Measuring the beneficial actions of the nitric oxide and amino acid neurotransmitters in chronic and acute pain in humans – A comparative study

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Abstract

Chronic pain although triggered by an injury or tissue damage, may be perpetuated by factors other than the cause of the pain itself thus becoming out of proportion to the original injury itself. Continual and prolonged stimulation by these neurotransmitters can result in chronic persistent pain. Acute pain is biologically useful as a warning system and subsides as healing signs of progress while chronic pain is more challenging, does not spontaneously resolve and serves no useful biological function. The amino acids are known to be involved in chronic pain mechanisms per se, the differential roles of these amino acid neurotransmitters and modulators in acute and chronic pain states have not been compared so far.

A standard amino acid mixture was prepared and tabulated each time with the CSF samples and analyzed using HPLC. After HPLC separation, the amounts of the different compounds in the CSF samples were calculated by comparing the peak areas in the HPLC run of the CSF sample with the corresponding peak areas in the desolated Std AA, which was also run on the same day. A duplicate analysis was performed for each CSF sample to minimize the variance.

All these three studies have consistently reported a positive correlation between age and CSF concentrations of aspartate, isoleucine, leucine, phenylalanine and valine. These amino acids were all reported to be significantly increased in CSF of elderly patients. In addition, Ferraro and Hare suggested that GABA and serine in CSF significantly decreased in elderly patients. Hence when evaluating the differential roles of these amino acids in chronic and acute pain states, the influence of age of the patients in these groups cannot be neglected.

These results highlight the important role of aspartate in unrelenting chronic pain and thus could be a signal for chronification of pain conditions. Hence this amino acid could also be a possible chronic pain indicator and thus support the role of NMDA receptor antagonists as reasonable targets for designing new effective drugs for chronic pain.

Key Words: standard amino acid mixture, chronic pain, antagonists, minimize the variance, prolonged stimulation

Introduction

Acute pain is elicited by substantial injury of body tissue and activation of nociceptive transducers at the site of local tissue damage. Chronic pain although triggered by an injury or tissue damage, may be perpetuated by factors other than the cause of the pain itself thus becoming out of proportion to the original injury itself (Loeser and Melzack, 1999). The EAAs glutamate and aspartate, IAAs glycine, GABA and taurine, NO, prostaglandins and other neurotransmitters play important roles in pain perception (Wen et al., 2003; Cronin et al., 2004; El Idrissi and Trenkner, 2004). Continual and prolonged stimulation by these neurotransmitters can result in chronic persistent pain. Acute pain is biologically useful as a warning system and subsides as healing signs of progress while chronic pain is more challenging, does not spontaneously resolve and serves no useful biological function.

Though amino acid analysis studies date back more than 50 years, there have been very few reports on CSF amino acid levels in pain states. Two of them have analyzed only the EAAs in headaches (Castillo et al., 1995; Gallai et al., 2003). A third report analyzed the CSF levels of the EAAs and also glycine, taurine, arginine and citrulline in fibromyalgia syndrome (Larson et al., 2000) and found an interesting correlation between the tender point index (pain scale) in these patients and some of these amino acid levels. Though these amino acids are known to be involved in chronic pain mechanisms per se, the differential roles of these amino acid neurotransmitters and modulators in acute and chronic pain states have not been compared so far.

In this study, three groups of subjects were chosen – acute pain, chronic pain and no pain control to study the roles of nine pain-related amino acids described in paper in the mechanisms of pain perception. The HPLC method, recently developed in our laboratory (Sethuraman et al., 2004) as described in paper for quantitatively measuring the physiological amino acids in CSF without pretreatment was applied. The levels of these neurotransmitters and modulators were quantitatively analyzed in the CSF of chronic pain patients and compared with their levels in the no pain control group and in the acute pain group to analyze which of these amino acids were involved in the establishment of chronic persistent pain. Statistically significant differences in the CSF levels of some amino acids between these groups were observed, which suggested that amino acid neurotransmitters were differentially involved in the two types of pain conditions.

Materials and Methods

Collection of CSF samples

The experimental protocol was approved by the Institutional Review Board. CSF samples were collected from patients after written informed consent was obtained. Three groups of patients

were involved in this study – (1) chronic pain female patients (n = 29) with chronic osteoarthritis of the knees undergoing joint replacement surgery or with chronic backache undergoing myelogram and (2) a no pain female control group (n =35) comprising of pregnant women scheduled for elective Cesarean delivery, womb removal or renal impairment patients (3) acute pain female patients (n = 46) comprising of acute labour pain patients and few with fractures.

Patients in the labour pain group received a combined spinal-epidural technique for pain relief. Patients in the non-pain group received spinal anaesthesia for their operative delivery or other surgery. Patients in the chronic pain group received spinal anaesthesia before their joint replacement surgery or the myelogram procedure. 0.5 to 1 ml of CSF was collected from the patients after successful subarachnoid puncture before intrathecal injection of analgesics. All the CSF samples were kept frozen at -80°C immediately after collection until final analysis. During the collection of samples, CSF was collected from both male and female patients. The summary of patient details from whom the CSF samples were collected and analyzed are summarized below:

Clinical Data of Patients

	Chronic Pain (n=48)	**Acute Pain (n=59)**	**No pain (n=60)**
Age Mean(range)	63.2(32-87)	40.7(20-97)	50.0(23-83)
Sex male/female	19/29	13/46	25/35
Clinical condition (no. of patients)	Knee pain/OA (30) Back pain (6) Others (11)	Labor pain (38) Fractures (7) Others (14)	Pregnant caesarean(30) TURP/prostate (12) Others (18)

The number of male subjects in all the three groups of patients was relatively lower than female subjects and the number of males in each group was not sufficient (n was small) to compare the data from these subjects, between the groups. Hence only the analysis data of female subjects were chosen for comparison.

HPLC analysis

CSF analysis was performed following the same protocol as explained in the paper. The damsel chloride derivatization procedure and the HPLC conditions used were discussed in paper (Sethuraman et al., 2004). A standard amino acid mixture (Std AA) was prepared and tabulated

each time with the CSF samples and analyzed using HPLC. After HPLC separation, the amounts of the different compounds in the CSF samples were calculated by comparing the peak areas in the HPLC run of the CSF sample with the corresponding peak areas in the desolated Std AA, which was also run on the same day. A duplicate analysis was performed for each CSF sample to minimize the variance.

Statistical analysis

All amino acid concentrations were expressed as mean ± SEM.

Statistical comparison of amino acids concentrations in the different groups of patients was performed using the student's *t*-test –

Labour pain vs. other acute pain

1. ***Acute male vs. acute female***
1. ***Chronic pain vs. control with no pain***
1. ***Chronic pain vs. acute pain***

SPSS (version 12.0) statistical software was used. A *P* value of less than 0.05 was considered statistically significant.

Results

Although labour pain is considered a typical acute pain (Melzack, 1993), we compared 38 labour pain CSF samples with the other acute female samples and found there were no significant differences between the corresponding amino acids except GABA. On the other hand, there were significant differences in many amino acids between male and female acute pain CSF samples. Therefore, in this study, we limited to female CSF sample data for comparison and labour pain and other acute pain samples were pooled into the acute pain group and applied to the comparison study. The typical separation of the compounds of interest in the CSF from the three groups of patients is shown in the chromatograms.

Statistical analysis results are discussed in two groups as indicated below –

(a) Chronic pain vs. control with no pain

(b) Chronic pain vs. acute pain

The amounts of the nine pain-related amino acids in the CSF of chronic pain (n = 29), no pain control (n = 35) and acute pain (n = 46) groups are shown in Table

Analysis of EAAs – aspartame, glutamate and their metabolites

(a) Aspartate and glutamate in the chronic pain group were significantly higher ($P < 0.01$) than that of the no pain control. Asparagine, the metabolite of aspartate in the chronic pain group was also significantly higher ($P < 0.01$) than in the control group. Glutamine levels were not significantly different between the chronic pain and no pain groups.

(b) Aspartate in the chronic pain group was significantly higher ($P < 0.01$) than that of the acute pain group while glutamate did not show any significant difference. Asparagine and glutamine, the respective metabolites of aspartate and glutamate, in the chronic pain group were also significantly higher ($P < 0.001$) than in the acute pain group.

Analysis of IAAs – glycine, GABA and taurine

(a) Glycine in the chronic pain group was significantly higher ($P < 0.01$) while the levels of taurine and GABA, though lower in chronic pain, were not significantly different as compared to the control group.

(b) GABA in the chronic pain group was significantly decreased ($P < 0.05$) as compared to the acute pain group while taurine and glycine were not significantly different between the two groups.

Analysis of citrulline and arginine (NO indicators)

(a) The level of citrulline, which is the by-product of NO formation from arginine, in the chronic pain group was significantly higher ($P< 0.001$) as compared to the no pain control group. Citrulline was detected in 17 out of the 29 chronic pain CSF samples (1.65 ± 0.36 µmol/L) and only 3 out of 35 CSF samples in the no pain control group showed a very small amount of citrulline. Arginine, which is the substrate of NO synthase was also significantly higher in the chronic pain group as compared to the control group ($P < 0.001$).

(b) No significant differences were observed in the concentrations of citrulline between the chronic pain and acute pain group. CSF arginine levels were significantly higher in the chronic pain group as compared to the acute pain group ($P < 0.001$).

Discussion

Acute and chronic pains though initiated by tissue damage or injury, are very different from each other. Chronic pain is more challenging and unrelenting. Prolonged activation of nociceptors evokes continuous release of EAAs, which in combination with co-released neuropeptides like substance P etc… play a pivotal role in the chronification of pain (Chizh, 2002). The levels of

various amino acids were determined in the CSF of the chronic pain group and compared with no pain control group to evaluate the role of these compounds in chronic pain per se. Then by comparing the concentrations of these amino acids in the chronic pain group with their levels in the acute pain group, the differential roles of these amino acids in these two types of pain were evaluated. The focus of this work was mainly on the amino acids, known to modulate pain.

In our pursuit to quantitatively analyze physiological amino acids in CSF, several samples from chronic pain, acute pain and no pain groups including both male and female subjects have been analyzed. Previous studies on the effect of gender on CSF amino acids concentrations, reported that only the arginine and tyrosine levels in CSF of males are significantly higher than in females (Ferraro and Hare, 1985) while Kornhuber et al (1988) stated that there was no consistent relation between gender and CSF amino acid levels. Before choosing the data for this comparative study, the samples in the different groups were separated based on their gender and statistically compared.

Statistically significant differences in many amino acids were observed between male and female samples in both acute pain and no pain control group. Hence to nullify the impact of gender in our study, it was decided to choose only the female subjects from both acute and no pain groups to compare with the chronic pain group also comprised of only female subjects.The acute pain group in our study had both labour pain patients and other non-pregnant patients similar to our no-pain group. Hence another comparison was performed between the amino acid levels in acute labour pain and other acute pain patient groups and since no statistically significant differences were seen between these groups except for GABA, it was decided to pool the labour pain and other acute pain samples.

The next concern was the age differences between the different groups. The chronic pain group in our study were all elderly patients as compared to the other two groups. The influence of age on CSF amino acids was investigated by different studies (Gjessing et al., 1974; Goodnick et al., 1980; Ferraro and Hare, 1985). All these three studies have consistently reported a positive correlation between age and CSF concentrations of aspartate, isoleucine, leucine, phenylalanine and valine. These amino acids were all reported to be significantly increased in CSF of elderly patients (Ferraro and Hare, 1985). In addition, Ferraro and Hare suggested that GABA and serine in CSF significantly decreased in elderly patients. Hence when evaluating the differential roles of these amino acids in chronic and acute pain states, the influence of age of the patients in these groups cannot be neglected.

Excitatory amino acids

The EAAs aspartate and glutamate were significantly higher in the chronic pain group ($P < 0.01$) as compared to the no pain group. This suggests the involvement of the EAAs, aspartate and glutamate in chronic pain mechanisms. Asparagine, the metabolite of aspartame was also higher ($P < 0.01$) in the chronic pain group as compared to the control. This is justified as asparagine concentration is projected to be higher in the pain group (Bergles et al., 1999) and further supports the involvement of aspartate in chronic pain. However, glutamine concentration was not significantly different between the two groups.

In chronic pain, aspartate ($P < 0.01$) was significantly higher than in acute pain suggesting aspartate may have important contributions to the establishment of a persistent chronic pain state. In chronic pain syndromes, the intensity of pain is out of proportion to the original injury or tissue damage (Loeser and Melzack, 1999) unlike acute pain and hence the observed higher aspartate in chronic pain patients should have stimulatory effects on the pain processing pathway. The chronic pain group consisted of elderly patients as compared to the acute labour pain group and aspartate levels have been reported to increase in CSF with the age of the patients but glutamate, glutamine and asparagine levels were not influenced by age of the subjects (Ferraro and Hare, 1985). Though the increase in aspartate observed in chronic pain patients could also be the effect of age, the significant increase observed in asparagine ($P < 0.001$) – the metabolite of aspartate in the chronic pain group further supports the hypothesis that aspartate levels are increased due to the chronic pain condition itself. Glutamate levels though higher in the chronic pain group as compared to acute pain (4.42 µmol/L vs. 2.86 µmol/L); this difference was not statistically significant. This could also be due to insufficient numbers (n = 29) in the chronic pain group. The glutamine levels, however, were significantly higher in the chronic pain group as against the acute pain group ($P < 0.001$) which hints that the glutamate levels could be significantly higher with an increase in the number of samples. Taken together, the above two sets of the comparison indicate, that the EAAs aspartate and glutamate have vital roles in chronic pain mechanisms. Aspartate in particular is important in the carbonification of pain states.

Inhibitory amino acids

IAAs glycine, GABA, and taurine are also known to modulate pain perception. However, a dual role has been suggested for glycine in pain perception. In addition to being the primary inhibitory neurotransmitter in the spinal cord and brain stem, it is an obligatory coagonist at the excitatory glutamate receptors of the NMDA subtype (Chatterton et al., 2002, Ahmadi et al., 2003) and the affinity of glycine to the NMDA receptors is much higher than its affinity to the strychnine-sensitive glycine receptors, which mediate the anti-nociceptive effect (Becker et al.,

1988). As discussed earlier in the discussion in the paper, synoptically released glycine might be detected in the CSF. Therefore, CSF glycine levels were determined to analyze if extracellular glycine in the CSF might reflect pain status clinically.

As shown in Table, glycine level in the chronic pain group was significantly higher than in the non-pain control group (10.80 µmol/L vs. 4.86 µmol/L, $P < 0.01$). This suggested the involvement of glycine in chronic pain via the NMDA subtype excitatory glutamate receptors. Though there was some increase in glycine in the chronic pain group as compared to the acute pain group, the increase was not statistically significant (10.50 µmol/L vs. 8.18 µmol/L). As already shown in the Paper, glycine is involved in acute pain mechanisms. However, higher glycine levels sustained in the chronic pain group as compared to acute pain patients suggested glycine might contribute to acute pain becoming chronic pain states.

The level of GABA was not significantly different between the chronic pain and the control group. In the second comparison between the chronic and acute pain groups, GABA levels were significantly lower ($P < 0.05$) in the chronic pain group. As mentioned earlier, in the comparison between labour pain and other acute pain, GABA was different between these groups. GABA might be affected by pregnancy and also the age of the patients (Ferraro and Hare, 1985). Hence the GABA levels in the chronic and acute pain groups in this study cannot be compared. Taurine levels were not different in both the comparisons – chronic pain vs. no pain control and chronic pain vs. acute pain group. This suggests taurine may not have important implications in chronic pain mechanisms.

Citrulline – Surrogate of NO

NO has been accepted as one of the neurotransmitters involved in pain perception (Yaksh et al., 1999; Lewin et al., 2004). NO was analyzed indirectly in this study too, by analyzing the amount of citrulline, a byproduct of NO synthesis from arginine. A similar method was applied to patients with fibromyalgia (Larson et al., 2000) and similarly, Perez-Neri et al. (2004) reported that the citrulline levels in CSF reflected actual NO biosynthesis. The citrulline and arginine levels in chronic pain patients were both significantly higher than the control no pain group ($P < 0.001$). NO is formed by nitric oxide synthase which oxidizes L-arginine to citrulline and NO and it has been shown that the reaction product NO acts as a feedback inhibitor of the neuronal enzyme nitric oxide synthase (Wang et al., 1994; Abu-Soud et al., 1995). Such a feedback inhibition by NO itself would account for the higher arginine levels in addition to high citrulline in chronic pain patients. This suggests NO is involved in the mechanism of pain perception in these patients.

Citruline levels in chronic pain patients were not significantly different from those in the acute pain group although the chronic pain patients had significantly high levels of arginine as compared to the acute pain group ($P < 0.001$). Citrulline and arginine levels in CSF were not influenced by the age of the subjects. Arginine transport and biosynthesis are both stimulated by cytokines such as interleukin-1 and interferon-γ (Simmons et al., 1996; Perez-Neri et al., 2004), so its concentration is expected to increase during any immune response. Also, arginine levels are regulated by several other mechanisms, such as metabolic consumption and efflux transport across the blood-brain barrier (Wiesinger, 2001). Since there were no significant differences in citrulline levels in this comparison, only the arginine increase cannot be taken as an indicator of NO involvement in chronic pain state. Hence NO, though involved in pain perception, may not contribute to the development of a persistent, chronic pain state.

Table - 1: Comparison of concentration of pain-related amino acids in CSF – Acute labor pain vs other acute pain group.

Amino acids	Acute labor pain[a] (µmol/L) (n = 38)	Other acute pain[b] (µmol/L) (n = 8)
Arginine	13.51 ± 0.56	23.64 ± 4.69
Asparagine	10.19 ± 0.51	13.89 ± 1.73
Aspartate	6.63 ± 0.41	7.78 ± 2.91
Citrulline	2.11 ± 0.42	1.52 ± 0.68
GABA	5.71 ± 0.73*	2.46 ± 0.86
Glutamate	2.48 ± 0.18	4.69 ± 2.54
Glutamine	583.65 ± 16.36	663.22 ± 103.08
Glycine	8.18 ± 1.26	8.17 ± 2.34
Taurine	9.85 ± 1.01	10.15 ± 2.76

Values are expressed as mean ± SEM
[a] Women with acute labor pain
[b] Women in other acute pain
* ($P < 0.05$) – Statistically significant in the comparison.

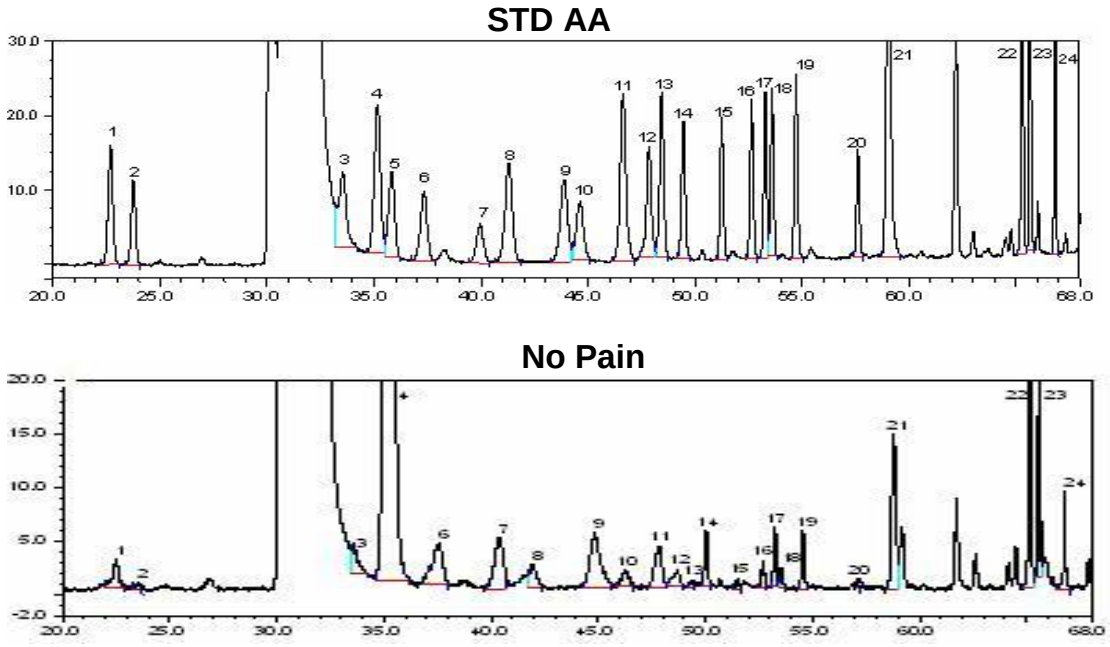
In summary, the EAAs – aspartate and glutamate, NO and Glycine are involved in the mechanism of chronic pain perception. Aspartate in particular seems to have a vital role in the establishment of persistent, chronic pain state from acute pain. Glutamate and NO though involved in pain perception may not directly contribute to pain chronification. The other IAAs GABA and taurine are not the major contributors to chronic pain mechanisms. These results highlight the important role of aspartate in unrelenting chronic pain and thus could be a signal for chronification of pain conditions. Hence this amino acid could also be a possible chronic pain indicator and thus

support the role of NMDA receptor antagonists as reasonable targets for designing new effective drugs for chronic pain.

Table - 2: Comparison of concentration of pain-related amino acids in CSF Acute pain MALE Vs Acute pain FEMALE group.

Amino acids	Acute pain MALE[a] (µmol/L) (n = 13)	Acute pain FEMALE[b] (µmol/L) (n = 46)
Arginine	15.27 ± 1.07**	33.22 ± 4.62
Asparagine	10.84 ± 0.55*	18.21 ± 2.38
Aspartate	6.83 ± 0.59	6.12 ± 1.56
Citrulline	2.00 ± 0.37	1.34 ± 0.44
GABA	5.14 ± 0.64	3.35 ± 0.64
Glutamate	2.86 ± 0.46	5.31 ± 1.32
Glutamine	597.49 ± 22.12***	1141.59 ± 115.89
Glycine	8.18 ± 1.11	10.50 ± 1.55
Taurine	9.90 ± 0.95*	18.26 ± 2.87

Values are expressed as mean ± SEM
[a] Male with acute pain
[b] Female in acute pain
* ($P < 0.05$), ** ($P < 0.01$) *** ($P < 0.001$) – Statistically significant in the comparison.



Actual Plan

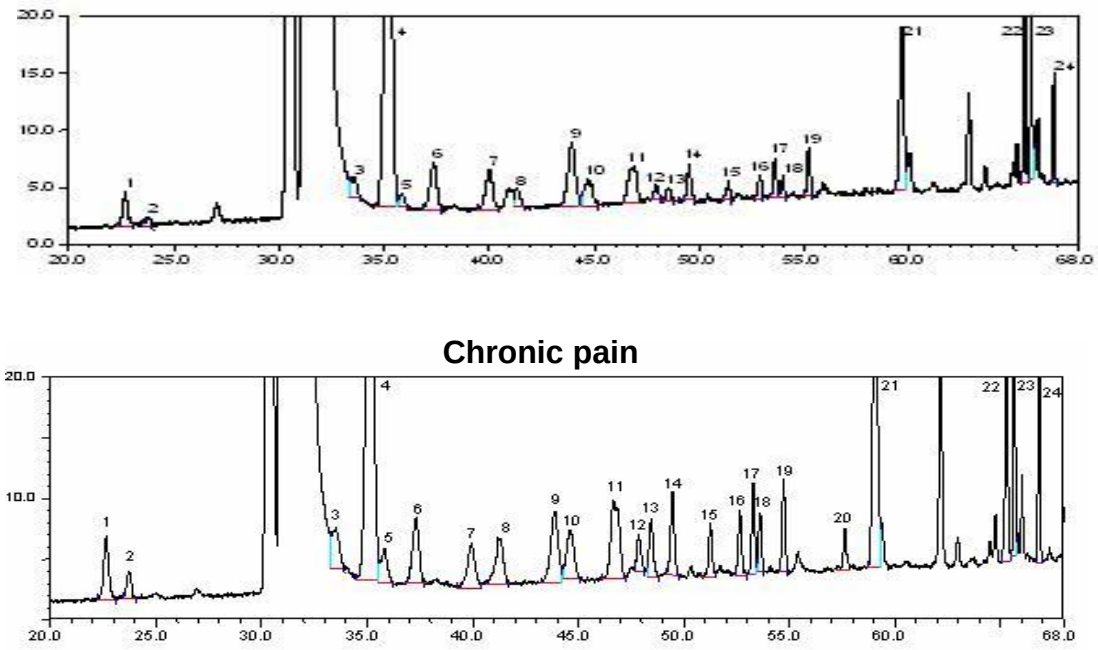


Figure: Chromatograms of CSF samples from the three patient groups

Peaks: 1-aspartate; 2-glutamate; 3-asparagine; 4-glutamine; 5-citrulline; 6-serine; 7-threonine; 8-glycine; 9-alanine; 10-arginine; 11-taurine; 12-GABA; 13-proline; 14-valine; 15-methionine; 16-isoleucine; 17-leucine; 18-tryptophan; 19-phenylalanine; 20-cystine; 21-ammonia; 22-lysine; 23-histidine; 24-tyrosine.