 106.97	286.74 ± 55.04
Glycyl-proline	8.92 ± 1.21	22.98 ± 2.59	46.10 ± 9.94
Proline	32.66 ± 9.82	NR	ND
5) Aspartate-, serine- and pyruvate-derived FAAs			
Aspartic acid	ND	1003.61 ± 265.86	ND
Asparagine	181.86 ± 31.83	NR	235.92 ± 6.53
Isoleucine	281.43 ± 64.43	187810.77 ± 28068.57	184.99 ± 84.41
Threonine	ND	NR	49.52 ± 15.43
Lysine	5351.34 ± 1462.52	18317.00 ± 4875.55	13778.29 ± 5757.93
Hydroxylysine	ND	51.05 ± 4.36	ND
Lysyl-alanine	ND	127222.47 ± 16481.34	513292.77 ± 161435.94
Serine	ND	21.15 ± 2.74	ND
Cysteine	131.62 ± 71.03	1087.05 ± 225.73	2922.35 ± 43.45
Cystine	693.00 ± 201.88	15475.08 ± 3889.80	77988.79 ± 16066.00
Cystathionine	ND	103.34 ± 21.05	3028.55 ± 682.19
Glycine	21.83 ± 7.06	15.13 ± 1.10	8.53 ± 1.31
Valine	539.44 ± 158.45	5197.60 ± 1372.70	1828.19 ± 483.91
Leucine	1281.14 ± 174.84	NR	1176.95 ± 315.86
Allo-leucine	ND	3450.96 ± 584.46	ND
Aminopimelic acid	ND	245.30 ± 55.20	ND

We extracted and derivatized FAAs and DPs from 200 µl CSF, 100 µl plasma and 200 µl urine (C) as described in Materials and methods. We performed $LCMS^2$ and calculated mole quantities (nmol/dl) of FAAs and DPs in samples from control subjects with no classifiable neurological disease. These data are mean ± SEM of 4 male and 4 female subjects with an average age of 79.5 + 1.93 years. Data are shown for all FAA and DPs detected in $n > 8$ samples
ND Not detected; *NR* not resolved

proach are illustrated in this study where different concentrations of various FAAs and DPs were measured in CSF, plasma or urine.

Perhaps the greatest advance in $LCMS^2$ is our ability to simultaneously measure several FAA and DPs in the same sample. This approach makes it possible to follow bio-

chemical pathways and to easily pinpoint metabolites and enzymes that are altered. This approach should have broad application in the study of metabolic diseases or of biochemical pathways that involve changes in the concentrations of several metabolites. We have used this approach to show changes in several metabolites in CSF, plasma or urine from pAD compared with CT subjects (Fonteh et al., 2007).

In most ELISA based methods, only one FAA is measured. Therefore, a bigger volume of sample is required to obtain comparable data as would be obtained in an $LCMS^2$ experiment. Experiments based on LC with UV detection alone are limited in specificity. Adding mass detection increases the specificity and makes it possible to detect dozens of FAA and DPs. Sensitivity is also increased when each FAA and DP is optimized for SRM studies and over 40 samples can be analyzed within a day on one instrument setup with an auto injector module. Thus, when sample amounts are limiting, significantly more data can be obtained using $LCMS^2$ than conventional ELISA and HPLC-UV methods.

Overall, $LCMS^2$ methods using a triple quadrupole mass spectrometer coupled with isotope dilution are specific and sensitive enough to quantify over 75 FAAs and DPs in a single run. These methods should be useful in elucidating biochemical pathways or discovering biomarkers of diseases even when sample amounts are limiting.

Acknowledgements

We thank Ryan Beck and Connie Chen for technical help. We thank LCF and all study participants who donated CSF and plasma for this research. We thank Drs. Andreas Huhmer and Roger Biringer (Thermo Electron, San Jose, CA) for their support and discussion of this research and Dr. James Riggins for review of this manuscript. This work was supported in part by NIH grants RO1# NS43295; the Norris, Jameson and Glide Foundations; and donations from the Dunlevy, Hezlep and Posthuma families.

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