Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 59 (2010) 14 – 19
Metabolism
Clinical and Experimental
www.metabolismjournal.com

The significance of D-isomers in stable isotope studies in humans is dependent on the age of the subject and the amino acid tracer

Chris Tomlinson[a,b,c], Mahroukh Rafii[b], Ronald O. Ball[c,d], Paul Pencharz[a,b,c,d,*]

[a]Department of Paediatrics, The Hospital for Sick Children, Toronto, M5G1X8 Canada
[b]Research Institute, The Hospital for Sick Children, Toronto, M5G1X8 Canada
[c]Department of Nutritional Sciences, University of Toronto, Toronto, M5S 3E2 Canada
[d]Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, T6G 2P5 Canada

Received 12 January 2009; accepted 29 June 2009

Abstract

D-Amino acids (D-AAs) in stable isotope tracers may result in erroneous estimates of enrichment, particularly if urine is used as a surrogate for plasma enrichment. Previous studies suggest that a D-AA content of less than 0.2% will not result in significant error in studies with adult humans. To describe the effects of D-AA content of less than 0.2%, in 3 different AA tracers, on isotope enrichment in urine and plasma, arginine, proline, and phenylalanine (Phe) tracers were given enterally to human neonates. Enrichment was measured in urine and plasma using chiral chromatography and tandem mass spectrometry. The Phe tracer was also given parenterally to human neonates and enterally to children and adults to further characterize the D-AA effect. All isotopes had a confirmed D-AA content of less than 0.2%. Labeled D-arginine resulted in an overestimate for enrichment of 20% in plasma and 87% in urine. A smaller effect was seen for D-Phe, which resulted in a 5% overestimate for plasma and 40% in urine. D-Proline had no significant effect. Using the same Phe tracer, a developmental effect was found, with a reduction in the overestimate in children compared with infants and no effect on enrichment in adults. Investigators using commercially produced, stable isotope–labeled AAs need to be aware that there is no safe level of D contamination; a D-AA content less than 0.2% may result in significant overestimate for enrichment, even in plasma, for infants and children. This source of error can be avoided by the use of chiral chromatography.

1. Introduction

D-Amino acids (D-AAs) are increasingly recognized as playing a biological role. D-Isoforms of AAs had previously been thought to be metabolically inert but have been shown to be linked with disease processes, for example, schizophrenia [1] and with aging [2]. D-Amino acids found in mammals are largely synthesized from intestinal microbes [3]. However, mammals (including humans) possess a D-AA oxidase (DAAO) to deaminate these isoforms to their keto acids [4]. The DAAO is highly expressed in kidney, such that individuals with renal failure have high levels of serum D-AAs [3].

Amino acids labeled with stable isotopes are now commonly used in human and animal research because they pose no known biological hazard and can provide dynamic information on metabolic processes. Most stable isotope–labeled compounds used for these studies are commercially available; and in the case of labeled AAs, there is usually a measure of their optical purity, which is commonly in the order of greater than 99% of the L-isoform.

In neonates, we and others have developed minimally invasive methods in which stable isotope–labeled AA tracers are given enterally or parenterally and enrichment in urine is used as a surrogate for plasma enrichment, principally to minimize venipuncture and blood loss in this vulnerable group. One drawback to this technique is the confounding effect of D-AA content of the tracer, which results in falsely elevated estimates of enrichments as the D- and L-isoforms are metabolized at different rates. This effect was first suggested by Waterlow [5] and by Waterlow and Stephen [6] and subsequently demonstrated by Zello et al [7] in adult humans.

We have previously published data demonstrating that D-phenylalanine (Phe) and D-lysine substantially increase the

* Corresponding author. University of Toronto, Toronto, Ontario, Canada M5G 1X8. Tel.: +1 416 813 7733; fax: +1 416 813 4972.
E-mail address: paul.pencharz@sickkids.ca (P. Pencharz).

0026-0495/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2009.06.024

AA enrichment in urine compared with plasma [8]. This would result in a falsely low flux estimate if the D contaminated enrichment value was used. In this observational study [8], 2 different Phe isomers were used with different D-AA content; and we concluded that, if the amount of D was less than 0.2%, this would not result in any significant reduction in Phe flux rates. These earlier results were obtained using a gas chromatography chiral column.

Recently, as part of new studies of arginine (Arg) metabolism in enterally fed neonates, we used a liquid chromatography chiral column. We uncovered a major problem with the ratio of enrichment in urine vs plasma for Arg in neonates and, to our surprise, a smaller problem with Phe. We therefore expanded our evaluation to the effects of small amounts of tracer D-Phe (<0.2%) in the urine of parenterally fed neonates (thereby controlling for the effects of gut absorption and metabolism) and to whether there was a developmental effect in the handling of D-Phe by studying enrichments in 6- to 10-year–old children and adults receiving the same tracer.

2. Methods

Four studies are presented.

2.1. Enteral studies in neonates

These studies were primarily performed to investigate Arg metabolism in preterm infants. Briefly, 7 eligible infants were studied at between 30 and 34 weeks postconceptual age, less than 21 days and fully enterally fed. All infants received 3 labeled AAs that were given enterally via nasogastric tube, 2 or 3 hourly, depending on feeding protocol, to simulate a constant infusion [9]. The dose of AA tracers were as follows: [1-^{13}C]Phe, 30 μmol/kg prime and 15 μmol/(kg h) infusion; [$^{15}N_2$-guanidino]Arg, 20 μmol/kg prime and infusion; and [^{15}N]proline (Pro), 20 μmol/kg prime and infusion. All 7 infants had urine collected at each diaper change. In 4 of the 7 infants, blood was collected by venipuncture at 10 or 12 hours after the prime dose depending on timing of nursing care to allow comparison of blood and urine enrichments. The study lasted 12 hours. This study has been partially presented in abstract form using only the L enrichments [10].

2.2. Parenteral studies in neonates

These studies were performed in parenterally fed infants, postsurgery for congenital gastrointestinal anomalies, primarily to investigate sulfur AA requirements. The study has been presented in detail elsewhere [11] using only the L enrichments. Briefly, an intravenous priming dose of [1-^{13}C]Phe was given at 15.6 μmol/kg over 15 minutes followed by a continuous intravenous infusion of 13 μmol/(kg h) for 23.75 hours. Three to 5 urine samples were collected at the isotopic plateau, between 12 and 24 hours after initiation of the isotope infusion. The urine of 5 subjects was reanalyzed for the current study.

2.3. Enteral studies in children

Six healthy school-aged children took part in these studies investigating methionine requirements [12]. Briefly, the [1-^{13}C]Phe tracer was given *per os* hourly for 4 hours with the hourly meals. Urine was collected at isotopic plateau at 2.5, 3, 3.5, and 4 hours. The priming dose was 6.55 μmol/kg, and infusion rate was 11.8 μmol/(kg h). Requirement estimates were based on breath $^{13}CO_2$ enrichments and are therefore not affected by the tracer D content. The urine of 5 subjects was reanalyzed for the current study.

2.4. Enteral studies in adults

Eight adult men took part in these studies investigating protein requirements. The full studies are published elsewhere [13]. Briefly, the [1-^{13}C]Phe tracer was given orally with a priming dose of 0.66 mg/kg followed by an hourly dose of 50 μmol/(kg h) for 4 hours. Urine was then collected at isotopic plateau at 2.5, 3, 3.5, and 4 hours. Urine samples of 5 of the subjects were retested for the current study.

2.5. Ethics approval

All the studies were approved by the Research Ethics Board at The Hospital for Sick Children, Toronto; and the neonatal enteral studies were also approved by the Research Ethics Board at St Michael's Hospital, Toronto. Written informed consent was obtained from one parent or from the research subject.

2.6. Isotopes

Isotopes were obtained from Cambridge Isotope Laboratories (Andover, MA). Three labeled AAs were used in the studies: [1-^{13}C]Phe, [^{15}N]Pro, and [guanidino-$^{15}N_2$]Arg, with a stated optical purity for content of the D-isoform of 0.1% for Phe, less than 0.5% for Pro, and 0.27% for Arg.

2.7. Sample collection and handling

Urine was collected in neonates using a bag and in children and adults by collection of urine in sterile containers. Urine was kept frozen at −20°C until analysis.

Blood was collected by sterile venipuncture and was centrifuged at 13 000 rpm for 10 minutes to obtain plasma. Plasma was stored at −20°C until analysis.

2.8. Analysis of enrichment and chiral separation

Fifty microliters of plasma or 60 μL of urine was deproteinized by the addition of 500 μL of methanol followed by centrifugation at 13 000*g* for 5 minutes. The supernatant was dried under N_2 and reconstituted in 0.1% formic acid. Isotope analysis was carried out using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) operated

in positive electrospray ionization mode. This was coupled to an Agilent 1100 high-performance liquid chromatography (HPLC) system (Agilent Technologies, Palo Alto, CA). All aspects of the system operation and data acquisition were controlled using the Analyst NT version 1.4.1 software (Applied Biosystems, Concord, Ontario, Canada). Maximum sensitivity for L- and D-Arg, -Pro, and -Phe was achieved by measuring product ions multiple reaction monitoring from the fragmentation of the protonated $[M + H]^+$ molecule for the L and D separation of each AA. Pure solutions of 5 μmol/L of L- and D-Arg, -Pro, and -Phe were injected into the mass spectrometer for maximum sensitivity of the $[M + H]^+$ precursor (parent) ions (m/z 175.1 for Arg, m/z 116.1 for Pro, and m/z 166.2 for Phe). The signal for the most abundant product (daughter) ions (m/z 70.1 for Arg, m/z 70.1 for Pro, and m/z 120.2 for Phe) was also optimized. Chiral separations were performed with a Chirobiotic T (teicoplanin) HPLC column (25 cm × 4.6 mm, 5 μm; Astec, Whippany, NJ) using an isocratic gradient with 95:5 (10 mmol/L NH4Ac, pH = 4.1):(2:1 MeOH:0.1% formic acid in acetontrile) buffer at 700 μL/min. The injection volume was 20 μL, with an overall analysis time (injection to injection) of 35 minutes. The retention times were 23.89 and 27.16 minutes, respectively, for L- and D-Arg; 6.38 and 9.46 minutes, respectively, for L- and D-Pro; and 7.23 and 7.86 minutes, respectively, for L- and D-Phe.

2.9. Expression of data

In Table 1, results are expressed as percentage enrichment of the tracer at steady state and are derived using the formula tracer/(tracee + tracer) ∗100.

In the other tables, the amount of labeled D-AA is expressed as a percentage of the total labeled AA (ie, D/[D + L] ∗ 100) for each AA isomer.

2.10. Statistics

Results are expressed as mean ± SD. Differences between total and L-only enrichments in urine and plasma were compared using paired Student t test. Difference in D-isomer percentage in urine between infants, children, and adults were compared using Student t test. Analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC).

3. Results

3.1. Analysis of tracer-labeled AAs

All of our isotopes had an optical purity greater than 99.8%, as stated on the certificate of analysis from the manufacturer. Analysis of the AA isotopes via the chiral column and tandem mass spectrometry confirmed the content of D-Pro M + 1 to be 0.08%, D-Arg M + 2 to be 0.14%, and D-Phe to be 0.1%.

3.2. Enteral studies in preterm infants [10]

The results of the enteral studies in preterm infants are shown in Table 1. As presented, the results compare enrichment when the total tracer amount is used and when only the L-isoform is used after chiral separation. Both Phe and Arg had a significant reduction in enrichment when this was calculated using the L-isoform only rather than the total enrichment (ie, the sum of L- + D-isoforms). This effect was most pronounced for Arg, in which the D-isomer was found to comprise 21% of the labeled Arg in plasma and 86% of the labeled AAs in urine. Sample chromatograms from one infant are shown in Fig. 1, highlighting the significance of the effect. Failure to use chiral separation would have resulted in estimates of flux of 80% for plasma and 15% for urine of the true value as estimated from the L-isoform alone. The effect on Phe was smaller but still statistically significant. When using the sum of the L- and D-isomers, enrichment was 5% higher in plasma and 40% higher in urine compared with enrichment using only the L-isomer. This would also result in significantly lower estimates of flux if chiral separation was not used.

The percentage of the tracer in plasma that was the D-isomer appeared to be greater for Arg (mean ± SD, 21.6% ± 18.3%) than for Phe (6.33% ± 5.27%); but because of wide variability, this was not statistically significant using paired t test. However, the percentage of D-isomer tracer in urine was greater for Arg (85.68% ± 6.86%) than for Phe (36% ± 9.98%) using paired t test ($P < .001$).

The Pro tracer appeared to show some D-isomer in both plasma and urine; however, this amount could not be quantified because the chromatogram peaks were too small to be accurately measured. Therefore, this effect

Table 1
Comparison of enrichment of AAs when using total tracer amounts (ie, L- + D-isoforms) and using only the L-isoform in plasma and urine of neonates (n = 4) with tracer given enterally [10]

	Pro M + 1 enrichment		Phe M + 1 enrichment		Arg M + 2 enrichment	
	Total (L + D)	L-Isoform only	Total (L + D)	L-Isoform only	Total (L + D)	L-Isoform only
Plasma (n = 4)	8.1%[a] ± 2.22%	8.1%[a] ± 2.2%	7.25%[a] ± 3.4%	6.9%[a,*] ± 3.5%	6.14%[a] ± 1.4%	4.9%[a,*] ± 2.0%
Urine (n = 4)	9.1%[a] ± 1.4%	9.1%[a] ± 1.4%	14.5%[b] ± 1.53%	8.7%[a,*] ± 0.9%	35.3%[b] ± 6.13%	5.1%[a,*] ± 1.0%

Values are percentage enrichment ± SD.

[a,b] Different superscripts in columns indicate significant difference between urine and plasma enrichments using paired t test ($P < .05$).

* L-Isoform enrichment significantly different from total enrichment in plasma or urine using paired t test ($P < .05$).

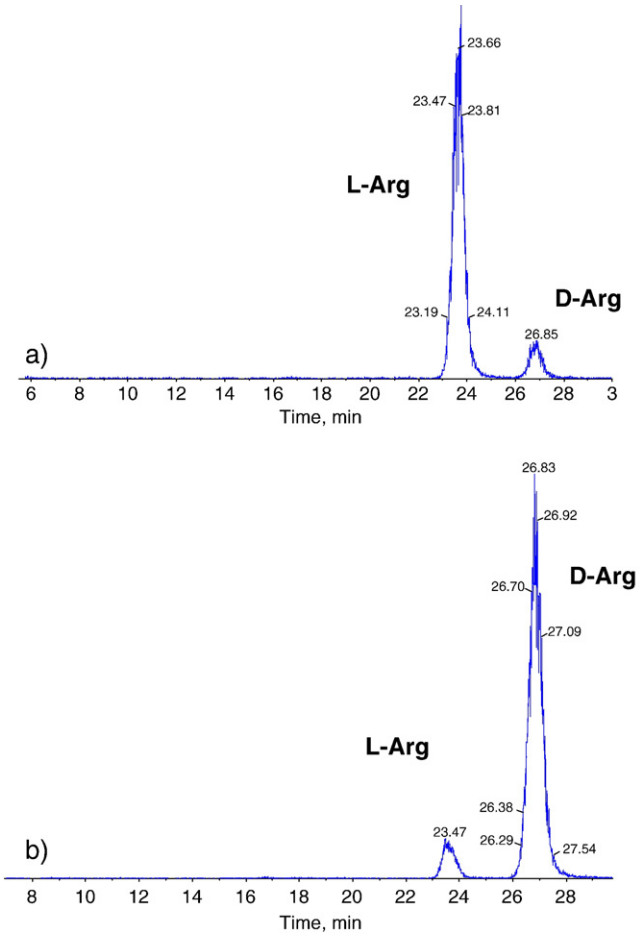

Fig. 1. Representative chromatograms of L- and D-[$^{15}N_2$]Arg from one patient (a) from plasma and (b) from urine.

was clearly not statistically or scientifically significant (data not shown).

Comparison was made of enrichment in plasma and urine of the 3 L-AAs after chiral separation using paired *t* test and showed no difference, indicating that urine enrichment may be used as a surrogate for plasma enrichment.

Naturally occurring M + 0 D-Arg was detected in the urine of all infants comprising a mean of 11% of total urinary Arg (SD, 13%). M + 0 D-Arg was not detected in plasma, and naturally occurring D-isomers of Pro and Phe were not detected in either plasma or urine.

3.3. Effect of route of administration of D-Phe tracer in neonates [10,11]

By comparing the results of the first 2 studies, we were able to assess the effect of route of administration on the percentage of the tracer in urine that was the D-isomer (Table 2). The mean D-isomer content of urine for the enterally fed neonates was 37% compared with 27% for those parenterally fed. Because of the small number of subjects, this is not statistically significant but suggests a trend ($P = .06$).

Table 2
Comparison of route of tracer in neonates and percentage of D M + 1 Phe in urine

Subjects	Route of tracer		% d M + 1/total M + 1	
		n	Mean	SD
Newborn	Oral [10]	7	37.2	7.3
Newborn	IV [11]	5	27.7	8.6

Difference between oral and intravenous groups approached significance ($P = .06$). From Tomlinson et al [10] and Courtney-Martin et al [11]. IV indicates intravenous.

3.4. Phe studies in neonates, children, and adults [10,12,13]

In children, as in the neonates, a significant percentage of the tracer in urine was found to be of the D-isoform (Table 3). However, the percentage of the D-isoform was significantly lower in children compared with neonates, at 20.9% and 37.2%, respectively ($P < .05$). No D-Phe was found in urine in any of the adult subjects.

4. Discussion

We have shown variable but consistent effects of D-AA contamination of stable isotope tracers on urine vs plasma enrichment in 3 human age groups, when the tracers are given both enterally or parenterally.

Three stable isotope AAs were given enterally to neonates, all with tracer D-AA less than 0.2%. The effect of minimal D content in the tracer on enrichment of Arg was most striking. This was found to be such that, if chiral methodology was not used, significant errors in flux estimates would have occurred in both plasma and urine.

Similar but less-pronounced effects were seen for Phe. When the tracer was given either enterally or parenterally, urine enrichment without chiral HPLC analysis would have resulted in a significant overestimate of tracer enrichments. We have noted this effect before in adults [8] but not if the D content was less than 0.2%. Hence, we were surprised to find that, when the tracer was given enterally, there was a significant amount of D-Phe in plasma, which would result in a 5% overestimate in enrichment. The earlier studies used a gas chromatography chiral column in which the separation of L- and D-Phe was only 6 seconds compared with 36 seconds with the liquid chromatography method. This allowed more precise measurement of the D content. In contrast, the

Table 3
Developmental effect of age on percentage of D M + 1 Phe in urine

Subjects	Route of tracer		% d M + 1/total M + 1	
		n	Mean	SD
Newborn	Oral [10]	7	37.2	7.3
Child	Oral [12]	5	20.9	5.2
Adult	Oral [13]	5	0	0

Difference between newborn and child groups is significant ($P < .05$).

enrichment of the Pro tracer, which had a similar D content to the other labeled AAs, was not significantly affected in either plasma or urine.

By using the same Phe tracer in different age groups, we have been able to show an age effect on the importance of D content of tracer. The effect of D-isomer was most striking in neonates and was less so, but still important, in children; and there was no effect at all in adults.

It has previously been thought that D-AAs are not biologically important [14]. Certainly, they are unable to be incorporated into proteins; and enzymes active against the L-isoform of an AA do not have significant reactivity against the D form [15]. Nevertheless, in recent years, it has become clear that D-AAs are present in the environment and do make up a small but significant portion of our diet, mostly from bacterial sources [14]. More importantly, some D-AAs, notably D-aspartate and D-serine, have been shown to be biologically active and have a role in pathophysiology, being implicated in the pathogenesis of schizophrenia, and in the effects of aging, as seen in the amount of D-aspartate in lens proteins [16]. Mammals, including humans, have a DAAO, which is widely expressed in tissues and catalyzes the deamination of D-AAs to its keto acid [17]. Keto acids exist in equilibrium between the L- and D-isoforms.

That D-AAs may affect isotope enrichment is well known. For example, in this Journal in 1982, Clarke and Bier [18], using a [^{13}C]tyrosine stable isotope that had 9% D, demonstrated that this comprised approximately 30% of plasma enrichment They used a combination of treatment with DAAO and chiral chromatography to remove the D-tyrosine. Similarly, our group has also shown that D-Phe of 0.4% in a stable isotope may add significant error to the apparent enrichment of the tracer in urine [8].

However, the current study indicates that even levels of D-isoform less than 0.2% can have a significant effect on stable isotope enrichment. In addition, by using 3 AAs with similar amounts of D-isomer, we have demonstrated that the magnitude of this effect depends upon the AA in question. This may in part be explained by the activity of DAAO against different AAs. Mammalian DAAO has been shown to be most active for the nonpolar AAs, including Pro and Phe. For example, the porcine form is most active against D-Pro, has moderate activity for D-Phe, and has little activity against D-Arg [2]. We may be observing in our studies the inability of DAAO to remove D-Arg and, to a lesser extent, D-Phe, resulting in an increase in the percentage of D measured in both plasma and urine for these AA tracers. However, little is known about the expression and activity of DAAO in human infants and children; and this needs to be elucidated.

We are unaware of any AA transfer system that has significant activity for D-isoforms of the AAs used in our study, although there is a transporter system with activity for D-serine [19]. D-Amino acids may be passively transferred across the gut by the paracellular route, which has been shown to occur for D peptides [20]. Alternatively, Oguri et al [21] have shown that uptake of D-AAs may occur through a sodium-dependent transporter system. It is clear, however, based upon our data in enterally fed subjects and that of others [17,21], that D-AAs are readily absorbed by the gastrointestinal tract. The splanchnic bed is known to significantly metabolize AAs on first pass. For example, Matthews et al [22] estimated that 30% of enteral Phe was sequestered by the splanchnic bed. Because the D-AA will not undergo similar first-pass metabolism, the result is an increase in the percentage of D tracer in plasma. This may account for the effect we observed on the differing effects of the tracer route (enteral or parenteral) on the magnitude of the effect of the D-Phe isomer.

We reason that, from blood, both D- and L-AAs are freely filtered at the glomerulus from which the L- but not the D-isoform is taken up through active transfer by the nephron, resulting in the further increase in percentage of D measured in urine. An additional effect, explaining the quantitative difference seen between AAs, may again be the activity of DAAO, as renal DAAO has been shown to be actively involved in the pharmacokinetics of D-leucine [23].

This is also the first study to demonstrate a developmental effect on the importance of D-AAs in tracers, with the effect being greatest in neonates and children. This may be explained in part by a dose effect, as the children were given a smaller hourly dose than the neonates. However, the adults were given a much larger dose per kilogram than children or neonates, suggesting another explanation, namely, that it may be possibly related to a change in the expression of DAAO with age.

We hypothesize that apparent isotope effects observed by others may be explained by D-AAs; for example, Krempf et al [24] noted different flux estimates with 3 different Phe tracers and suggested that this may be explained by proton exchange among the molecules. Given the data in our study, some of the differences in older studies attributed to the isotope effect, that is, the difference in mass and charge between the isotopes, may in fact be explained by contamination with D-AAs.

In conclusion, we have shown that very low concentrations of D-AAs in stable isotope tracers may cause significant errors in measurement of enrichment and hence in AA kinetics. This effect is variable, dependent on the AA used, the age of the patient, the route of administration, and the site of sampling. A researcher performing stable isotope–labeled AA studies, particularly in children, must consider using chiral chromatography, a relatively simple method, to ensure that this potential error is identified and eliminated.

Acknowledgment

The study was supported by grant MOP12928 from the Canadian Institutes for Health Research.

We gratefully acknowledge the contributions of the infants and their parents, and the volunteers who participated in these studies.

The authors' responsibilities were as follows—CT: study design, subject recruitment, data collection, sample and data analysis, and manuscript writing; MR: study design, sample and data analysis, and manuscript writing; ROB and PBP: study design, data analysis, and manuscript writing.

None of the authors have any conflicts of interest to declare.

References

[1] Fuchs SA, Berger R, Klomp LW, de Koning TJ. D-Amino acids in the central nervous system in health and disease. Mol Genet Metab 2005;85:168-80.

[2] Tishkov VI, Khoronenkova SV. D-Amino acid oxidase: structure, catalytic mechanism, and practical application. Biochemistry (Mosc) 2005;70:40-54.

[3] Nagata Y, Sato T, Enomoto N, Ishii Y, Sasaki K, Yamada T. High concentrations of D-amino acids in human gastric juice. Amino Acids 2007;32:137-40.

[4] Pollegioni L, Piubelli L, Sacchi S, Pilone MS, Molla G. Physiological functions of D-amino acid oxidases: from yeast to humans. Cell Mol Life Sci 2007;64:1373-94.

[5] Waterlow JC. Lysine turnover in man measured by intravenous infusion of L-[U-14C]lysine. Clin Sci 1967;33:507-15.

[6] Waterlow JC, Stephen JM. The measurement of total lysine turnover in the rat by intravenous infusion of L-[U-14C]lysine. Clin Sci 1967;33:489-506.

[7] Zello GA, Marai L, Tung AS, Ball RO, Pencharz PB. Plasma and urine enrichments following infusion of L-[1-13C]phenylalanine and L-[ring-2H5]phenylalanine in humans: evidence for an isotope effect in renal tubular reabsorption. Metabolism 1994;43:487-91.

[8] Darling PB, Bross R, Wykes LJ, Ball RO, Pencharz PB. Isotopic enrichment of amino acids in urine following oral infusions of L-[1-(13)C]phenylalanine and L-[1-(13)C]lysine in humans: confounding effect of D-[13C]amino acids. Metabolism 1999;48:732-7.

[9] Wykes LJ, Ball RO, Menendez CE, Pencharz PB. Urine collection as an alternative to blood sampling: a noninvasive means of determining isotopic enrichment to study amino acid flux in neonates. Eur J Clin Nutr 1990;44:605-8.

[10] Tomlinson C, Rafii M, Sgro M, Ball R, Pencharz P. Arginine is synthesized from proline not glutamate in enterally fed preterm human infants. FASEB J 2008;22(1_MeetingAbstracts):869.28.

[11] Courtney-Martin G, Chapman KP, Moore AM, Kim JH, Ball RO, Pencharz PB. Total sulfur amino acid requirement and metabolism in parenterally fed postsurgical human neonates. Am J Clin Nutr 2008;88:115-24.

[12] Humayun MA, Turner JM, Elango R, Rafii M, Langos V, Ball RO, et al. Minimum methionine requirement and cysteine sparing of methionine in healthy school-age children. Am J Clin Nutr 2006;84:1080-5.

[13] Humayun MA, Elango R, Ball RO, Pencharz PB. Reevaluation of the protein requirement in young men with the indicator amino acid oxidation technique. Am J Clin Nutr 2007;86:995-1002.

[14] Fujii N. D-Amino acids in living higher organisms. Orig Life Evol Biosph 2002;32:103-27.

[15] Man EH, Bada JL. Dietary D-amino acids. Annu Rev Nutr 1987;7:209-25.

[16] Hamase K, Morikawa A, Zaitsu K. D-Amino acids in mammals and their diagnostic value. J Chromatogr B Analyt Technol Biomed Life Sci 2002;781:73-91.

[17] D'Aniello A, D'Onofrio G, Pischetola M, D'Aniello G, Vetere A, Petrucelli L, et al. Biological role of D-amino acid oxidase and D-aspartate oxidase. Effects of D-amino acids. J Biol Chem 1993;268:26941-9.

[18] Clarke JT, Bier DM. The conversion of phenylalanine to tyrosine in man. Direct measurement by continuous intravenous tracer infusions of L-[ring-2H5]phenylalanine and L-[1-13C] tyrosine in the post-absorptive state. Metabolism 1982;31:999-1005.

[19] Hatanaka T, Huang W, Nakanishi T, Bridges CC, Smith SB, Prasad PD, et al. Transport of D-serine via the amino acid transporter ATB (0,+) expressed in the colon. Biochem Biophys Res Commun 2002;291:291-5.

[20] Pappenheimer JR, Dahl CE, Karnovsky ML, Maggio JE. Intestinal absorption and excretion of octapeptides composed of D amino acids. Proc Natl Acad Sci U S A 1994;91:1942-5.

[21] Oguri S, Kumazaki M, Kitou R, Nonoyama H, Tooda N. Elucidation of intestinal absorption of D,L-amino acid enantiomers and aging in rats. Biochim Biophys Acta 1999;1472:107-14.

[22] Matthews DE, Marano MA, Campbell RG. Splanchnic bed utilization of leucine and phenylalanine in humans. Am J Physiol 1993;264(1 Pt 1):E109-18.

[23] Hasegawa H, Matsukawa T, Shinohara Y, Konno R, Hashimoto T. Role of renal D-amino-acid oxidase in pharmacokinetics of D-leucine. Am J Physiol Endocrinol Metab 2004;287:E160-5.

[24] Krempf M, Hoerr RA, Marks L, Young VR. Phenylalanine flux in adult men: estimates with different tracers and route of administration. Metabolism 1990;39:560-2.