IN VITRO INVESTIGATION OF D- AND L-ENANTIOMER SYNERGISTIC EFECTS OF SOME AMINO ACIDS

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Abstract: D-amino acids can arise from endogenous microbial flora, from ingestion with the diet or from spontaneous racemization of L-amino acids during ageing. In this work, the behavior of methionine, homocysteine and cysteine enantiomers was investigated in human serum *in vitro* during 0-72 h at incubation temperature 37 °C. The separation of enantiomers was realized in two dimensional *on-line* system (the connection of an achiral column Purospher RP-18 endcapped and a chiral column Chirobiotic TAG). This system allowed simultaneous monitoring all tested amino acids and their enantiomers. The possible effect D-enantiomer on the behavior of its L-enantiomer (the synergistic effect) was evaluated during incubation time. The first results have showed that no synergistic effect of D-enantiomer on its L-isomer has been observed in our experimental conditions *in vitro*.

Key words: amino acid; enantiomers; serum; synergism

1. Introduction

FUCHS *et al.* (2005) reported that D-amino acids can arise from endogenous microbial flora, from ingestion with the diet or from spontaneous racemization of L-amino acids incorporated in polypeptides during ageing.

From all mentioned origin of D-amino acids, food is the most significant source. There are formed D-isomers from common L-amino acids either in the production processes or as a consequence of changes in the microbiological quality of the diet (FRIEDMAN, 1999). Some animal experiments have demonstrated that D-methionine (Met) acts an otoprotective agent and its oral administration can be useful in mitigating hearing loss from platinum based chemotherapy, aminoglycosides and excessive noise (CAMPELL *et al.,* 2007). On the other hand, other studies have showed that D-Met is poorly utilized in humans (EFRON *et al.,* 1969; KIES *et al*., 1975; ZEZULKA and CALLOWAY, 1976; PRINTEN *et al.,* 1979; FRIEDMAN and GUMBMANN, 1984).

The conversion of D-Met into L-Met in rats was also indicated using a stable isotope methodology in the work of HASEGAWA *et al.* (2005). Their results showed that almost all (more than 90 %) of administered D-Met has been converted into L-enantiomer *in vivo.* The conversion of D-Met can be possible through oxidative deamination by D-amino acid oxidase to form α-keto-γ-methiolbutyric acid and after that α-keto-γ-methiolbutyric acid is stereospecifically reaminated by transaminases to form L-Met.

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D-cysteine (Cys) has significantly depressed the growth of mice and therefore it can be considered nutritionally antagonistic or toxic. After oral administration of D-Cys, the concentration of sulfate in rat urine was shown to increase (about 55 % of the dose), but the concentration of taurine in the serum did not increase indicating that it is probably not or only slowly converted to taurine. The administration of L-Cys caused an increase the concentration of sulfate in the urine (about 33 % of the dose) as well as the concentration of taurine in the serum (KRIJGSHELD *et al.,* 1981; FRIEDMAN and GUMBMANN, 1984). Other studies have showed that D-Cys has beneficial effects in acute alcohol intoxication and in cancer therapeutics and it shows no mutagenicity for humans (GLATT and OESCH, 1985; TSUKAMOTO *et al.,* 1990; ROBERTS, 1995; OANCEA and FORMAGGIO, 2008).

This work originated from the effort to simulate processes *in vivo* by using *in vitro.* We focused on investigation of the behavior of selected amino acid enantiomers in human serum and for these purposes two dimensional *on-line* system was used. This system allowed simultaneous monitoring all tested amino acids as well as their enantiomers.

2. Materials and methods

2.1 Chemicals

Acetonitrile, sodium phosphate monobasic monohydrate, perchloric acid, sodium borohydride, D-Met, DL-homocysteine (HCy) and D-Cys were purchased from Sigma-Aldrich (USA). Ortho-phosphoric acid and 1-octanesulfonic acid sodium salt were purchased from Fluka Biochemika (Switzerland). Sodium hydroxide and methanol were obtained from Merck (Germany). D-HCy was not available and its racemate was used for measurements.

2.2 Preparation of working solutions

All standard solutions were prepared in water. Doubly deionized water (≥ 18 MΩ cm) was produced on the apparatus Rodem 6 (Rodem Water s.r.o., Slovakia). Working solutions of these amino acids were obtained by mixing of stock solutions. All solutions were stored at -80 °C until use.

2.3 Calibration curves

To determine molar concentrations, regression equations $y = 159.5x + 737$ ($R^2 = 0.999$) for Met, $y = 381.7x – 1681$ ($R^2 = 0.995$) for HCy and $y = 327.7x - 7746$ ($R^2 = 0.984$) for Cys were used. Calibration curves were constructed in the range of 5-200 µmol/L for Met, 2.5-30 µmol/L for HCy and 25-500 µmol/L for Cys.

2.4 Blood collection and sample pretreatment

The blood was obtained from two healthy female volunteers (age 28 and 29). Venous blood samples were collected after 12 h overnight fast. Within 1 h of

collection, the blood was centrifuged at 2200 g for 15 min. at 4 °C. Obtained serum was storaged at -80 °C until the analysis. Serum samples were pre-treated according to GARAIOVA *et al.* (2013). Shortly, 40 µL of sodium borohydride (1 mol/L in 0.1 mol/L NaOH) was added to 100 µL of the serum, mixed and incubated at 50 °C for 30 min. 100 µL of perchloric acid (0.6 mol/L in water) was added and samples centrifuged at 14 000 g for 15 min. Non-diluted supernatant (20 µL) was injected into the chromatographic system.

2.5 Instrumentation and chromatographic conditions

The chromatographic system consisted of an isocratic pump (DeltaChrom SDS 030, Watrex, Praha, Czech Republic) and an electrochemical detector (Coulochem II, ESA, Chelmsford, UK). The detector was composed of guard cell (Model 5020) and analytical cell (Model 5010A, ESA, Chelmsford, UK) with porous graphite electrodes. Analytical cells had potential +0.7 V (E1) and +0.9 V (E2), and the guard cell potential was set to +1.4 V.

Achiral separations were achieved on Purospher RP-18 endcapped 250-4 mm (5 µm) (Merck, Darmstadt, Germany) used together with a pre-column Purospher STAR RP-18e (5 µm) (Merck, Darmstadt, Germany). Chiral separations were performed on Chirobiotic TAG 250-4.6 mm (5 µm) column (ASTEC, USA) in an *on-line* system. The mobile phase contained 25 mmol/L phosphate buffer, 1 mmol/L octanesulfonic acid (pH 2.7), acetonitrile and methanol with ratio 94 : 3 : 3 (v/v/v). The separation system was proposed in our previous work (DEÁKOVÁ *et al.,* 2015). The flow rate was 0.4 mL/min. A nylon filter (47 mm in diameter) was used for mobile phase filtering. Both columns were thermostated with JET STREAM II Plus HPLC Column Thermostat (WO Industrial Electronics, Austria) at 20 °C. Sample incubations were realized by using two thermostats - thermostat 1 (Bibby Stuart Scientific Block heater, UK) set to 50 °C for the sample treatment and thermostat 2 (Eppendorf Thermomixer 5437, Germany) set to 37 °C for sample incubation.

2.6 Experiment design

The serum sample was divided into 2 aliquots (2 × 500 µL) and incubated at 37 °C during 0, 24, 48 and 72 h for total time of the analysis (150 min.). In first aliquot, only L-amino acids were present (without D-amino acids addition). In second aliquot, standards of D-enantiomes were artificially added to the serum (10 µL of D-enantiomers to 500 µL of the serum). D-enantiomers were added so that their final concentrations were at equimolar (or two times higher) concentrations of their corresponding L-enantiomers. In given time, 100 µL of the serum was picked up and pre-treated as described above.

Measurements of both aliquots were realized on the same day and concentration changes were evaluated in dependence on incubation time. The synergistic effect was estimated via differences in the behavior of L-enantiomer with and without D-enantiomer presence. The significance was determined by Student´s *t*-test that was chosen according to Fisher *F*-test results. Statistically significant results were obtained

when *p*-values were lower than 0.05. Measurements were realized on two independent samples in two replicates.

3. Results and discussion

GIBSON and WISEMAN (1951) reported that L-forms of amino acids disappeared from intestine of rats more rapidly than their corresponding D-enantiomers and therefore they regarded existence of a stereochemically specific mechanism for active absorption of L-amino acids. Later results (VISEMAN, 1953; AGAR *et al*., 1953; MATTHEWS and SMYTH, 1954) showed that transport of L-amino acids involves an active process and it was assumed that this did not apply to D-amino acids. On the other hand, JERVIS and SMYTH (1959) showed that competition between some D-histidine and L-Met was possible using *in vivo* as well as *in vitro* techniques (WISEMAN, 1955). In the later work, JERVIS and SMYTH (1960) suggested that the transfer mechanism for L-Met has not been absolutely stereochemically specific and that D-Met is able to utilize this mechanism *in vitro.*

Utilization of D-Met during parenteral nutrition in adult rats was investigated by CHO and STEGINK (1979). Infusion of the solution containing DL-Met significantly increased plasma Met levels during protein sparing therapy with accumulation of the D-isomer. There were also urinary Met losses small as well as losses of methionine sulfoxide and alpha-keto-gamma-methiolbutyrate. In contrast to humans, adult rats utilized more than 99% of parenterally administered D- or L-Met.

Data obtained from the study focusing on the feeding of young infants by the formulas containing DL-Met showed that substantial concentrations of D-Met circulated in the plasma (STEGINK *et al.,* 1971).

These findings have indicated that the nutritional utilization of different D-amino acids varies widely in animals and humans. Some D-amino acids may be both beneficial and deleterious. Thus, whereas D-Met is largely utilized as a nutritional source of its L-isomer, D-serine induces histological changes in the rat kidney. Because D-amino acids are consumed by animals as well as humans as part of their normal diets, there exists a need for understanding of their roles in nutrition, food safety, microbiology, physiology and medicine (FRIEDMAN, 1999).

In the present study, L-amino acid concentrations of Met, HCy and Cys were estimated in the serum and concentration changes of L-enantiomers were evaluated in dependence on incubation time. The same procedure was realized in the presence of D-enantiomers which have equimolar concentrations of corresponding L-amino acids. Differences in the behavior of L-enantiomers in the presence and absence of D-enantiomers were evaluated statistically.

Results showed that D-enantiomers had not significant influence on the behavior of their L-enantiomers ($p = 0.087$ for Met, $p = 0.177$ for HCy and $p = 0.173$ for Cys). For confirmation of these results, two times higher concentrations of D-enantiomers were added to the serum. We have assumed that higher concentrations of D-enantiomers could have had larger effect on their L-enantiomers. However, results did not confirm our assumptions ($p = 0.319$ for Met, $p = 0.453$ for HCY and $p = 0.731$ for Cys). More detailed results are shown in Table 1. Representative chromatograms of human serum after addition of D-amino acids at time 0 h and 24 h are shown in Fig. 1.

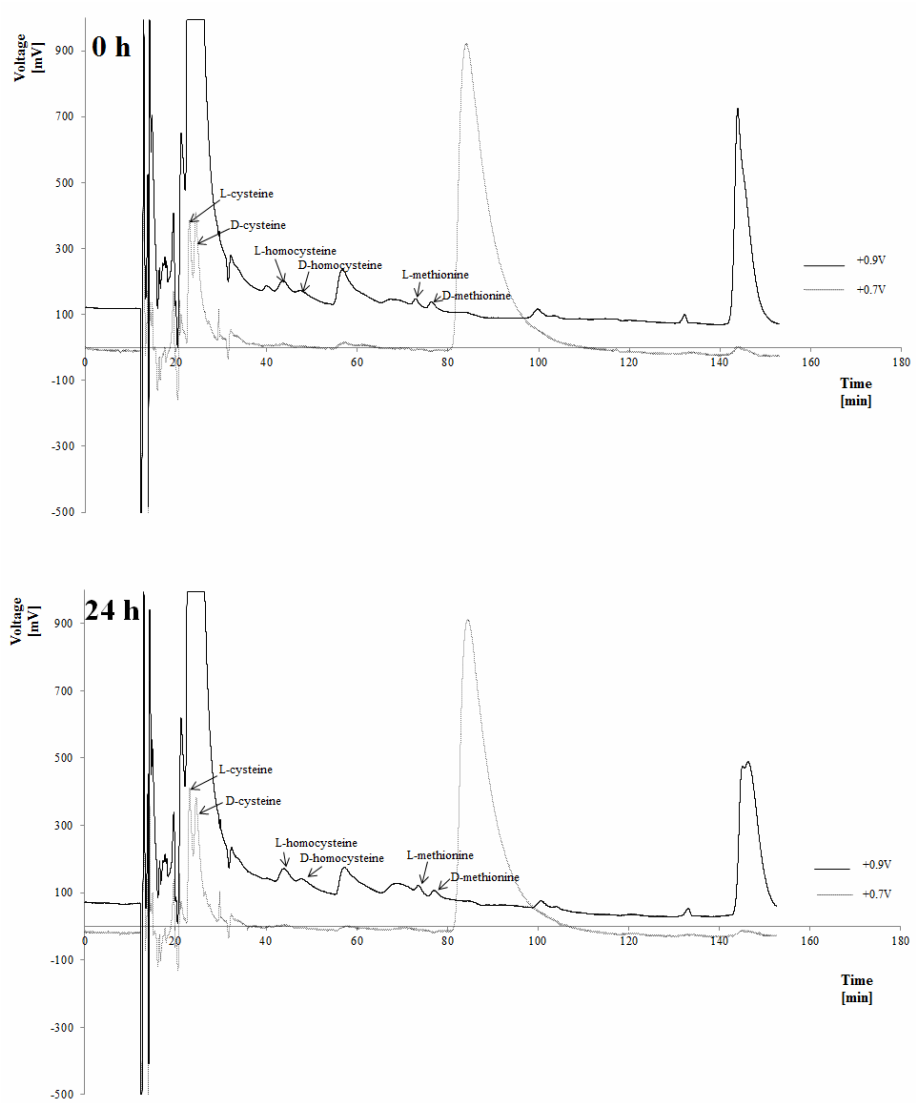

Fig. 1. Chromatograms of human serum after addition of D-amino acids at time 0 h and 24 h. D-amino acids were added to the serum at equimolar concentrations of their corresponding L-enantiomers. Cysteine enantiomers were determined at electrode cell potential +0.7 V and homocysteine as well as methionine enantiomers were determined at +0.9 V.

Obtained data were also used for the comparison of the behavior of L- and D-enantiomers in human serum *in vitro*. There were used values of L-enantiomers and also D-enantiomers that were added to the serum so that they had equimolar concentrations to their corresponding L-enantiomers. The behavior of L- and D-isomers of same amino acid was evaluated at chosen incubation time. However, the

standard of D-HCy was not available and its racemate was used for measurements, in the order to compare the behavior of these amino acid enantiomers, we calculated concentrations of L-HCy when racemate was used as total concentrations of L-HCy minus concentrations of D-HCy. Results of *t*-test have showed no significant differences in the behavior of L- and D-enantiomers ($p = 0.285$ for Met, $p = 0.067$ for HCy and $p = 0.071$ for Cys) and therefore probably no stereospecific behavior of enzymes was observed in these experimental conditions.

Table 1. Amino acid level changes with and without D-amino acids addition into serum.

Amino acid	Unit	Incubation time			
		0 h	24 h	48 h	72 h
L-Met	µmol/L	22.7	21.9	15.1	15.2
L-Met (D)	µmol/L	21.6	16.5	12.1	10.8
L-HCy	µmol/L	7.5	6.6	5.6	5.4
L-HCy (D)	µmol/L	7.8	7.2	6.4	5.8
L-Cys	µmol/L	269.7	225.6	213.2	213.5
L-Cys (D)	µmol/L	270.6	211.9	206.4	203.1

D-amino acids were added to the serum at two times higher concentrations as their corresponding L-enantiomers had. Data are present as average values of two measurements (n=2, n-number of volunteers)
Abbreviations: L-Met is a nomenclature for L-Met without the presence of D-Met and L-Met (D) is a nomenclature for L-Met in the presence of D-Met.

Basing on obtained results, we can only hypothesize that necessary enzymes inclusive in the methionine metabolism should be not active in our experimental conditions or however, D-enantiomers had not the influence on the behavior of their L-isomers, the presence of L-enantiomers might have inhibitory effect on D-enantiomers as it published JERVIS and SMYTH (1960).

4. Conclusions

Obtained results can provide the first view for us on *in vitro* behavior of amino acid enantiomers in human serum. No effect of D-enantiomers on the behavior of their L-enantiomers (no synergistic effect) was observed during chosen incubation time. Also, no conversion of selected D-amino acids to their L-forms and no differences in the behavior of D- and L-enantiomers were observed.

Taking into consideration knowledge of methionine metabolism, it is hard to simulate processes in the living organism by using *in vitro* system because the behavior of amino acids *in vivo* and *in vitro* is apparently different. Moreover, there probably exists the difference between the utilization (behavior) of amino acids in humans and animals and therefore it is hard to generalize our conclusions.

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References

AGAR, W.T., HIRD, F.J.R., SIDHFU, G.S.: The active absorption of amino-acids by the intestine. J. Physiol., 121, 1953, 255-263.

CAMPELL, K.C.M., MEECH, R.P., KLEMENS, J.J., GERBERI, M.T., DYRSTAD, S.S.W., LARSEN, D.L., MITCHELL, D.L., EL-AZIZI, M., VERHULS, S.J.T., HUGHES, L.F.: Prevention of noise- and drug-induced hearing loss with D-methionine. Hearing Res., 226, 2007, 92-103.

DEÁKOVÁ, Z., ĎURAČKOVÁ, Z., ARMSTRONG, D.W., LEHOTAY, J.: Two-dimensional high performance liquid chromatography for determination of homocysteine, methionine and cysteine enantiomers in human serum. J. Chromatogr A, 1408, 2015, 118-124.

EFRON, M.L., MCPHERSON, T.C., SHIH, V.E., WELSH, C.F., MAC CREADZ, R.A.: D-methioninuria due to DL-methionine ingestion. Am. J. Dis. Chid., 117, 1969, 104-107.

FRIEDMAN, M.: Chemistry, Nutrition, and Microbiology of d-Amino Acids. J. Agric. Food Chem., 47, 1999, 3457-3479.

FRIEDMAN, M., GUMBMANN, M.R.: The utilization and safety of isomeric sulfur-containing amino acids in mice. J. Nutr., 114, 1984, 2301-2310.

FUCHS, S.A., Berger, R., Klomp, L.W.J, De Koning T.J.: D-Amino acids in the central nervous system in health and disease: Minireview. Mol. Genet. Metab., 85, 2005, 168-180.

GARAIOVA, I., MUCHOVA, J., NAGYOVA, Z., MISLANOVA, C., ORAVEC, S., DUKAT, A., WANG, D., PLUMMER, S.F., DURACKOVA, Z.: Effect of a plant sterol, fish oil and B vitamin combination on cardiovascular risk factors in hypercholesterolemic children and adolescents: a pilot study. Nutr. J., 12, 2013, 1-8.

GIBSON, Q.H., WISEMAN, G.: Selective absorption of stereo-isomers of amino-acids from loops of the small intestine of the rat. Biochem. J., 48, 1951, 426-429.

GLATT, H., OESCH, F.: Mutagenicity of cysteine and penicillamine and its enantiomeric selectivity. Biochem. Pharmacol., 34, 1985, 3725-3728.

HASEGAWA, H., SHINOHARA, Y., AKAHANE, K., HASHIMOTO, T.: Direct Detection and Evaluation of Conversion of D-Methionine into L-Methionine in Rats by Stable Isotope Methodology. J. Nutr., 35, 2005, 2001-2005.

CHO, E.S., STEGINK, L.D.: D-Methionine utilization during parenteral nutrition in adult rats. J. Nutr., 109, 1979, 1086-1093.

JERVIS, E.L., SMYTH, D. H.: Competition between enantiomorphs of amino acids during intestinal absorption. J. Physiol., 145, 1959, 57-65.

JERVIS, E.L., SMYTH, D. H.: The active transfer of D-methionine bz the rat intestine in vitro. J. Physiol., 151, 1960, 51-58.

KIES, C., FOX, H., APRAHAMIAN, S.: Comparative value of L-, DL- and D-methionine supplementation of an oat-based diet for humans. J. Nutr., 105, 1975, 809-814.

KRIJGSHELD, K.R., GLAZENBURG, E.J., SCHOLTENS, E., MULDER, G.J.: The oxidation of L- and D-cysteine to inorganic sulfate ad taurine in the rat. Biochim. Biophys. Acta, 18, 1981, 7-12.

MATTHEWS, D.M., SMYTH, D.H.: The intestinal absorption of amino acid enantiomorphs. J. Physiol., 126, 1954, 96-100.

OANCEA, S., FORMAGGIO, F.: Biological role of D-α-amino acids and their occurrence in foodstuffs – review. Acta Universitatis Cibiniensis Series E: FOOD TECHNOLOGY, 12, 2008, 3-18.

PRINTEN, K.J., BRUMMEL, M.C., ERICSON, M.S., STEGINK, L.D.: Utilization of D-methionine during total parenteral nutrition in post-surgica patients. Am. J. Clin. Nutr., 32, 1979, 1200-1205.

ROBERTS, J.C.: Stereoisomers of cysteine and its analogs Potential effects on chemo- and radioprotection strategies. Amino Acids, 8, 1995, 113-124.

STEGINK, L.D., SCHMITT, J.L., MEYER, P.D., KAIN, P.H.: Effect of diets fortified with DL-methionine on urinary and plasma methionine levels in young infants. J. Pediatr., 79, 1971, 648-655.

TSUKAMOTO, S., KANEGAE, T., NAGOYA, T., SHIMAMURA, M., MIEDA, Y., NOMURA, M., HOJO, K., OKUBO, H.: Effects of amino acids on acute alcohol intoxication in mice-concentrations of ethanol, acetaldehyde, acetate and acetone in blood and tissues. Arukoru Kenkyuto Yakubutsu Ison (JAPAN), 25, 1990, 429-440.

WISEMAN, G.: Absorption of amino-acids using an in vitro technique. J. Physiol., 120, 1953, 63-72.

WISEMAN, G.: Preferential transference of amino acids from amino-acid mixtures by sacs of everted small intestine of the golden hamster (*Memocricetus auratus*). J. Physiol., 127, 1955, 414-422.

ZEZULKA, A.Y., CALLOWAY, D.H.: Nitrogen retention in men fed isolated soybean supplemented with L-methionine, D-methionine, N-acetyl-L-methionine or inorganic sulfate. J. Nutr., 106, 1976, 1286-1291.

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