Simultaneous Determination of the Neurotransmitters and Free Amino Acids in Rat Organs by LC-MS Analysis

Naoyuki Maeda[1, 2※], Michiko Sato[2], Satoko Haeno[2] and Hiroshi Yokota[2]

[*1]Safety Research Institute for Chemical Compounds Co.,Ltd, Sapporo, Hokkaido 004-0839, Japan.
[2]Laboratory of Veterinary Biochemistry, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan.

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[※]Corresponding Author: Naoyuki Maeda, Ph.D.
Safety Research Institute for Chemical Compounds Co., Ltd
Sapporo, Hokkaido, Japan
Tel 81-11-885-5031
Fax 81-11-885-5313
Email: maeda-naoyuki@ka-anken.co.jp

Abstract

Free amino acids were kept at constant levels, and their alterations reflect the real state of energy metabolisms in the cells and living body. Neurotransmitters synthesized from certain amino acids fluctuated along with the current neuro-functional viability. Their content levels in each tissue give us substantial information about their state of health and disease in mammals. The determination method of free amino acids without any derivative or labeling reactions and neurotransmitters in the various rat organs was developed using a single column. The levels of free amino acids and neurotransmitters in the organs could be simultaneously and directly obtained within 80 min. Amino acids and neurotransmitters could be separated within 20 min by using an "Intrada Amino Acid" column and identified by LC-TOF MS analysis, and they were quantitatively analyzed by LC-MS/MS. Leucine and isoleucine could be completely separated by column chromatography of the LC-MS analysis. The tissue specific localization of the amino acids and neurotransmitters obtained in this study can be physiologically explained. Additionally, the muscle specific branched-chain amino acids localization was found and the reasons were discussed.

Keywords: Simultaneous assay, Free amino acid, Neurotransmitters, LC-MS analysis, Rat organs.

Introduction

Abnormal plasma and urinary concentrations of free amino acids reflect the biosynthesis and catabolism of proteins and energy metabolism in the cells of living bodies (1). Amino acid metabolism, involving serine and glycine, can provide the essential precursors for the synthesis of protein, nucleic acids, and lipids that are crucial to cancer metabolism (2, 3). Neurotransmitters and their metabolites are widely distributed in the brain and peripheral body fluids of mammals (4, 5). They are well known to play a significant role in the nervous system, and they consist of amino acid neurotransmitters, such as glutamine and γ-aminobutyric acid; monoamine neurotransmitters, such as 5-hydroxy-L-tryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, L-dihydroxy-phenylalanine, dopamine, norepinephrine, epinephrine and melatonin; and others, such as acetylcholine. Neurotransmitter imbalances caused by disturbances in the monoamine

metabolism and transporter have been linked to various neuronal abnormalities (6, 7). It would be possible to recognize more real conditions of each cell if we could determine the concentrations of the free amino acids and neurotransmitters contained in the tissues and organs. The monitoring of neurotransmitters and their metabolites is an essential tool for elucidating the normal and pathological neuronal system activities. Assays of the amino acids and neurotransmitters were performed by HPLC, and they were detected by the ultraviolet (UV) absorbance or derivative methods. However, accurate and higher sensitivities could not be obtained by UV methods. Additionally, suitable stabilities were not obtained by derivative methods (9 - 12) because these methods were abandoned in response to their individual reactivities with derivatization reagents (13). Recently, using LC-MS analysis, some investigators developed a method with lower variability, good recovery and accuracy in the simultaneous determination of neurotransmitters (14 - 16). Only neurotransmitters were measured with using HPLC with ECD or GC/MS and LC-MS/MS previously reported (8, 15). However the comprehensive and simultaneous data on the levels of free amino acids and their metabolite neurotransmitters were significant for understanding metabolite disorders and neuronal diseases.

We developed an assay method with accurate and highly sensitivity at the same levels as those described above for free amino acids and neurotransmitters contained in the various tissues of rat using LC-TOF MS and LC-MS/MS, and their simultaneous actual levels and new findings on muscular branched-chain amino acids (BCAA; leucine, isoleucine and valine) were obtained.

Materials and Methods

Chemicals and reagents - The following were purchased from Wako Pure Chemical Industries (Osaka, JPN): amino acids - alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cystine ((Cys)$_2$), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), valine (Val), and 4-aminobutanoic acid (GABA); stable isotopes - GABA-d$_6$; indoleamines - Serotonin (5-HT), 5-hydroxytryptphan (5-HTP), 5-Hydroxyindoleacetic Acid (5-HIAA), melatonin (MLT) and catecholamines - dopamine (DA), epinephrine (EP), norepinephrine (NE), L-3,4-dihydroxyphenylalanine (L-DOPA) and neurotransmitter- acetylcholine (AC) were purchased from Sigma Aldrich (St. Louis, MO). Stable isotopes, 5-HT-d$_4$ and DA-d$_4$, were purchased from Taiyo Nippon Sanso (Tokyo, JPN). An acetonitrile and hexane for pesticide residue analysis were purchased from Kanto Chemical (Tokyo, JPN). LC-MS grade acetonitrile, tetorahydrofulan, ammonium formate and formic acid were purchased from Thermo Fisher scientific (San Jose, CA).

Preparation of rat organs - Male Sprague-Dawley (SD) rats (weight, 280 ± 20 g; age, 8 - 10 weeks) were fed, housed and allowed to adapt to their environments for one week prior to the experiments. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85 - 23, revised 1996). The blood and organs were prepared from the animals by exsanguination under isoflurane anesthesia. After dissection, the organ samples that were excised post-mortem were weighed, immediately frozen and stored at -25 °C until use.

Preparation of samples for MS analysis - Male Sprague-Dawley rat organs were obtained on ice immediately after death in the animal laboratory and transported in liquid nitrogen to our laboratory; they were frozen immediately at -80 °C until biochemical analysis. The rat serum and brain tissue was homogenized in ice-cold 4:6 acetonitrile: water with 0.1 % formic acid (per gram of tissue by adding 4.0 mL of solution) after they were precisely weighed in the 50 mL centrifuge tube. Then, 1.0 mL of 100 μ mol /L of DA, 5-HT and GABA of the internal standard were added to the tube and centrifuged at 8,000 rpm (10,160 ×g, 7780II, Kubota Tokyo, JPN) for 20 min. Supernatant (1.0 mL) was pipetted into a 2.0 mL centrifuge tube and 1 mL of hexane was added (saturated with acetonitrile); the solution was vortex-mixed for 10 sec, centrifuged at 3,000 rpm (700 ×g, Kitman-18, TOMY Tokyo, JPN) for 5 min, and finally centrifuged at 12,000 rpm (11,400 ×g) for 5 min using PVDF 0.22 μm Ultrafree-MC (Millipore, Billerica, MA) filtrated. The filtrated sample was injected into the LC-TOF MS and LC-MS/MS analysis (Fig. 1).

Separation of the free amino acids and neurotransmitters - The separation was achieved using an Intrada Amino Acid (100 × 3 mm, 3-μm particle size, Imtakt Kyoto, JPN) at a 400 μL/min flow rate at 35 °C. A 5 μL aliquot was used for the autosampler injection. The positive ion mode scanning of a gradient mobile phase, consisting of (A) acetonitrile: THF: 25 m mol/L ammonium formate: formic acid 10: 80: 10: 0.4 [v/v] solution and (B) acetonitrile: 100 m mol/L ammonium

formate 20:80 solution, was used. For the gradient elution, (A)/(B) ratios were used from 100/0 hold at 1 min, to 83/17 and 0 / 100 for between 1 and 6.5 min and between 6.5 and 10 min, respectively, which was followed by an 8 min hold at 100 % (B) and a final return to 100 % (A) within 4 min for a total 22 min running time.

Quantification of free amino acids and neurotransmitters expressed in tissues - For the quantification of the free amino acids and neurotransmitters, a Xevo TQD triple-stage quadrupole mass spectrometer connected to an acuity H-class (Waters Midford, MA) and an ESI ion source device was constructed (LC-MS/MS). The MS system and data were operated and analyzed using targetlynx software (waters version 4.1). The instrumental parameters were optimized during the direct infusion of the compounds with 50 % solvent (0.1 % acetic acid in water/acetonitrile at 1:1 [v/v]) at a flow rate of a 5 µL/min. The $[M+H]^+$ ions of the compounds were identified by LC-MS, with the MS1 operated in the full scanning mode in the range of 50 - 800 m/z. A product ion spectrum was obtained for each compound (Table 1). Multiple reaction monitoring (MRM) was used for the quantitative analysis.

Calibration curves - Stock solutions of indoleamine, catecholamine and intact amino acid were used to prepare the working standards of each compound (concentrations of 1 - 5,000 µ mol/L) by serial dilution in 4:6 acetonitrile: water with 0.1 % formic acid; isotopes of the internal standards GABA-d_6, DP-d_4 and 5-HT-d_4 were prepared at a concentration of 25.0 µ mol/L. The calibration curves of amino acids were plotted using the absolute calibration curve method, and the concentration ratios of the analytes to the internal standards (GABA/GABA-d4, DA, L-DOPA, EP and NE/DA-d_4, 5-HT, 5-HTP and HIAA/5-HT-d_4) as the x axes and the peak area ratios of the analytes to the internal standards as the y axes. The calibration curves for each compound showed excellent linearity over the concentration range used (R > 0.995), and the detection limits of these were shown in Table 2.

Overall method recovery - Recovery tests were performed to search for the recovery rates and assess the accuracy of the method. Several concentration mixtures of the standards and internal GABA-d_6, DA-d_4 and 5-HT-d_4 standards were added to 100 µ mol/L. The samples were measured and evaluated for the recovery rate and relative standard deviation (RSD %) using LC-MS/MS. The accuracy and precision of the entire analytical procedure were evaluated by spiking the serum samples (n=3 - 5) with 100 µ mol/L. The levels of free amino acids and neurotransmitters were subtracted from the spiked levels of the analysis in the male rat serum. Accurate data with 70 - 120 % recovery and RSD under 25 % and limits of detection (LOD; signal / noise [S/N] > 3) were obtained, respectively, as previously indicated by the FDA as a guideline (17).

Identification of free amino acids and neurotransmitters - The HPLC system was a UFLC Nexera (Shimadzu, JPN) instrument, consisting of a vacuum degasser, autosampler, binary pump and column oven. The mass analyzer was a micrOTOF - QII time of flight mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with an orthogonal electro - spray (ESI) source and a 6 - port diverter valve. The instrument was operated in the positive ion mode, using a range of 50 - 500 m/z. The capillary voltage of the ion source was set to 4,500 V; then, the nebulizer gas flow was 1.6 bar and the dry gas flow was 8 L/min. The dry temperature was set to 180 °C. Instrument calibration was performed prior to each sequence using 10 m mol/L sodium formate/2-propanol (1:1, v/v). The post-run internal mass scale calibration for the individual samples was performed using data acquired during a calibration injection at the beginning of the run via a 6 - port diverter valve equipped with a 20 µL loop. The calibrant was also injected at the end of each run to verify the calibration stability. The instrument calibration and post-run internal mass scale calibrations were performed using sodium formate ions Na (NaCOOH) $_{1-7}$, ranging from 90.976645 to 498.901189 m/z, with an accuracy of 5 ppm. The data processing was performed using Data Analysis software (version 4.0, Bruker Daltonics). The base mass peak (after background subtraction) was measured after the proton subtraction in the compounds (10 ppm tolerance). For each retrieved chemical formula, the mass error (difference between the measured and theoretical masses) and SigmaFit^{TM} (a parameter, were calculated using Bruker software, accounting for the difference between the theoretical and measured isotonic pattern; the lower the sigma value (usually < 0.05), the better the matching.

Results

Certification of the analytical method developed - Extraction and preparation procedures of the free amino acids and neurotransmitters are shown in Fig. 1. These compounds were extracted with acetonitrile/water (4:6) solution containing 0.1 % formic acid as the final concentration for efficient positive ionization. Standard compounds, such as deuterium-substituted DA, 5-HTP and GABA, were added into the homogenates for the internal standards. Hexane was added into the supernatant solution to remove hydrophobic contaminants, as shown in Fig. 1. High-performance liquid chromatography profiles using an

"Intrada Amino Acid" column without sample modification are shown in Fig. 2. For the LC-MS chromatography monitoring at 132 m/z, Leu and Ile were separated completely within 7 min with the column (Fig. 3 A and B). Because a high Ile peak was observed from the chromatography monitoring of $132 \rightarrow 70$ m/z ion without a Leu peak (Fig. 3 A), the amino acid could be quantitatively determined. Leu peaks with a molecular mass at $132 \rightarrow 43$ m/z could also be determined quantitatively (Fig. 3 B). A Leu peak with $132 \rightarrow 43$ m/z and Ile peak with $132 \rightarrow 70$ m/z were recognized as the first target ions respectively, as shown in Table 1. By using the present method, 20 kinds of amino acids and neurotransmitters were completely separated within 20 min, as shown in Fig. 2, and the levels were determined in each tissue, indicating that the metabolic relationships between amino acids and neurotransmitters consisting of amino acids can be discussed. Identification processes of amino acids, such as Leu, contained in the muscle extract are shown in Fig. 4. By monitoring with 132.101905 ± 0.05 Da, a dabble peaks extracted from the muscle was detected on the MS chromatography profile as shown in Fig. 4 B, and it had same retention time (5.80 min) with standard Leu, as shown in Fig. 4 A, which depicts chromatogram monitoring with the same mass. After analysis of the components with a retention time at 5.80 min, as shown in Fig. 4 A and B, with the LC-TOF MS, the resulting LC-TOF chromatography is shown in Fig. 4. C and D. The theoretical profile of Leu is shown in E panel. After comparison with the theoretical value, the "MS errors" of the standard and extracted Leu were only 5.0 and 9.2 mDa, and sigma values of them were 0.5 and 1.0, respectively, as shown in Fig. 4. C, D and E, showing that the peak obtained in panel B was identified as Leu. Identification of neurotransmitters was performed as shown in 5-HT. The chromatography patterns of the standard 5-HT (Fig. 5) and extracted 5-HT from the rat muscle (B) were shown using LC-TOF MS monitoring with 177.102239 ± 0.05 (m/z). The mass spectrum of the peak that had a retention time at 10.80 min in the standard shown in A panel (C) and in the extracted peak in the B panel (D) using LC-TOF MS analysis. The mass spectrum of the theoretical pattern of standard 5-HT is also shown (E). A small d-value (sigma value) was observed at 0.00188, and the mass error was only 3.2 mDa in the D panel, which was identified as 5-HT. Other neurotransmitters were all identified with the SigmaFit analysis as in the figure. The recovery and LOD with a RSD are shown in Table 2. Accurate data with 78.3 - 112.1 % recovery,

RSD with less than 5 % and LOD as previously indicated by FDA as a guideline (FDA 2001) were obtained as shown in Table 2. These data indicate that the present assay method is available for measuring all free amino acids and neurotransmitters contained in organs.

Concentration of amino acids and neurotransmitters in rat organs - Free amino acids and neurotransmitters contained in the various rat organs were assayed by the method developed in the present study, and the content levels are shown in Table 3 and 4. The levels of the free amino acids in the pituitary glands were especially higher than those in the other organs (Table 4). The Gln had higher level content in all organs (m mol / L order) compared with other amino acid, even in the blood, indicating that Gln is a final acceptor and transporter of amino residues in the degradation of amino acids. In the data on the neurotransmitters, AC was distributed in all organs, even in the blood, but the GABA levels were higher in the brain, and GABA was not observed in the blood. 5-HT was only detected in the cerebrum of the brain tissues (Table 4). And high levels of EP, NE, DA and L-DOPA were observed in the adrenal glands (Table 3). The BCAA content levels in the gastrocnemius muscle (GM) and extensor digitorum longus muscle (EDL) were lower than those of the soleus muscle (SM) as shown in Table 5. Furthermore, the BCAA content in the GM was drastically lower than that in the EDL. Leu, which plays important roles in muscle regeneration, mostly had a higher level in the SM.

Discussion

Progression of the method development in this study - The information on the real state of metabolism was obtained by determining the free amino acids contents. Amino acid analysis has presented an analytical challenge in terms of the sample preparation, separation, and detection. Among the separation methods, liquid chromatography has prevailed in the amino acid analysis field with either pre-column or post-column labeling techniques to improve either the separation of amino acids or selectivity and sensitivity of the analysis (13). More simplicity and ease were necessary for automatic analysis. We developed a simultaneous determination method of free amino acids without any derivative or labeling reactions in the rat various organs. We developed an accurate determination method with 70 - 120 % recovery, RSD under 5 %, the lowest LOD, and signal/noise (S/N)>3 (Table 2). These data were within the limits indicated by the FDA guidelines (17). A recent method was reported to have higher

sensitivity and selectivity when combined with post-column labeling techniques for amino acid analysis; however, the chromatography process of that method takes 200 minutes (13). The procedure developed in this study included the preparation of samples for 40 min, a complete separation of 20 amino acids and neurotransmitters with the "Intrada Amino Acid" column and identification by LC-TOF MS analysis within 20 min, as shown in Fig. 2. Our direct method developed in this study does not require consideration of the reagent stability, reproducibility or compatibility, which are important risk factors in amino acid labeling techniques. The "Intrada Amino Acid" column could separate isoforms, Leu and Ile, within 7 min, as shown in Fig. 2. Most current assay methods require the derivatization or labeling of amino acids for qualitative and quantitative analysis. For simple and fast performance, several procedures without any derivatization or labeling of amino acids were developed (18, 19); however, these methods could not separate all of the amino acids in a chromatography performance with LOD levels. We developed a method in this study that can simultaneously analyze all 20 amino acids by using a column " Intrada Amino Acid" with higher sensitivities (n mol/L levels LOD). The concentration of neurotransmitters is known to fluctuate along with the current neuro-functional viability. The content levels in each tissue provide substantial information about their state of health and disease. In the present study, the levels of neurotransmitters, such as GABA, 5-HT, DA and AC in the various organs, could also be separated and determined on the chromatography and detected with the MS analysis. This method completes the simultaneous assay within 80 min and has the lowest LOD values compared with other methods (20, 21). The monitoring of neurotransmitters, such as AC, MLT, GABA, 5-HT, EP, NE, DA and their metabolites, is essential to elucidating the neuronal functions and pathological states. Analysis of the neurotransmitters levels could aid in the disease diagnosis, prognosis and monitoring of treatments. Several HPLC or LC-MS methods have been reported to determine the levels of various neurotransmitters (21, 22); however, few studies have reported the simultaneous determination of all of these neurotransmitters and their metabolites in the tissues. Recently, several studies on simultaneous determination methods using LC-MS analysis were reported to have lower variability, good recovery and accuracy (14-16). The LOD of these methods was in the ng/mL level, corresponding to the μ mol/L levels of neurotransmitters. In this study, we developed a new method for measuring neurotransmitters and their metabolites in rat tissues with a similar LOD, accuracy and recovery, which could simultaneously be evaluated with amino acids analysis using a single column.

Local levels of amino acids and neurotransmitters in rat organs - Higher levels of Gln , Glu and Ala were observed in the skeletal muscle compared with other amino acid, indicating that these amino acids were tissue specific acceptors of amino residues from the deamination of other amino acids (Table 3). The free amino acid levels in the skeletal muscle were lower than in other tissues except for these three amino acids (Gln, Glu and Ala), indicating an extensive scale of amino acid metabolism in the organ (Table 3). Two types of the neurotransmitter localization were observed, as shown in Table 3 and 4. GABA and AC were detected in all tissues, and other neurotransmitters were specifically located in each tissue. The 5-HT was only detected in the cerebrum of the brain tissues, reflecting the localization of the 5-HT neuron mainly in the cerebrum (Table 4). There were high levels of EP and its precursors, NE, DA and L-DOPA, in the adrenal gland (Table 3), which was certainly from the presence of the synthetic epinephrine system in the glands. The reason for the higher level of GABA in the cerebellum was clearly because of the high number of GABA neurons in that region. The higher level of 5-HT in the skeletal muscles coincided with the localization of the 5-HT2A receptor in the tissue, where 5-HT might act as an intracellular signal (23), and facilitate muscle spasms after spinal cord injury (24).

BCAA in the muscle types - The levels of BCAA in the GM and EDL were lower than in the SM (Table 5). The levels are inversely correlated with the content rates of fast muscle (Type II), which accounts for approximately 60 - 100 % of the GM, 96 % of the EDL and 2.7 % of the SM (25). This may be reflected by the properties of fast muscle, which has a higher turnover rate and frequently incorporates BCAA into the muscle proteins as a result of the higher glucocorticoid receptor (GR) expression (26). It is interesting, though the cause is unknown, that the BCAA content in the GM was drastically lower than that in the EDL, suggesting that BCAA has important roles in protein metabolism or muscle regeneration. A higher level of Leu was found in the SM, which consists of slow type muscle (Table 5). High Leu levels can inhibit protein degradation in skeletal muscles and induce muscle protein synthesis by enhancing its sensitivity to insulin (27). The degradation rate of muscle proteins is slow due to the lower expression of the GR in the SM (26).

A higher level of Leu might be involved in the slow turnover of the muscle protein in the SM.

Conclusion - Evaluation of the tissue-specific localization and concentration of amino acids and neurotransmitters was performed using the method developed in this study. Several new observations on the metabolisms of amino acids, such as BCAA, and neurotransmitters in the skeletal muscle were made.

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Legends

Table 1. MS/MS conditions in SRM transitions employed for the determination of amino acids and neurotransmitters.

Compound	Precursor ion (m/z)	Cone voltage (CV)	Quantitative ion		First target ion	
			Product ion (m/z)	Collision energy (CE)	Product ion (m/z)	Collision energy (CE)
Alanine (Ala)	90	20	44	10	-	-
Arginine (Arg)	175	30	70	20	116	10
Asparagine (Asn)	133	20	74	10	46	20
Aspartic acid (Asp)	134	30	88	10	74	10
Cystine (Cys)$_2$	241	20	120	20	152	10
Glutamine (Gln)	147	20	84	15	130	10
Glutamic acid (Glu)	148	20	85	10	131	10
Glycine (Gly)	76	30	30	10	-	-
Histidine (His)	156	30	110	10	83	20
Isoleucine (Ile)	132	20	70	20	86	10
Leucine (Leu)	132	20	43	20	30	10
Lysine (Lys)	147	20	84	20	130	10
Methionine (Met)	150	20	56	20	61	20
Phenylalanine (Phe)	166	20	103	30	120	10
Proline (Pro)	116	30	70	10	43	20
Serine (Ser)	106	20	60	10	42	20
Threonine (Thr)	120	30	74	10	56	10
Tryptophan (Trp)	205	20	118	30	91	40
Tyrosine (Tyr)	182	20	136	10	91	30
Valine (Val)	118	20	72	10	55	20
4-Aminobutanoic acid (GABA)	104	20	87	10	69	15
Acetylcholine (AC)	146	30	87	14	43	24
Dopamin (DA)	154	20	137	10	91	20
Serotonin (5-HT)	177	20	160	10	132	30
Epinephrine (EP)	184	20	166	10	107	20
Norepinephrine (NE)	170	20	152	10	107	20
5-Hydroxytryptphan (5-HTP)	221	30	204	10	162	20
5-Hydroxyindoleacetic Acid (5-HIAA)	191	30	146	10	91	30
L-3,4-dihydroxyphenylalanine (L-DOPA)	198	20	152	10	107	30
Melatonin (MLT)	233	30	174	10	159	30
Isotope						
GABA d$_6$	110	20	93	10	49	20
DA d$_4$	158	10	95	20	141	10
5-HT d$_4$	181	20	136	20	118	30

Table 2. Recovery tests of additive indoleamine, catecholamine and intact amino acid into the serum.

Compound	Serum (1.0 ml)		LOD (nmol/L)
	Recovery(%)	RSD(%)	
Ala	84.5	1.0	0.21
Arg	96.3	4.7	0.18
Asn	88.8	3.1	2.06
Asp	101.5	3.9	2.26
(Cys)$_2$	95.9	1.5	2.37
Gln	86.0	4.0	0.11
Glu	92.0	3.2	0.99
Gly	87.2	2.5	3.62
His	78.3	0.5	0.34
Ile	96.8	0.7	1.17
Leu	87.6	1.7	0.51
Lys	83.2	4.1	0.07
Met	83.6	1.8	0.13
Phe	95.1	2.7	0.48
Pro	91.2	1.5	0.23
Ser	84.5	2.7	0.46
Thr	90.5	4.9	0.26
Trp	84.7	4.5	0.21
Tyr	78.5	0.5	0.35
Val	100.1	0.4	0.28
GABA	100.4	4.1	2.00
AC	93.0	3.4	9.20
DA	96.3	3.5	61.6
5-HT	100.1	0.8	6.51
EP	110.3	2.8	8.78
NE	91.7	3.5	41.4
5-HTP	112.1	2.0	17.0
5-HIAA	90.0	4.2	0.27
L-DOPA	101.7	3.0	61.2
MLT	98.9	4.6	0.11

LOD: limits of detection
RSD: Relative Standard Deviation
Data presented mean values of n=3 - 5

Recovery data obtained were shown with relative standard deviation (RSD). The accurate data having 70 - 120 % recovery and RSD under 25 % and limits of detection (LOD; signal / noise [S/N]>3) were obtained respectively as previously indicated by FDA as a guideline (FDA 2001). The accuracy and precision of the entire analytical procedure were evaluated by spiking the serum samples (n=3 - 5) with 100 μ mol / L.

Table 3. Amino acids and neurotransmitters in various tissues of adult male rats.

Compound	Serum	Testis	Liver	Kidney	Adrenal glands	Skeletal muscle
Ala	612 ± 25	978 ± 95	444 ± 263	521 ± 36	4.34E+03 ± 9.03E+02	1.80E+03 ± 3.10E+02
Arg	232 ± 10	404 ± 47	376 ± 121	860 ± 28	1.19E+03 ± 2.04E+02	112 ± 21
Asn	67.5 ± 1.7	72.8 ± 19.8	367 ± 89	1.27E+03 ± 2.25E+02	394 ± 165	24.1 ± 1.3
Asp	30.1 ± 1.3	440 ± 57	251 ± 40	1.14E+03 ± 1.32E+02	776 ± 49	34.9 ± 2.3
(Cys)$_2$	3.10 ± 0.82	8.00 ± 4.02	6.20 ± 1.20	297 ± 73	12.1 ± 2.6	51.1 ± 12.2
Gln	5.20E+04 ± 1.40E+03	2.60E+04 ± 1.01E+03	1.46E+04 ± 3.80E+03	1.62E+04 ± 1.60E+02	2.11E+05 ± 5.07E+03	9.70E+03 ± 8.03E+02
Glu	140 ± 13	1.00E+03 ± 1.02E+02	299 ± 63	1.32E+03 ± 1.05E+02	1.89E+03 ± 3.00E+02	2.80E+03 ± 4.20E+02
Gly	225 ± 9	1.00E+03 ± 1.11E+02	980 ± 169	3.11E+03 ± 2.32E+02	2.13E+03 ± 7.08E+02	783 ± 52
His	46.3 ± 2.4	141 ± 16	249 ± 69	245 ± 18	123 ± 30	95.0 ± 6.2
Ile	165 ± 8	154 ± 30	328 ± 50	856 ± 59	444 ± 112	41.7 ± 9.0
Leu	153 ± 10	366 ± 55	584 ± 104	1.15E+03 ± 1.21E+02	926 ± 164	56.6 ± 10.0
Lys	417 ± 22	490 ± 95	587 ± 130	551 ± 29	2.23E+03 ± 1.45E+02	36.8 ± 5.9
Met	61.8 ± 4.0	183 ± 28	290 ± 56	479 ± 38	184 ± 95	24.3 ± 2.4
Phe	89.4 ± 7.1	299 ± 37	452 ± 94	579 ± 11	763 ± 144	66.7 ± 11.5
Pro	485 ± 18	765 ± 97	272 ± 82	556 ± 34	3.05E+03 ± 3.12E+02	303 ± 11
Ser	145 ± 5	484 ± 72	545 ± 104	1.10E+03 ± 1.38E+02	592 ± 94	186 ± 26
Thr	260 ± 13	390 ± 74	382 ± 85	448 ± 36	887 ± 163	209 ± 23
Trp	150 ± 7	76.7 ± 12.1	95.0 ± 23.3	241 ± 20	220 ± 44	14.2 ± 3.2
Tyr	127 ± 7	241 ± 37	328 ± 66	273 ± 13	570 ± 176	59.9 ± 8.4
Val	207 ± 6	142 ± 12	78.3 ± 19.6	78.1 ± 3.2	858 ± 192	70.9 ± 6.0
GABA	—	53.5 ± 15.4	15.4 ± 9.9	166 ± 17	60.0 ± 5.0	4.40 ± 0.54
AC	63.7 ± 10.5	578 ± 50	1.14E+04 ± 4.18E+02	2.12E+03 ± 3.09E+02	48.2 ± 22.4	1.01E+03 ± 1.02E+02
EP	—	—	—	—	6.27E+04 ± 6.31E+03	—
NE	—	—	47.8 ± 13.7	71.7 ± 18.3	5.52E+04 ± 1.36E+04	30.5 ± 6.6
DA	56.0 ± 3.2	—	—	—	3.24E+04 ± 5.02E+03	—
5-HT	636 ± 193	—	55.6 ± 10.8	87.6 ± 17.3	—	444 ± 156
5-HTP	—	—	—	—	—	—
5-HIAA	—	—	—	—	—	—
L-DOPA	—	—	—	—	255 ± 55	—
MLT	—	—	—	—	—	0.16 ± 0.08

Concentration of amino acids and neurotransmitters in rat organs were assayed by the method developed in this study as described in "materials and methods".
Data were shown as the means ± S.D. for 5 animals (μ mol / L or g of tissue weight). " – " mean as "LOD".

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Table 4. Amino acids and neurotransmitters in brain tissues of adult male rats.

Compound	Cerebrum			Cerebellum			Hipocampus			Pituitary glands		
							Brain					
Ala	935	±	143	2.30E+03	±	3.36E+02	2.52E+03	±	5.07E+02	2.12E+03	±	1.20E+03
Arg	420	±	76	793	±	109	618	±	87	1.64E+03	±	6.21E+02
Asn	83.2	±	19.9	124	±	28	180	±	27	2.01E+03	±	7.01E+02
Asp	2.11E+03	±	4.02E+02	2.30E+03	±	2.08E+02	2.97E+03	±	1.40E+02	4.62E+03	±	9.05E+02
(Cys)$_2$	16.7	±	4.4	44.8	±	12.2	27.2	±	4.2	190	±	52
Gln	2.74E+04	±	3.60E+02	1.33E+05	±	9.40E+02	2.09E+05	±	3.87E+02	1.90E+05	±	5.02E+03
Glu	2.67E+03	±	4.20E+02	5.10E+03	±	7.37E+02	6.03E+03	±	9.74E+02	1.78E+03	±	6.60E+03
Gly	676	±	77	1.02E+03	±	2.08E+02	977	±	205	1.21E+03	±	5.70E+03
His	165	±	37	107	±	30	73.7	±	20.1	274	±	47
Ile	168	±	40	264	±	74	287	±	27	2.22E+03	±	1.51E+03
Leu	267	±	54	434	±	123	343	±	24	3.43E+03	±	1.31E+03
Lys	299	±	43	571	±	96	433	±	62	3.84E+03	±	1.15E+03
Met	137	±	44	176	±	69	102	±	16	888	±	264
Phe	266	±	43	521	±	75	444	±	57	5.89E+03	±	1.87E+03
Pro	742	±	157	1.89E+03	±	4.20E+02	2.03E+03	±	2.04E+02	3.47E+03	±	1.08E+03
Ser	541	±	101	651	±	99	869	±	60	3.96E+03	±	2.42E+03
Thr	557	±	96	940	±	158	984	±	206	4.16E+03	±	2.27E+03
Trp	64.2	±	15.6	142	±	22	120	±	24	1.21E+03	±	5.07E+02
Tyr	183	±	58	344	±	30	362	±	72	3.13E+03	±	1.50E+03
Val	182	±	34	444	±	78	520	±	53	5.69E+03	±	2.20E+03
GABA	929	±	181	2.97E+03	±	3.09E+02	3.48E+03	±	2.78E+02	1.17E+03	±	8.07E+02
AC	3.59E+03	±	2.01E+02	2.46E+03	±	2.45E+02	427	±	35	203	±	93
EP		–			–			–			–	
NE	104	±	14	90.0	±	51.7		–			–	
DA		–			–			–		1.82E+03	±	4.04E+02
5-HT	66.4	±	18.9		–			–			–	
5-HTP		–			–			–			–	
5-HIAA		–			–			–			–	
L-DOPA		–			–			–			–	
MLT	0.09	±	0.03	0.20	±	0.02		–			–	

Concentration of amino acids and neurotransmitters in rat brain were assayed by the method developed in this study as described in "materials and methods". Data were shown as the means ± S.D. for 5 animals (μ mol / L or g of tissue weight). " – " mean as "LOD".

Table 5. Contents of BCAA in rat muscle type and Tukey-Kramer Multiple Comparisons

Muscle type	BCAA Contents (n mol / g of Tissue)			Total
	Leu	Ile	Val	
Soleus muscle (SM)	410.8 ± 86.6	261.8 ± 63.1	266.3 ± 33.7	938.8 ± 182.1
Extensor digitorum longus muscle (EDL)	246.2 ± 21.2	155.7 ± 12.1	214.3 ± 20.7	616.2 ± 53.6
Gastrocnemius muscle (GM)	24.0 ± 0.9	17.1 ± 0.5	14.8 ± 0.5	55.3 ± 2.7

Tukey-Kramer multiple comparisons

Muscle type	Leu	Ile	Val
	Absolute Difference		
GM : SM	15.03**	13.20**	22.04**
GM : EDL	8.64**	7.47*	17.49**
SM : EDL	6.4*	5.72*	4.55

*: $p < 0.05$

**: $p < 0.01$

Extraction procedure

Weighing of tissue

added per gram of tissue by adding 4.0 mL solution
(acetonitrile : water with 0.1% formin acid ; 4:6, v/v)
added 1.0 mL of 100 μ mol / L DA-d$_4$, 5-HT-d$_4$ and GABA-d$_6$
homogenized with biotechnology mixer
centrifuged at 8,000 rpm (10,160 × g) for 20 min

Supernatant

1.0 mL of supernatant

added 1 mL of hexane (saturated with acetonitrile)
mixed by vortex for 10 sec and centrifuged at 3,000 rpm (700 × g) for 5 min
filtrated by centrifugation at 12,000 rpm (11,400 × g) for 5 min
(Ultra free-MC 0.22 μm pore size, Millipore)

Sample for LC-MS/MS and LC-TOF MS analyses

Fig. 1   **Preparation procedure for the MS analyses from rat organs**

Amino acids and catecholamine were extracted from rat organs with acetonitrile solution, and the internal standards (DA, 5-HT and GABA transferred 4 molecules of hydrogen to 4 of deuterium (DA-d$_4$), 4 of hydrogen (5-HT-d$_4$) and 6 of hydrogen (GABA-d$_6$), respectively) were added into the preparations.
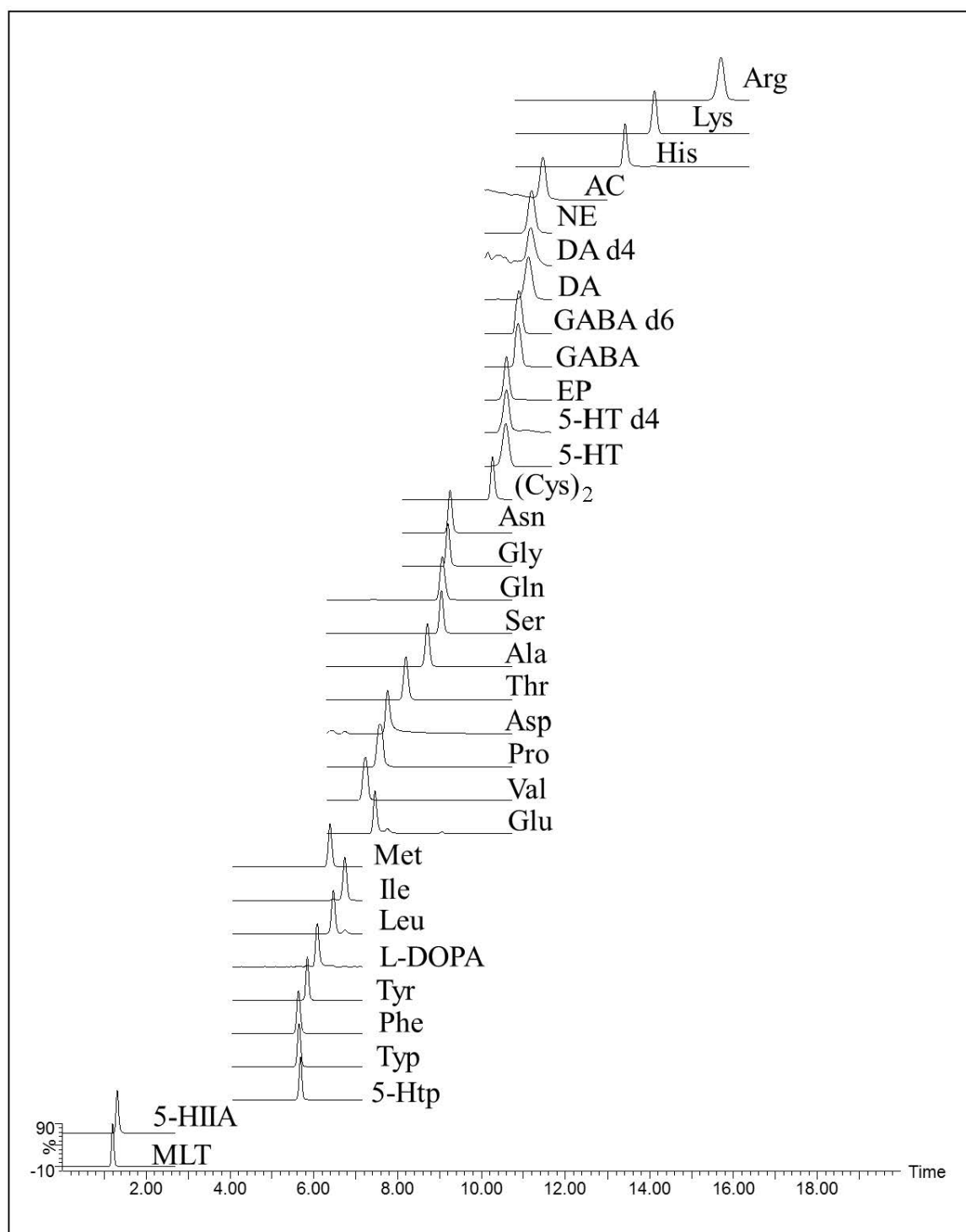
Fig. 2 **Separation of amino acids and neurotransmitters by LC-MS using an "Intrada Amino Acid" column**

The quantitative ions of each compound determined in Table 1 were monitored with positive ion mode scanning of a gradient mobile phase, as described in the Materials and Methods. All compounds were analyzed within 20 min by the analytical method.
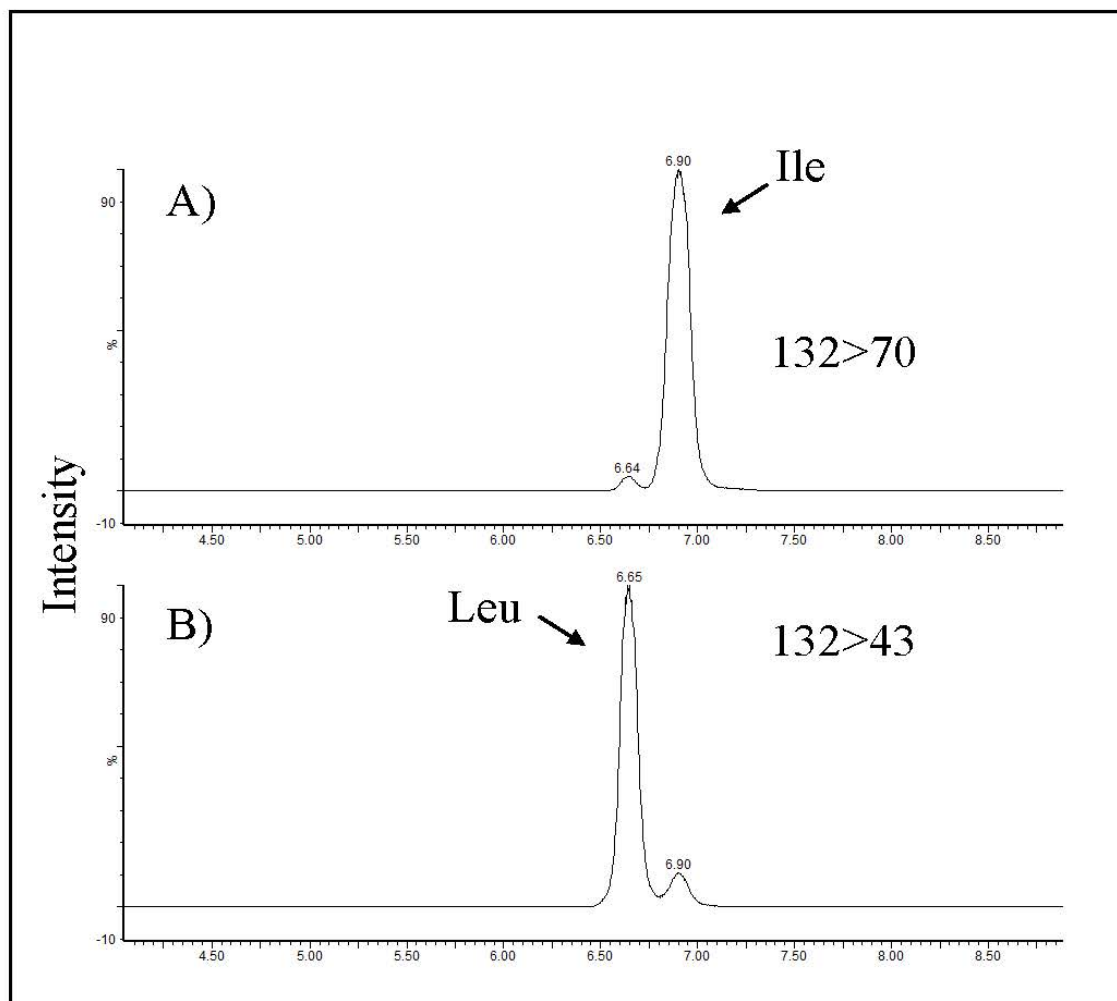
Fig.3   **Separation of Leu and Ile by LC-MS using "Intrada Amino Acid" column**

Standard Leu and Ile were separated completely within 7 min. The positive ions having 70 m/z derived from precursor Ile and 43 m/z peak derived from Leu showed a single maximum peak, respectively (A,B). The quantitative and first target ions of the all compounds tested determined as showing in the figure and listed in Table 1.
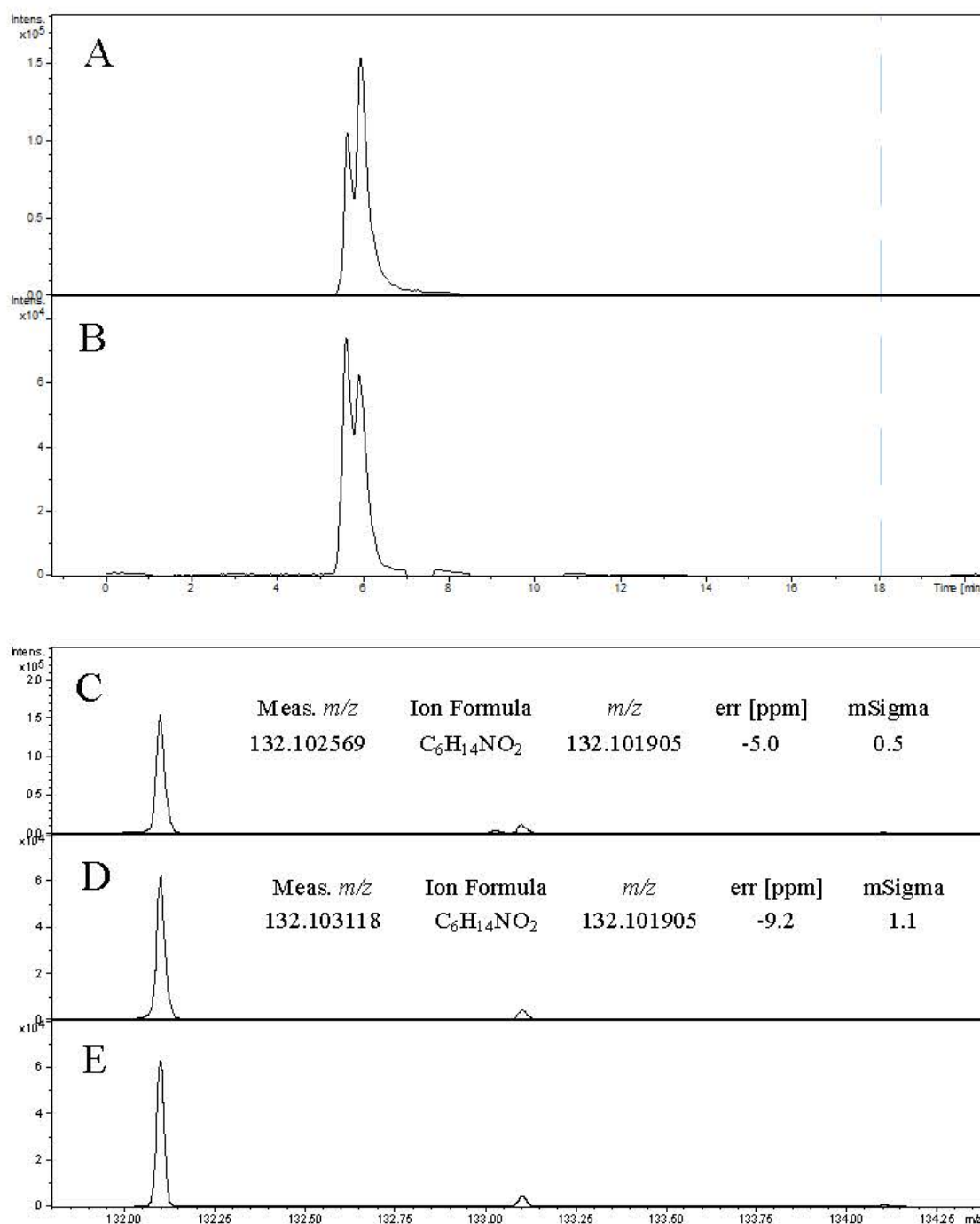
Fig. 4   **Identification of Leu by LC-TOF MS analysis**

The chromatography patterns of the standard Leu (A) and extracted Leu from the rat muscle (B) were shown using LC-TOF MS monitoring with 132.101905 ± 0.05 (m/z). The mass spectrum of the peak that had a retention time at 5.80 min in the standard shown in A panel (C) and in the extracted peak in the B panel (D) using LC-TOF MS analysis. The mass spectrum of the theoretical pattern of standard Leu is also shown (E). A small d-value (sigma value) was observed at 0.00011, and the mass error was only 9.2 mDa in the D panel, which was identified as Leu. Other compounds were all identified with the SigmaFit analysis as in the figure.
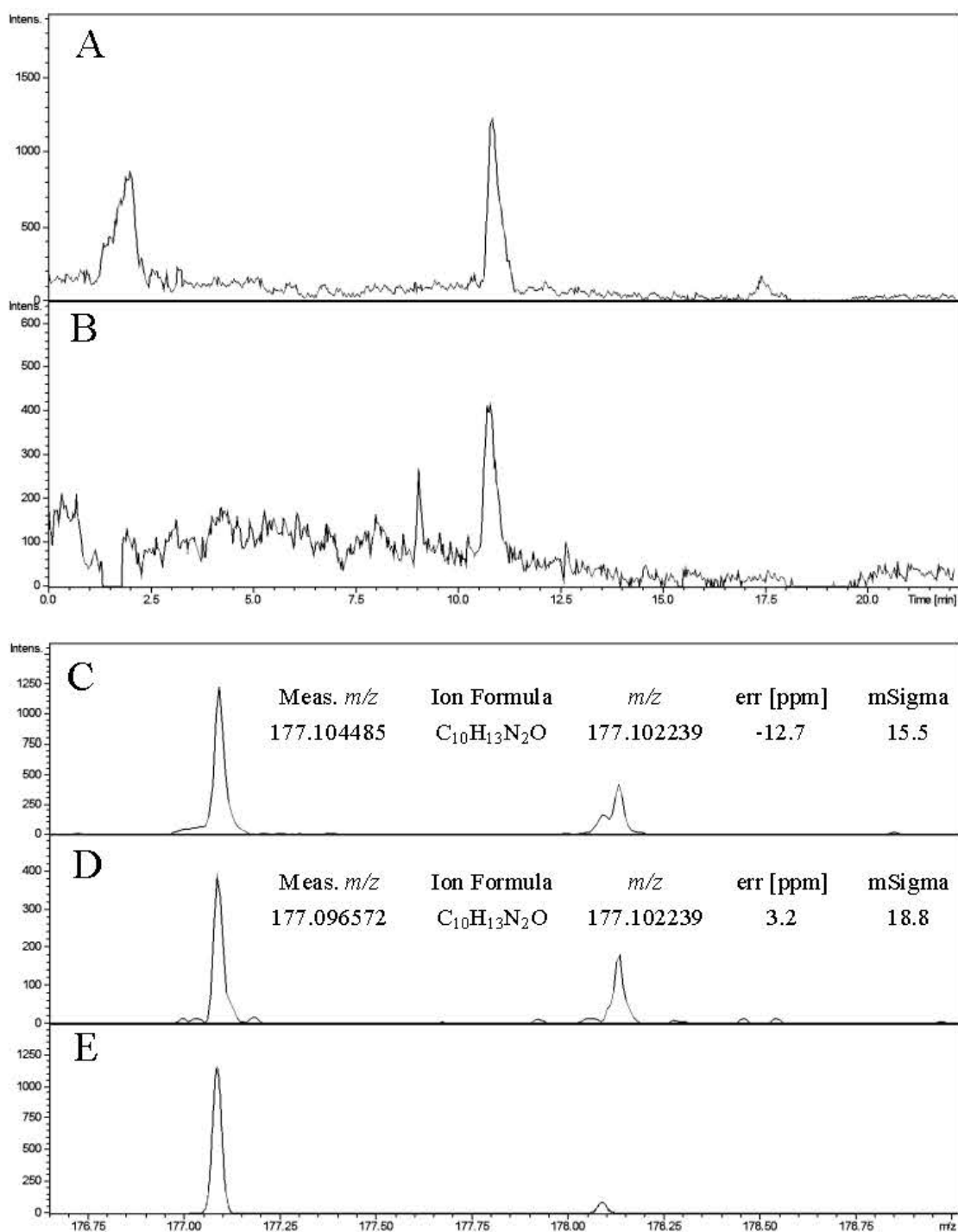
Fig.5 **Identification of 5-HT by LC-TOF MS analysis**

The chromatography patterns of the standard 5-HT (A) and extracted 5-HT from the rat muscle (B) were shown using LC-TOF MS monitoring with 177.102239 ± 0.05 (m/z). The mass spectrum of the peak that had a retention time at 10.8 min in the standard shown in A panel (C) and in the extracted peak in the B (D) panel using LC-TOF MS analysis. The mass spectrum of the theoretical pattern of standard 5-HT is also shown (E). A small d-value (sigma value) was observed at 0.00188, and the mass error was only 3.2 mDa in the D panel, which was identified as 5-HT. Other compounds were all identified with the Sig-maFit analysis as in the figure.