Pharmacological Research 53 (2006) 1–5

Pharmacological research

www.elsevier.com/locate/yphrs

The effect of aspartame metabolites on human erythrocyte membrane acetylcholinesterase activity

Stylianos Tsakiris [a,*], Aglaia Giannoulia-Karantana [b], Irene Simintzi [a], Kleopatra H. Schulpis [b]

[a] *Department of Experimental Physiology, Medical School, University of Athens, P.O. Box 65257, GR-154 01 Athens, Greece*
[b] *Institute of Child Health, Research Center, Aghia Sophia Children's Hospital, GR-115 27 Athens, Greece*

Accepted 19 July 2005

Abstract

Studies have implicated aspartame (ASP) with neurological problems. The aim of this study was to evaluate acetylcholinesterase (AChE) activity in human erythrocyte membranes after incubation with the sum of ASP metabolites, phenylalanine (Phe), methanol (met) and aspartic acid (aspt), or with each one separately. Erythrocyte membranes were obtained from 12 healthy individuals and were incubated with ASP hydrolysis products for 1 h at 37 °C. AChE was measured spectrophotometrically. Incubation of membranes with ASP metabolites corresponding with 34 mg/kg, 150 mg/kg or 200 mg/kg of ASP consumption resulted in an enzyme activity reduction by −33%, −41%, and −57%, respectively. Met concentrations 0.14 mM, 0.60 mM, and 0.80 mM decreased the enzyme activity by −20%, −32% or −40%, respectively. Aspt concentrations 2.80 mM, 7.60 mM or 10.0 mM inhibited membrane AChE acitivity by −20%, −35%, and −47%, respectively. Phe concentrations 0.14 mM, 0.35 mM or 0.50 mM reduced the enzyme activity by −11%, −33%, and −35%, respectively. Aspt or Phe concentrations 0.82 mM or 0.07 mM, respectively, did not alter the membrane AChE activity. It is concluded that low concentrations of ASP metabolites had no effect on the membrane enzyme activity, whereas high or toxic concentrations partially or remarkably decreased the membrane AChE activity, respectively. Additionally, neurological symptoms, including learning and memory processes, may be related to the high or toxic concentrations of the sweetener metabolites.

Keywords: Acetylcholinesterase; Aspartame; Phenylalanine; Methanol; Aspartic acid

1. Introduction

Although aspartame (ASP) received Food and Drug Administration (FDA) approval in 1981 and has been judged safe by medical groups such as the AMA Counsil on Scientific Affairs [1], there has been persistent concern that the use of ASP, an *O*-methyl ester of the dipeptide L-a-aspartyl-L-phenylalanine, may result in adverse neurologic symptoms or other abnormalities [1,2]. Clinical studies have demonstrated that administration of ASP can alter some aspects of blood chemistry, in particular plasma phenylalanine (Phe) levels [3–5] and brain wave activity among epileptic children [6,7]. Moreover, since ASP's approval numerous case reports have implicated ASP in the occurrence of such problems as seizures [7], memory loss [8], headache [9] and hypersensitivity reactions [7,9]. In addition, most of clinical studies investigating ASP have used as subjects healthy adults [9] and individuals likely to use large amount of ASP, like diabetics or in weight reduction [10,11]. Furthermore, other short-term studies have been conducted and none of these have suggested any relationship between ASP consumption and memory loss [3,11]. In contrast, various neurochemical effects due to ASP ingestion have been reported [8]. Certain brain amino acid levels, including aspt or Phe, have been shown to be increased after the consumption of the sweetener [12]. Taken collectively, these studies suggest that ASP might affect neurotransmitters and receptors and these effects may become more prominent with long-term consumption [3,12–14].

* Corresponding author. Tel.: +30 210 7462662; fax: +30 210 7462571.
E-mail address: stsakir@cc.uoa.gr (S. Tsakiris).

1043-6618/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phrs.2005.07.006

In our previous in vitro study [15], acetylcholinesterase (AChE) activity of rat brain homogenates or pure enzyme of eel E. electricus showed a decrease up to 20% ($P < 0.01$) after preincubation with Phe concentrations 7–30 mg dl^{-1} (or 0.4–1.8 mM, Phe normal values: 1–2 mg dl^{-1} or 0.06–0.12 mM). These concentrations are usually measured in the blood of PKU patients "off diet". Moreover, we reported [16] that the above mentioned high Phe concentrations, caused a 30% inhibitory effect on AChE from the rat diaphragm. Although, there is no literature relating to AChE inhibition by Phe and clinical symptoms in PKU patients, we found not only a significant inhibition (about 60%, $P < 0.001$) of the erythrocyte membrane AChE from PKU patients "off diet" but also a significant reduction of their plasma neurotransmitter concentrations [17].

Since ASP is immediately hydrolyzed in the intestinal lumen giving Phe, methanol (met) and aspartic acid (aspt) [3], it was important to evaluate AChE activity in the erythrocyte membranes of healthy individuals after incubation with various concentrations of each or the sum of ASP metabolites.

2. Subjects and methods

The study was approved by the Greek ethics committee and conducted according to the principles expressed in the declaration of Helsinki.

2.1. Subjects

The study population consisted of 12 healthy individuals, mean age 24 ± 5 years old.

2.2. Erythrocyte membrane preparation

Venous blood samples (7.0 ml) were collected into heparinized blood collection tubes from the individuals. Within 2 h of collection, the erythrocytes were sedimented by centrifugation at 2000 × g for 30 min at 4 °C, washed three times, after three similar centrifugations, with a buffer solution (250 mM, tris (hydroxymethyl) aminomethane–HCI (Tris–HCI), pH 7.4, 140 mM NaCl, 1 mM $MgCl_2$, 10 mM glucose. The erythrocytes were then resuspended in 1.0 ml of this buffer and stored at 4 °C for up to 24 h before erythrocyte membrane preparation. The washed erythrocytes were lysed after freeze (−80 °C) thawing (50 °C) for five times, as described by Galbraith and Watts [18] and Kamber et al. [19]. The hemolysate was centrifuged at 35,000 × g for 30 min with 40–60 volumes of cold 0.1 M Tris–HCl pH 7.4, four times until white pink color appeared. Membranes were suspended in 0.1 M Tris–HCl, pH 7.4 to a final concentration of 2 mg protein ml^{-1}. The protein content was determined, as described previously [20]. Membranes stored at −40 °C retained the enzyme activities, for at least 2 weeks. The minor Hb that remained attached to the membrane surface was measured with the kit 527-A (Sigma Chemicals Co., St. Louis, USA), and the value was substracted from the total protein concentration.

Table 1
The effect of the sum of ASP metabolites on the erythrocyte membrane AChE activity

	AChE activity ($\Delta\overline{OD}$ min^{-1} mg^{-1} protein)
Control	3.01 ± 0.12 a
ASP (10 mg kg^{-1}) Mix 1	3.0 ± 0.09 b (NS)
ASP (18 mg kg^{-1}) Mix 2	2.80 ± 0.10 f (−7%)
ASP (34 mg kg^{-1}) Mix 3	2.02 ± 0.10 c (−33%)
ASP (150 mg kg^{-1}) Mix 4	1.78 ± 0.05 d (−41%)
ASP (200 mg kg^{-1}) Mix 5	1.29 ± 0.07 e (−57%)

The inhibition and its degree have been observed in each subject ($N = 12$). Values are expressed: means ± S.D. a/b, a/f = NS; NS: non statistically significant; a/c, a/d, b/d, b/c, c/d, c/e = $P < 0.01$; a/e, b/e = $P < 0.001$. The concentrations of ASP metabolites used in Mixes 1–5 are described in details in Section 2.

2.3. Measurement of erythrocyte membrane acetylcholinesterase (AChE) activity after incubation with ASP metabolites

Erythrocyte membranes were incubated with ASP metabolites at 37 °C, as shown in Tables 1 and 2.

ASP follows a hydrolysis process in the laboratory giving met, aspt and Phe 10:40:50 (w/w/w) [1]. Furthermore,

Table 2
The effect of each one of the aspartame metabolites on the human erythrocyte membrane acetylcholinesterase activity

Treatment	AChE activity ($\Delta\overline{OD}$ min^{-1} mg^{-1} protein)
Control	3.01 ± 0.12 a
Methanol (met) w (mM)	
Not detected	3.11 ± 0.15 f (NS)
0.07	2.90 ± 0.10 b (NS)
0.14	2.41 ± 0.10 g (−20%)
0.60	2.05 ± 0.11 h (−32%)
0.80	1.81 ± 0.09 i (−40%)
Aspartate (aspt) w (mM)	
0.82	3.05 ± 0.06 k (NS)
1.40	2.95 ± 0.10 c (NS)
2.80	2.41 ± 0.13 l (−20%)
7.60	1.96 ± 0.05 m (−35%)
10.00	1.59 ± 0.09 n (−47%)
Phenylalanine (Phe) w (mM)	
0.07	2.97 ± 0.09 o (NS)
0.08	2.92 ± 0.09 d (NS)
0.14	2.68 ± 0.05 P (−11%)
0.35	2.02 ± 0.10 s (−33%)
0.50	1.95 ± 0.08 t (−35%)

The inhibition and its degree have been observed in each subject ($N = 12$). W: ASP metabolite concentrations measured in blood after ingestion of various (10, 18, 34, 150 or 200 mg kg^{-1}) doses of the sweetener. Values represent means ± S.D. met: a/b, a/f, g/f, g/h, h/i = NS, a/g = $P < 0.05$, a/h, f/h, g/i = $P < 0.01$, a/i, f/i, = $P < 0.001$; aspt: a/c, a/k, l/m, m/n = NS, a/l, k/l = $P < 0.05$, a/m, k/m, l/n = $P < 0.01$, a/n, k/n = $P < 0.001$; Phe: a/d, a/o, o/p, s/t = NS, a/p, o/s, p/s = $P < 0.05$, a/s, a/t, o/t, p/t = $P < 0.01$. NS: non statistically significant.

the concentrations of ASP hydrolysis products measured in the human plasma, after its ingestion, are different than those found in the laboratory and reported by Stegink [3,4]. So, we decided to use the concentrations of ASP degradation parts (Phe, met, aspt), which are commonly measured in the blood of ASP consumers as follows [3]: ASP ingestion of 10 mg kg^{-1} Mix 1 (met = undetected + aspt 0.82 mM + Phe 0.07 mM) represents the additive and its components when drinking a beverage (12 oz). Mix 2 (met 0.07 mM + aspt 1.40 mM + Phe 0.08 mM) represents the sweetener and its components when drinking a beverage of 16 oz. In addition, ASP intake of 34 mg kg^{-1} Mix 3 (met 0.14 mM + aspt 2.80 mM + Phe 0.14 mM) represents the 99th percentile of projected daily ingestion, whereas ASP 150 mg kg^{-1} Mix 4 (met 0.60 mM + aspt 7.60 mM + Phe 0.35 mM) or 200 mg kg^{-1} Mix 5 (met 0.80 mM + aspt 10.00 mM + Phe 0.50 mM) may be taken by accident [3]. For the sake of comparison with the results of our previous reports [17,22], we decided to incubate the erythrocyte membranes with the mixtures (Mixes 1–5) or each one of ASP degradation products for 1 h, as we previously did.

After 1-h incubation at 37 °C, determination of AChE activity was performed spectrophotometrically.

AChE activity was determined according to the method of Ellman et al. [21] as modified by Tsakiris et al. [17]. The reaction mixture (1 ml) contained 50 mM Tris–HCl, pH 8.0, 240 mM sucrose and 120 mM NaCl in the presence of 80–100 μg protein of erythrocyte membranes as well as in the presence or absence of ASP, met, aspt or Phe. Quinidine sulfate (2.10^{-2} mM) was added in the mixture, in order to inhibit pseudocholinesterase activity. Finally, 0.030 ml 5,5′-dithionitro-benzoic acid (DTNB) and 0.050 ml acetylthiocholine iodide, which was used as substrate, and the reaction was started. The final concentrations of DTNB and substrate were 0.125 and 0.5 mM, respectively. The reaction was measured spectrophotometrically by the increase in absorbance ($\Delta\overline{OD}$) at 412 nm.

2.4. Statistical analysis

Data were analyzed by one-way ANOVA. All analyses were performed with an IBM PC using the SPSS 10.0 statistical package. *P*-values <0.05 were considered statistically significant.

3. Results

As illustrated in Table 1, the amount of ASP ingestion, which correlates with the consumption of a 12 oz beverage (Mix 1) or 16 oz beverage (Mix 2), when incubated with erythrocyte membranes, did not alter AChE activity. On the contrary, ASP metabolite concentrations which correspond to the 99th percentile of the daily intake of the sweetener, which corresponds to the consumption of four to five beverages (12 oz) (Mix 3) as well as toxic doses (150 mg kg^{-1} and/or 200 mg kg^{-1}) (Mixes 4 and 5, respectively), resulted in a remarkable decrease of the human erythrocyte membrane enzyme activity.

As shown in Table 2, the metabolite met, significantly statistically inhibited AChE activity after incubation of the erythrocyte membranes with met concentrations usually determined in blood and related to ASP consumption of 34 mg kg^{-1} 24 h^{-1}) or with those measured in blood when taken toxic doses of ASP (150 or 200 mg ASP kg^{-1}). In addition, incubation of the erythrocyte membranes with concentrations of the dicarboxylic acid aspt, which correlated with ASP ingestion 34, 150, or 200 mg kg^{-1}, resulted in a reduction of AChE activity by −20%, −35%, and −47%, respectively.

Also, significant inhibition of the membrane enzyme activity was observed after 1-h incubation with high concentrations of the aromatic acid Phe, corresponding to Phe blood levels, which were found after ingestion of abuse doses of the sweetener. Lower concentrations of Phe had no or minor effect on AChE activity (Table 2).

4. Discussion

ASP is one of the artificial sweeteners added to many soft beverages, cakes etc., and its usage is increasing in health-conscious societies. Upon ingestion, this artificial sweetener is immediately absorbed from the lumen and metabolized to Phe, aspt and met. Their concentrations are found increased in the blood stream [3,4].

In speculating about parallel changes in brain and blood, both aspt and Phe are transported into CNS in competition with other amino acids. Movement of large neutral amino acids, such as Phe, across the blood brain barrier is mediated by a common high affinity, low capacity transport system. A large excess of Phe, as found in our previous study [22] and probably in ASP consumers [3], will saturate this carrier system excluding other amino acids, such as tyrosine (Tyr) from entry into the brain. Thus, high levels of Phe interfere with the conversion of Tyr to the biogenic amines dopamine and noradrenaline affecting AChE activity as found previously [22].

Aspt, the dicarboxylic amino acid may exert toxic effects when administered at very high doses, although species susceptibility varies considerably. Neonatal rodents administered large doses of aspt may develop hypothalamic neuronal necrosis. However, there is disagreement over the ability of aspt to produce neuronal necrosis in infant primates [3,4,23]. It is not suprising that the per oz loading with large amounts of ASP is claimed to be the cause of some ailments [9]. Human studies have affirmed the safety of ASP [23], although suggest that ASP consumption might constitute a hazard to humans [24,25]. An important question is whether the ingestion of ASP at abuse doses is still safe to humans, because consumers, including children, might unintentionally take a large amount of ASP commonly used in many products. The effect

of ASP on humans is probably dependent on its metabolite concentrations.

Of the ASP metabolites, met is a toxicant that causes systematic toxicity [26]. The primary metabolic fate of met is the direct oxidation to formaldehyde and then into formate. The severity of clinical findings in met intoxication correlated better with formate levels [27]. Mean peak blood methanol concentrations exceeded 2 mg dl^{-1} in subjects administered abuse doses of ASP and were still lower than those reported in met intoxication [4]. However, another study [28] revealed that the cellular glutathione content and activity of glutathione-related enzymes were decreased in met intoxication. In this study, the observed inhibition of the erythrocyte membrane AChE activity after incubation with high and/or toxic met concentrations may be due to the production of oxidative stress as a consequence of the cellular glutathione reduction [29].

Additionally, we observed a slight decrease of AChE activity when the membranes were incubated with Phe concentrations, usually measured in humans after ingestion a dose of ASP corresponding to the 99th percentile of ASP daily dose (Table 2), whereas no effect was observed on the enzyme activity when incubated with lower Phe concentrations (Table 2). On the contrary, a remarkable inhibition by the erythrocyte membrane enzyme activity was measured when the membranes were incubated with Phe concentrations related to abuse doses of the sweetener (Table 2). Almost the same degree of the enzyme inhibition was observed in the erythrocyte membrane AChE activity of phenylketonuric (PKU) patients "off diet", in whom their plasma neurotransmitter levels were found very low, whereas Phe levels were determined very high in their blood [17,30]. The high degree of AChE inhibition in erythrocyte membranes from healthy individuals after incubation with high Phe [17] concentrations may be caused by the indirect influence of the high concentrations of the amino acid on AChE in the membrane bilayer through lipid(s)–protein interactions as shown in our previous report [17]. Furthermore, we cannot exclude that the observed decrease of the membrane AChE activity may be due to an oxidative stress as observed in phenylketonuric patients "off diet" [17,30].

Aspartic acid (aspt), another ASP metabolite, is a dicarboxylic amino acid which may exert toxic effects when administered at very high doses although species susceptibility varies considerably [4]. In this study, high or toxic doses of aspt, when incubated with the erythrocyte membranes resulted in a significant (−20%) or very significant reduction (−50%) of AChE activity, respectively (Table 2). The observed enzyme activity inhibition may be due to an increase of reactive oxygen species (ROS) production and/or increases of intracellular Ca^{2+} by the amino acid as reported by Sureda et al. [31].

Orally ingested ASP components are immediately absorbed from the lumen and reach the portal blood in a manner similar to that of amino acids and met arising from dietary protein or polysaccharides [4,25]. The inhibitory action of ASP metabolites (Table 1) may simultaneously work on the erythrocyte membrane AChE activity either by producing an oxidative stress and/or through the indirect influence of the metabolites on AChE in the membrane bilayer through lipid(s) peroxidation, as suggested in our previous in vivo findings: It was reported that the erythrocyte membrane AChE activity was evaluated significantly decreased in PKU patients "off diet" with low antioxidant status [17,30].

Numerous studies have implicated muscarinic cholinergic receptors and memory [32,33]. In the rabbit, elevated muscarinic binding has been shown in the anterodorsal nucleus early in the learning process. It has been suggested that nicotinic transmission may be important in delayed response tasks, while the muscarinic system may be involved, in general working memory processes [34–36]. These studies lead us to hypothesize that it might exist an alteration in brain muscarinic system after high or toxic doses of ASP consumption and that the most available human tissue for AChE study was the human erythrocyte membranes. Also, it might be suggested that elevations of ASP hydrolysis products in the blood, especially aspt and Phe concentrations, may take place, in some degree, in the brain inducing modulations not only on the enzyme activity but also in the neurotransmitter levels, including acetylcholine and biogenic amines, as found in our previous work [17], resulting in learning difficulties and memory loss [4,8].

5. Conclusions

ASP metabolites may partially inhibit the erythrocyte membrane AChE activity, when incubated with their concentrations corresponding to the 99th percentile of the daily intake of the sweetener, whereas a remarkable decrease of the enzyme activity was observed after incubation with ASP hydrolysis products related to abuse doses of the additive. ASP metabolite concentrations commonly found in beverages had no effect on AChE activity. Oxidative stress and/or an indirect effect of ASP metabolites on the membrane bilayer through lipid(s) peroxidation may contribute to the reduction of the human erythrocyte membrane AChE activity.

Neurological symptoms, memory loss and/or learning process, headaches, confusion, visual difficulties and dizziness, observed after high or abuse doses of ASP consumption, may be related to the partial or remarkable decrease of the AChE activity.

Acknowledgements

This work was funded by the University of Athens and Institute of Child Health in Aghia Sophia Children's Hospital, Athens. Many thanks are expressed to the postgraduate student Theodore Parthimos and the medical students Nick

Mavromatis and Maria Stoupaki for their significant assistance.

We are very grateful to Mrs. Kalliopi Tassopoulou for her carefully typing of this manuscript.

References

[1] Pardridge WM. Potential effects of the dipeptide sweetener aspartame on the brain. Nutr Brain 1986;7:199–241.
[2] Tollefson L, Barnard RJ. An analysis of EDA passive surveillance reports of seizures associated with consumption of aspartame. Am J Clin Nutr 1986;43:464–9.
[3] Stegink LD, Filer Jr LJ, Baker GL. Repeated ingestion of aspartame—sweetened beverage effect on plasma amino acid concentrations in normal adults. Metabolism 1988;37:246–51.
[4] Stegink LD. The aspartame story: a model for the clinical testing of a food additive. Am J Clin Nutr 1987;46:204–15.
[5] Moller SE. Effect of aspartame and protein administered in phenylalanine equivalent doses, on plasma neutral amino acids, aspartate, insulin and glucose in man. Pharmacol Toxicol 1991;68:408–12.
[6] Camfield PR, Camfield CS, Dooley JM, Gordon K, Jollymore S, Weaver DF. Aspartame exacerbates EEG spike-wave discharge in children with generalized absence epilepsy: a double blind controlled study. Neurology 1992;42:1000–3.
[7] Walton RG. The possible role of aspartame in seizure induction. In: Wurthman RJ, Ritter-Walker E, editors. Dietary phenylalanine and brain function. Boston: Birkhauser; 1988. p. 159–62.
[8] Moser RH. Aspartame and memory loss. JAMA 1994;272:1543.
[9] Van Den Eeden SK, Koepsell TD, Longstreth Jr WT, van Belle G, Daling JR, McKnight B. Aspartame ingestion and headaches: a randomized crossover trial. Neurology 1994;44:1787–93.
[10] Stern SB, Bleicher SJ, Flores A, Gombos G, Recitas D, Shu J. Administration of aspartame in non insulin—dependent diabetics. J Toxicol Environ Health 1976;2:429–39.
[11] Knopp RH, Brandt K, Arky RA. Effects of aspartame to young persons during weight reduction. J Toxicol Environ Health 1976;2:417–28.
[12] Spiers PA, Sabounjian L, Reiner A, Myers DK, Wurtman J, Schomer DL. Aspartame: neuropsychologic and neurophysiologic evaluation of acute and chronic effects. Am J Clin Nutr 1998;68:531–7.
[13] Goerss AL, Wagner GC, Hill WL. Acute effects of aspartame on aggression and neurochemistry in rats. Life Sci 2000;67:1325–9.
[14] Dailey JW, Lasley SM, Burger RL, Bettendorf AF, Mishra PK, Jobe PC. Amino acids, monoamines and audiogenic seizures in genetically epilepsy-prone rats: effects of aspartame. Epilepsy Res 1991;8:122–33.
[15] Tsakiris S, Kouniniotou-Krontiri P, Schulpis KH, Stavridis J. L-Phenylalanine effect on rat brain acetylcholinesterase and Na^+, K^+-ATPase. Z Naturforsch [C] 1998;53:163–7.
[16] Tsakiris S, Kouniniotou-Krontiri P, Schulpis KH. L-Phenylalanine effect on diaphragma acetylcholinesterase and Na^+K^+-ATPase. Z Naturforsch [C] 1998;53:1055–60.
[17] Tsakiris S, Schulpis KH, Tjamouranis J, Michelakakis H, Karikas GA. Reduced acetylcholinesterase activity in erythrocyte membranes from patients with phenylketonuria. Clin Biochem 2002;35:615–9.
[18] Galbraith DA, Watts DC. Changes in some cytoplasmic enzymes from red cells fractionated into age groups by centrifugation in Ficoll/Triosil gradients. Comparison of normal humans and patients with Duchenne muscular dystrophy. Biochem J 1980;191:63–70.
[19] Kamber K, Poyiagi A, Delikonstantinos G. Modifications in the activities of membrane-bound enzymes during in vivo ageing of human and rabbit erythrocytes. Comp Biochem Physiol B 1984;77B:95–9.
[20] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;393:265–75.
[21] Ellman GL, Courtney D, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:85–8.
[22] Schulpis KH, Karikas GA, Tjamouranis J, Michelakakis H, Tsakiris S. Acetylcholinesterase and biogenic amines in phenylketonuria. Clin Chem 2002;48:1794–6.
[23] Ranney RE, Oppermann JA. A review of the metabolism of the aspartyl moiety of aspartame in experimental animals and man. J Environ Pathol Toxicol 1979;2:979–85.
[24] Tephly TR. Comments on the purported generation of formaldehyde and adduct formation from the sweetener. Life Sci 1999;65:PL157–60.
[25] Trocho C, Pardo R, Rafecas I, Virgili J, Remesar X, Fernadez-Lopez JA, et al. Formaldehyde derived from dietary aspartame binds to tissue components in vivo. Life Sci 1998;63:337–49.
[26] Kruse JA. Methanol poisoning. Intensive Care Med 1992;18:391–7.
[27] Osterloh JD, Pond SM, Grady S, Becker CE. Serum formate concentrations in methanol intoxication as a criterion for hemodialysis. Ann Intern Med 1986:2000–3.
[28] Skrzydlewska E, Farbiszewski R. Glutathione consumption and inactivation of glutathione-related enzymes in liver, erythrocytes and serum of rats after methanol intoxication. Arch Toxicol 1997;71:741–5.
[29] Oyama Y, Sakai H, Arata T, Okano Y, Akaike N, Sakai K, et al. Cytotoxic effects of methanol formaldehyde and formate on dissociated rat thymocytes: A possibility of aspartame toxicity. Cell Biol Toxicol 2002;18:43–50.
[30] Schulpis KH, Tsakiris S, Karikas GA, Moukas M, Behrakis P. Total antioxidant status in patients with phenylketonuria on diet. Eur J Clin Nutr 2003;57:383–7.
[31] Sureda F, Camins A, Trullas R, Camarasa J, Escubedo E. A flow cytometric study of *N*-methyl-D-aspartate effects on dissociated cerebellar cells. Brain Res 1996;723:110–4.
[32] Russell RW. Continuing the search for cholinergic factors in cognitive dysfunction. Life Sci 1996;58:1965–70.
[33] Christian B, McConnaughey K, Bethea E, Brantley S, Coffey A, Hammond L, et al. Chronic aspartame affects T-maze performance, brain cholinergic receptors and Na^+, K^+-ATPase in rats. Pharmacol Biochem Behav 2004;78:121–7.
[34] Granon S, Poucet B, Thinus-Blanc C, Changeux JP, Vidal C. Nicotinic and muscarinic receptors in the rat prefrontal cortex: differential roles in working memory, response selection and effortful processing. Psychopharmacology (Berl) 1995;119:139–44.
[35] Van der Zee EA, Luiten PG. Muscarinic acetylcholine receptors in the hippocampus, neocortex and amygdala: a review of immunocytochemical localization in relation to learning and memory. Prog Neurobiol 1999;58:409–71.
[36] Guiso G, Diomede L, Romano M, Caccia S, Sarati S, Salmona M. Effect of tyrosine on the potentiation by aspartame and phenylalanine of metrazol-induced convulsions in rats. Food Chem Toxicol 1991;29:855–7.