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# Assessment of plasma amino acid profile in autism using cation-exchange chromatography with postcolumn derivatization by ninhydrin

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**Background/aim:** Autism is a heterogeneous neurodevelopmental disorder. This study aimed to assess the clinical significance of amino acid profile assay in autism using cation-exchange chromatography with ninhydrin postcolumn derivatization.

**Materials and methods:** This study included 42 autistic children and 26 apparently healthy children. All participants were subjected to the assay of plasma amino acids (essential, nonessential, and nonstandard) using cation-exchange chromatography with postcolumn derivatization by ninhydrin.

**Results:** The levels of most of the essential amino acids were significantly lower in autistic children than controls. As regards nonessential amino acids, significantly lower levels for plasma cysteine, tyrosine, and serine and significantly higher levels for plasma glutamic acid were recorded in autistic children than controls. Finally, the autistic group demonstrated significantly lower levels of α-aminoadipic acid, carnosine, and β-alanine and significantly higher levels of hydroxyproline, phosphoserine, β-amino-isobutyric acid, and ammonia as compared to controls.

**Conclusion:** The study revealed that autistic children exhibit distinct alterations in the plasma levels of some amino acids, which can in turn participate in the disease etiology and can be applied as a diagnostic tool for early detection of autism.

**Key words:** Autism, amino acids, cation-exchange chromatography

## 1. Introduction

Autism is a set of pervasive heterogeneous neurodevelopmental disorders characterized by early onset of impairment of reciprocal social interactions and communication development along with extremely restricted and repetitive stereotyped behaviors (1). The worldwide prevalence of autism is about 1% (2).

There is substantial evidence implicating genetic heritability and environmental factors in addition to oxidative stress, inflammation, and immune dysregulation in the pathogenesis of autism. Although no single coherent explanation has emerged (3), some recent research studies suggested the implication of abnormalities in plasma amino acids in the etiology of autism (4–6).

The aim of the present study was to assess the clinical significance of plasma amino acid profile assay in autism using cation-exchange chromatography with ninhydrin postcolumn derivatization.

## 2. Material and methods

This study was conducted from February to August 2014 at the Medical Genetics Center, Ain Shams University Hospitals, Cairo, Egypt. The study protocol was approved by the ethical committee of the Faculty of Medicine of Ain Shams University.

### 2.1. Subjects

The study included Group 1, which included 42 autistic Egyptian children (34 males and 8 females), aged 2–14 years old. The diagnosis of autism was confirmed by DSM-IV criteria (7). The diagnosis was in accordance with Autism Diagnostic Interview-Revised (ADI-R) (8). In addition, Group II included 26 age- and sex-matched apparently healthy children serving as healthy controls. Patients with psychotic disorders, active substance dependence, evidence of liver disease, seizure disorder, unstable hypertension, cardiac disease, or kidney disease were excluded from the study. Each autistic child included in the study was subjected to full history taking with special stress on history of vaccinations, drug intake, infection,

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and developmental motor and mental milestones and clinical examination with special emphasis on the neurological examination and psychiatric evaluation. All participants included in the study were subjected to the quantitative determination of plasma amino acids (essential, nonessential, and nonstandard amino acids).

**2.2. Sampling**

Three milliliters of overnight fasting venous blood was withdrawn into sterile EDTA vacutainers under completely aseptic conditions. Plasma was separated immediately by centrifugation and was stored in aliquots at –80 °C until the assay. Any hemolyzed or lipemic sample was discarded.

**2.3. Amino acid assay**

The quantitative determination of plasma amino acids was performed by a high-performance liquid chromatography (HPLC) technique using cation-exchange resin and postcolumn derivatization with ninhydrin (9).

**2.3.1. Sample preparation**

An acid precipitation method was applied immediately for the precipitation of proteins and peptides in the sample. In this method, 800 µL of plasma was added to 200 µL of sulphosalicylic acid (10%). After being stored at 4 °C for 30 min, the sample was centrifuged at 13,000 rpm for 10 min and the resulting upper clear solution was dissolved in an equal volume of the sample diluent buffer (lithium citrate). If not analyzed immediately, the sample was stored at –80 °C.

**2.3.2. Working external standards**

Ready-to-use aqueous standard solutions were available for all the measured amino acids. The stock solution of the amino acids was prepared in 0.1% N HCL and 0.1% phenol. The working standard solution was prepared by adding 2 mL of the standard solution to 8 mL of sample diluent buffer (pH 2.20); this was followed by preparation of serial dilutions of the different working standards.

**2.3.3. Ninhydrin preparation**

First, 0.8 g of reducing agent hydrantin was dissolved in 30 mL of ethylene glycol monomethyl ether and was added to the ninhydrin solution. The solution was then flushed with $N_2$ gas for 5 min in order to get rid of oxygen and dissolved gases. The ninhydrin was then stored under continuous nitrogen pressure for 2–3 days before the analysis.

**2.3.4. Buffer system**

The ready-to-use buffer system consisted of three lithium buffers (A, B, and C) (i.e. lithium as the cationic counter-ion).

**2.3.5. Chromatographic analysis**

The assay was performed on the Sykam amino acid analyzer (S-433; Eresing, Germany). The equipment consisted mainly of a solvent delivery system with fully programmable quaternary gradient pumps, a degasser and reagent organizer to keep the solutions free of oxygen and dissolved gases, an autosampler, a separation column filled with cation-exchange resin, a reagent dosing pump for ninhydrin delivery and flushing of the reaction coil after each run, a high-temperature reactor for the color reaction of the amino acid–ninhydrin complex, a dual-channel photometer for amino acid detection at 440 nm and 570 nm wavelengths, and a software system for data integration.

**2.3.6. Principle of amino acid separation and quantification**

Amino acids are zwitterions that include both amino and carboxyl groups in their structures. Therefore, the higher the acidity (more likely to form anions) during the cation exchange, the faster the elution, whereas the more basic the solution, the slower the elution. Hence, the separation of the mixture of amino acids is based on their partitioning behavior between the buffer system and the stationary phase. This phenomenon depends on the different pH values of the buffer solutions, where the activity of buffer A leads to the separation of amino acids from phosphoserine up to glutamic acid, while the separation of α-aminoadipic acid up to α-amino butyric acid is due to the mixture between buffers A and B. The separation of amino acids from valine up to tyrosine was due to the activity of buffer B while the mixture between buffers B and C led to the separation from phenylalanine up to tryptophan. The activity of only buffer C led to the separation from ammonia up to arginine. The quantification of amino acids was based on the postcolumn derivatization of heated amino acid with ninhydrin. All amino acids having a free alpha amino group yield a purple product while proline, which has an imino group, yields a yellow product. Under appropriate conditions, the intensity of the color produced is proportional to the amino acid concentration. The chromatographic profile corresponding to the elution pattern of different amino acids is shown in the Figure.

**2.3.7. Calculation of results**

The final concentrations of amino acids in the samples were obtained from a calibration curve constructed by plotting the peak area of different dilutions of working external standards against their concentrations.

**2.4. Statistical analysis**

Statistical analysis was carried out on a personal computer using SPSS 8 (SPSS Inc., Chicago, IL, USA). Nonparametric qualitative data were expressed in numbers and percentages while nonquantitative parametric data were expressed as medians and interquartile ranges (IQRs). Comparative statistics of qualitative data were obtained using the chi-square test while comparison of quantitative data was done by Wilcoxon's rank sum test. $P < 0.05$ was considered significant and $P < 0.01$ was considered highly significant.

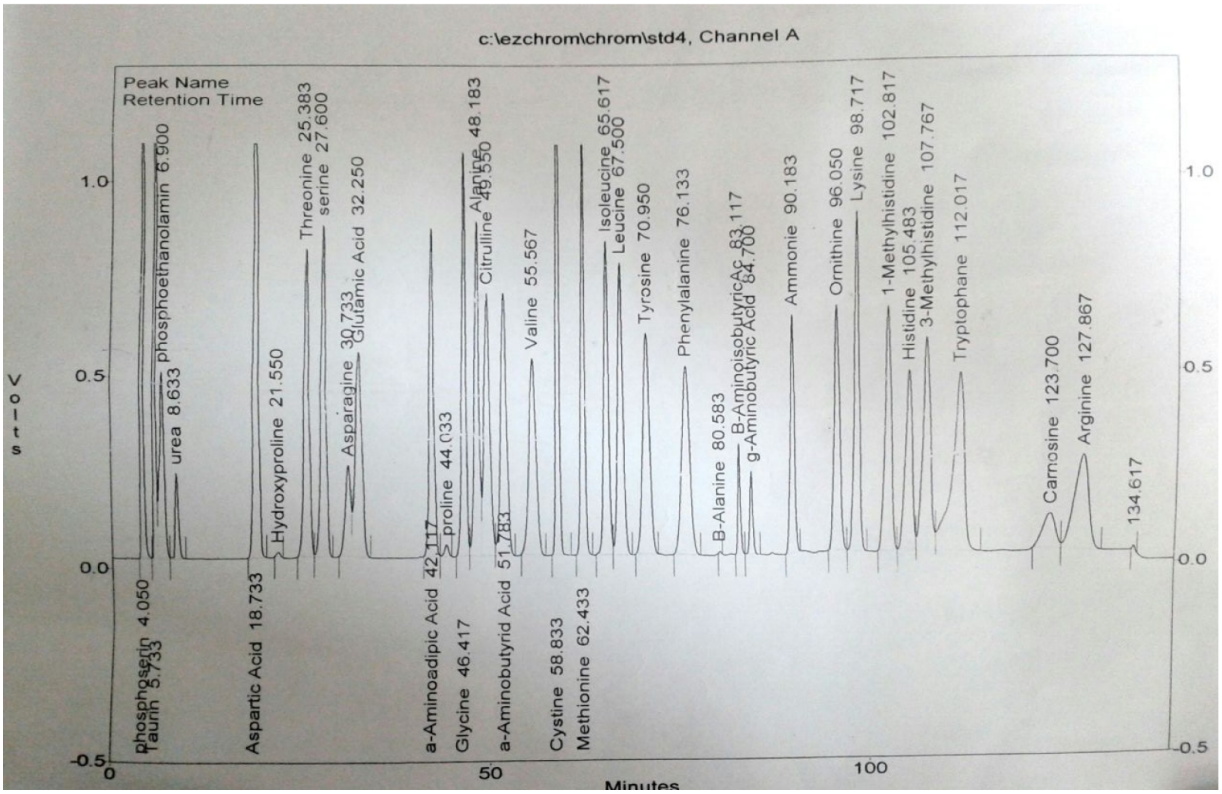


**Figure.** The chromatographic profile corresponding to the elution pattern of different studied plasma amino acids.

## 3. Results

The results of the present study are shown in Tables 1–5. Table 1 shows that 81% of the studied patients were male. The age at onset of disease was below 3 years in 83.3% of children. However, none of the children experienced any antenatal insult and only 9.5% had a postnatal history of fever, infection, fits, or hypoxia. The first alarming manifestation in 66.7% of autistic children was delayed speech, while others experienced loss of eye contact (14.3%), 9.5% like to play alone, and 9.5% lost their attention to their mothers.

Table 2 reveals the presence of a highly significant difference between the groups as regards some clinical data ($P < 0.001$), where 61.9% of autistic children had abnormal social development, 76% had delayed continence, 61.9% had stereotypic movements, 71.4% were hyperactive, and 71.4% had mild to moderate mental retardation.

Table 3 demonstrates a highly significant difference between the groups as regards the essential amino acids methionine, leucine, and phenylalanine ($P < 0.01$ for all), while a significant difference was found in the levels of isoleucine, histidine, tryptophan, and arginine ($P < 0.05$ for all), all being lower in patients as compared to controls.

As regards nonessential amino acids, statistical comparison between the studied groups revealed the presence of a highly significant difference in the level of cysteine ($P < 0.01$) and a significant difference in the levels of tyrosine and serine ($P < 0.05$ for both), all being lower in the autistic children as compared to healthy controls. However, the plasma glutamic acid level was significantly higher in autistic children than controls (Table 4).

Table 5 shows the descriptive and comparative statistics of nonstandard amino acids between patients and control group. Within the autistic group, significantly lower levels were reported regarding α-aminoadipic acid and carnosine ($P < 0.01$ for both) and β-alanine ($P < 0.05$). Meanwhile, significantly higher levels of phosphoserine ($P < 0.01$) and hydroxyproline, β-amino-isobutyric acid, and ammonia ($P < 0.05$ for all) were recorded in autistic children.

## 4. Discussion

Analysis of the demographic data of autistic children included in the present study revealed that most of autistic patients were male. Similar finding were reported by Chang et al. (10) and Majewka et al. (11), who suggested that sex steroids may influence the expression of specific genes in association with autism or may modulate the activity of neurotransmitters in the brain. Moreover, 83% of patients were diagnosed before the age of 3 years old with no antenatal or postnatal history of insult exposure. These data were consistent with those of Atladóttir et al. (12) and Tu et al. (5).

**Table 1.** Descriptive statistics of the demographic and clinical data of autistic patients.

| | Number | % |
|---|---|---|
| Sex<br>Male | 34 | 81 |
| Age at onset<br>Less than 3 years | 35 | 83.3 |
| Antenatal history<br>Drug intake/STORCH infection/radiation exposure | 0 | 0 |
| Normal vaginal delivery | 33 | 78.6 |
| Postnatal history<br>Fever/infection/fits/hypoxia | 4 | 9.5 |
| First alarming specific manifestation<br>Delayed speech<br>Loss of eye contact<br>Likes to play alone<br>Inattention to mother | 28<br>6<br>4<br>4 | 66.7<br>14.3<br>9.5<br>9.5 |

**Table 2.** Descriptive and comparative statistics of the clinical data between patients and control group using chi-square test.

| | Patient group (n = 42) | | Control group (n = 26) | | $\chi^2$ | P-value |
|---|---|---|---|---|---|---|
| | N | % | N | % | | |
| Delayed motor development<br>Head support/sitting/walking | 6 | 14.3 | 2 | 7.7 | 0.34 | 0.56 |
| Abnormal social development<br>Delayed waving 'bye-bye'<br>Afraid of strangers | 26<br>28 | 61.9<br>66.7 | 0<br>24 | 0<br>92.3 | 13.03<br>2.93 | 0.000**<br>0.08 |
| Delayed age of continence | 32 | 76.2 | 4 | 15.4 | 11.92 | <0.001** |
| Intolerance to pain | 38 | 90.5 | 0 | 0 | 26.66 | 0.000** |
| Stereotypic movements | 26 | 61.9 | 0 | 0 | 13.03 | 0.000** |
| Hypotonia/hyporeflexia | 8 | 19 | 0 | 0 | 2.81 | 0.09 |
| Hyperactivity | 30 | 71.4 | 2 | 7.7 | 11.04 | <0.001** |
| Mild to moderate mental retardation | 30 | 71.4 | 0 | 0 | 21.19 | 0.000** |

**Highly significant difference.

Regarding the disease manifestations, all patients included in the study had defects in communication skills manifested mainly by delayed speech. In addition, most of the patients had abnormal social development, intolerance to pain, stereotypic repetitive movements, hyperactivity, delayed continence, and mild to moderate mental retardation. These findings are strengthened by the works of other researchers (1,13,14) who concluded that the previous findings represented the core symptoms of autism with mental retardation being a common comorbidity.

In the present study, the plasma amino acid profile was assessed using cation-exchange chromatography with postcolumn derivatization by ninhydrin. This technique is the most preferred one for amino acid quantification as it requires minimum sample preparation and ensures elimination of interferences from the sample matrix prior to quantification, thus providing maximum accuracy (9).

As regards the studied essential amino acids, the present study elucidated the fact that autistic children are suffering from essential amino acid deficiency, mainly of methionine, tryptophan, leucine, phenylalanine,

**Table 3.** Descriptive and comparative statistics of essential amino acids (nmol/mL) between patients and control group using the Wilcoxon rank sum test.

| | Patient group (n = 42) | Control group (n = 26) | Z | P-value |
|---|---|---|---|---|
| | Median (IQR) | Median (IQR) | | |
| Threonine | 19 (1.1–38) | 34 (8–52 .45) | –1.067 | 0.286 |
| Valine | 66 (40.5–84.5) | 75 (42–123.5) | –1.453 | 0.146 |
| Methionine | 3.9 (0.9–9.3) | 17 (10.1–23.5) | –3.262 | 0.001** |
| Isoleucine | 13 (2.85–23.5) | 29 (17.5–23) | –2.447 | 0.014* |
| Leucine | 32 (8.75–45.5) | 64 (49.5–85.5) | –3.314 | 0.001** |
| Lysine | 27 (12.50–91) | 66 (8.90–78) | –0.675 | 0.499 |
| Phenylalanine | 15 (9.8–32.5) | 44 (34–53) | –3.387 | 0.001** |
| Histidine | 0.01 (0.01–3.30) | 9.40 (0.01–53.50) | –2.266 | 0.023* |
| Tryptophan | 0.1 (0.01–0.95) | 1.30 (0.35–5.0) | –2.167 | 0.03* |
| Arginine | 0.1 (0.01–6.8) | 16 (2–39) | –2.148 | 0.032* |

IQR = Interquartile range.
**Highly significant difference, *significant difference.

**Table 4.** Descriptive and comparative statistics of nonessential amino acids (nmol/mL) between patients and control group using the Wilcoxon rank sum test.

| | Patient group (n = 42) | Control group (n = 26) | Z | P-value |
|---|---|---|---|---|
| | Median (IQR) | Median (IQR) | | |
| Aspartic acid | 2.3 (0.01–5.85) | 1.7 (0.30–7.75) | 0.444 | 0.657 |
| Serine | 32 (5–54) | 62 (26–131) | –2.144 | 0.032* |
| Asparagine | 49 (0.05–289.5) | 46 (0.01–186) | 0.865 | 0.387 |
| Glutamic acid | 29 (10.0–90.5) | 4.70 (0.10–23.5) | 2.141 | 0.031* |
| Proline | 0.1 (0.01–6.7) | 5.2 (0.01–50.3) | –1.346 | 0.178 |
| Glycine | 86 (53–121) | 89 (51–113.5) | –0.071 | 0.943 |
| Alanine | 95 (0.05–151) | 185 (36–568) | –1.955 | 0.051 |
| Tyrosine | 11 (5.45–34.5) | 33 (34–53) | –2.34 | 0.019* |
| Cysteine | 0.1 (0.01–2.2) | 14 (6.85–19) | –2.857 | 0.004** |

IQR = Interquartile range.
**Highly significant difference, *significant difference.

isoleucine, histidine, and arginine. These results were confirmed by Adams et al. (15), Tirouvanziam et al. (16), and Tu et al. (5). Such deficiency is attributed to the presence of gastrointestinal problems in autistic children such as malabsorption, maldigestion, gastric dysfunction caused by intrinsic factor deficiency or vitamin B12 deficiency, and food intolerance in addition to their picky and selective eating (17,18). As drawbacks of some essential amino acid deficiencies, Lakhan and Vieira (19) and Kałużna-Czaplińska et al. (20) demonstrated that deficiency of the amino acids tryptophan, phenylalanine, and methionine can induce many mood disorders, including depression and irritability. Naushad et al. (18) attributed such findings to the fact that tryptophan is the precursor of serotonin; moreover, they highlighted the importance of methionine in the production of brain neurotransmitters and in the

**Table 5.** Descriptive and comparative statistics of nonstandard amino acids (nmol/mL) between patients and control group using the Wilcoxon rank sum test.

| | Patient group (n = 42) | Control group (n = 26) | Z | P-value |
|---|---|---|---|---|
| | Median (IQR) | Median (IQR) | | |
| Phosphoserine | 7.7 (4.45–13.0) | 3.1 (2.65–6.05) | 3.013 | 0.003** |
| Taurine | 62 (46.5–117) | 91.20 (62–170.5) | –1.666 | 0.096 |
| β-Alanine | 55 (0.01–377.5) | 235 (68.5–796.5) | –2.012 | 0.044* |
| Phosphoethanolamine | 0.01 (0.01–0.40) | 0.10 (0.025–1.55) | –1.658 | 0.097 |
| Urea | 1261 (880.50–1622.5) | 1204 (896.5–1885) | –0.23 | 0.818 |
| Hydroxyproline | 0.1 (0.01–1.4) | 0.01 (0.01–0.01) | 2.282 | 0.022* |
| Citrulline | 0.01 (0.01–0.10) | 0.01 (0.01–3.67) | –0.894 | 0.371 |
| α-Aminoadipic acid | 0.01 (0.01–0.10) | 1.50 (0.01–79.35) | –2.445 | 0.014* |
| α-Aminobutyric acid | 0.01 (0.01–0.45) | 0.01 (0.01 – 0.01) | 1.414 | 0.157 |
| δ-Aminobutyric acid | 7.9 (2.95–27.5) | 11 (4.9 – 21.0) | 1.17 | 0.242 |
| β-Amino-isobutyric acid | 12 (3.55–19) | 3.6 (0.65 – 6.5) | 2.093 | 0.031* |
| Carnosine | 0.1 (0.01–0.70) | 13 (0.11–33) | –2.674 | 0.007** |
| Ammonia | 1.6 (1.2–2.6) | 0.1 (0.01–2.25) | 2.086 | 0.037* |
| Ornithine | 89 (59–162.5) | 100 (69.5–164.5) | –0.532 | 0.595 |
| 1M Histidine | 57 (37–84.5) | 4.10 (0.01–114) | –1.581 | 0.114 |
| 3M Histidine | 17 (0.01–24.5) | 25 (0.65–36.5) | –0.483 | 0.629 |

IQR = Interquartile range.
**Highly significant difference, *significant difference.

methylation of DNA and proteins by being a precursor of S-adenosylmethionine, the universal methyl donor. All the previous findings raised serious concerns regarding the long-term effects of restricted diets and emphasized the importance of early dietary intervention for the improvement of the developmental outcome in autism (21,22).

Regarding the measured nonessential amino acids, the present study recorded a deficiency in the amino acids tyrosine, cysteine, and serine. These findings could be interpreted based on the deficiency of the phenylalanine precursor of tyrosine and deficiency of methionine, the sulfur donor for endogenous synthesis of cysteine, and they could be also related to genetic alteration of the biochemical synthesis pathways (5,19,23). Possible symptoms of low plasma tyrosine would be learning, memory, or behavioral disorders and autonomic dysfunction (19). Studies performed by Main et al. (24) considered cysteine as the rate-limiting amino acid for the synthesis of glutathione, which plays a key role in detoxification processes. Such a finding elucidates the association between autism and the presence of oxidative stress in such patients (15). Serine is a critical component in the biosynthesis of acetylcholine, an important CNS neurotransmitter used in memory function and a mediator of parasympathetic activity, and its deficiency was reported to be associated with behavioral alteration (25).

Moreover, in the present study, autistic children showed a significantly higher level of glutamic acid as compared to healthy controls. Similar results were reported by Ghanizadeh (6), who elucidated the role of glutamic acid as a major excitatory neurotransmitter in the brain and considered this hyperglutamatergic state as an etiology of autism being easily passed through the blood–brain barrier, hence causing excitotoxicity, neurodegeneration, and inflammation. Peripherally, glutamate was found to affect taste sensation, skin pain sensation, and pancreatic exocrine function (15). The rise in serum glutamic acid was in part attributed to the failure of its conversion to glutamine due to vitamin B6 deficiency in such patients (15), in addition to the modulatory effect of neuroactive steroids on glutamate level (11). Such findings of high glutamate led some researchers to consider glutamate as a diagnostic tool for early detection of autism (26).

The present study demonstrated significantly lower levels of the nonstandard amino acids α-aminoadipic acid,

carnosine, and β-alanine and significantly higher levels of hydroxyproline, phosphoserine, β-amino-isobutyric acid, and ammonia in autistic children as compared to healthy controls. A similar finding of high levels of β-amino-isobutyric acid was reported by Adams et al. (15), who added that this amino acid is the product of thymine catabolism; hence, its elevation pointed either to the increased rate of DNA turnover in autism or to the inhibition of the conversion of β-amino-isobutyric acid into its intermediates that eventually lead to the citric acid cycle. Regarding high levels of plasma ammonia, our finding was strengthened by Cubała-Kucharska (17), who attributed this to the impairment of many biochemical pathways in autism with subsequent failure to detoxify neuroendotoxins such as ammonia, hence leading to behavioral and cognitive changes in autism. In contrast to our study, which reported a nonsignificant difference in plasma taurine between the two studied groups, Adams et al. (15) reported lower levels of taurine in autism and attributed this to the increased wasting of taurine in urine. Nevertheless, Ghanizadeh (6) demonstrated increased taurine levels in autism, which was attributed to a compensatory phenomenon for the increased glutamate level.

In conclusion, the present study assessed the plasma amino acid profile in autistic children by cation-exchange chromatography using postcolumn derivatization by ninhydrin. The assay revealed that autistic children exhibit distinct alterations in the plasma levels of some amino acids, which can in turn participate in the disease etiology and hence can be applied as a diagnostic tool for early detection of autism.

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