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-[^{13}C]Amino Acids

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Urine sampling of the free amino acid pool serves to reflect plasma enrichment and is used as a noninvasive means to determine isotope enrichment in studies of amino acid metabolism. We determined the effect of D-[^{13}C]phenylalanine and D-[^{13}C]lysine content of tracers on urinary amino acid enrichment following oral infusion of L-[^{13}C]phenylalanine in 18 preterm infants and L-[1-^{13}C]lysine in seven healthy adult females. Urinary [^{13}C]phenylalanine enrichment was higher ($P < .0001$) for L-[^{13}C]phenylalanine containing 0.4% D-[^{13}C]phenylalanine (28.6 ± 7.1) versus L-[1-^{13}C]phenylalanine that contained undetectable D-[^{13}C]phenylalanine (10.2 ± 1.5). D-[^{13}C]phenylalanine, measured by chiral column gas chromatography–mass spectrometry (GC-MS), accounted for 10% to 30% (20.5% ± 7%) of total phenylalanine in the urine of infants who received 0.4% D-[^{13}C]phenylalanine, and was absent from the urine of infants receiving tracer with undetectable [^{13}C]phenylalanine. Urinary L-[^{13}C]phenylalanine enrichment did not differ between tracer groups (9.8 ± 1.5 and 9.8 ± 2.5). In adult females, the use of L-[1-^{13}C]lysine (1.6% D-lysine) resulted in a higher ($P < .02$) urine total L,D-[^{13}C]lysine enrichment compared with plasma enrichment (40.8 ± 4.1 *v* 11.1 ± 0.7). This study demonstrates the significant presence of D-[^{13}C]amino acids in urine that originate as contaminants from commercially manufactured tracers, as a result of renal tubular discrimination of D-amino acids. A tracer containing detectable amounts of D-[^{13}C]isomer cannot be recommended for any study in which urine will be used to reflect enrichment in the arterial plasma pool.

THE USE OF STABLE ISOTOPES to quantify various aspects of human amino acid metabolism in vivo has rapidly expanded over the past three decades. The reasons for this expansion include the technological advances that facilitate analysis and the increasing availability of commercially prepared amino acid tracers.[1] Recently, amino acid tracer infusion studies have begun to use less invasive procedures, such as oral instead of intravenous tracer infusion and urine as opposed to blood sampling.[2-5] These developments have opened the way to making amino acid metabolism studies practical, ethical, and therefore accessible to infants, children, and other vulnerable groups.

Urine collection, as a means of sampling the free amino acid pool, has been validated in several studies involving infusions of amino acid tracers by direct comparison of the isotopic enrichment of free amino acids in plasma and urine.[2,5-7] Similarity between phenylalanine enrichment in plasma and urine was shown in infants after oral infusion of L-[1-^{13}C]phenylalanine[6] and in adults after either intravenous[7] or oral[5] L-[1-^{13}C]phenylalanine.

During a recent investigation examining phenylalanine and threonine metabolism in preterm infants,[4] we were surprised to observe an unusually high urinary phenylalanine enrichment associated with one particular lot of L-[1-^{13}C]phenylalanine. One possible explanation was that the elevated urinary enrichment was due to the presence of D-[1-^{13}C]phenylalanine, which originated as a contaminant from the tracer.[8] The problem was accentuated by a second observation of a high urinary amino acid enrichment that occurred during another recent investigation in adults using L-[1-^{13}C]lysine.[5] In this second study, the enrichment ratio of urine to plasma lysine was 2.6-fold higher than expected.

The possibility of D-isomer contamination was first raised by Waterlow and Stephen in studies with constant infusion of L-[U-^{14}C]lysine in humans[8] and rats.[9] A small amount of highly radioactive material that was not L-lysine was observed in urine but not in plasma; it was assumed to be D-[U-^{14}C]lysine that originated in the tracer. However, direct evidence for the presence of D-lysine in urine was not presented in these studies.

The purpose of this study was therefore to investigate quantitatively the confounding effects of D-[1-^{13}C]amino acids originating from commercially manufactured tracers on the urinary amino acid enrichment recently encountered in our laboratory. The effect of D-[1-^{13}C]amino acid content of the phenylalanine tracer on the [^{13}C] enrichment of metabolites such as tyrosine in urine and breath CO_2 was also examined.

SUBJECTS AND METHODS

Materials

L-[1-^{13}C]phenylalanine (99% 1-^{13}C) was obtained from Merck, Sharpe & Dohme (Montreal, Quebec, Canada; lots A, B, and C) and from Cambridge Isotope Laboratories (Woburn, MA; lot D). Enrichment was verified by gas chromatography–mass spectrometry (GC-MS). Optical rotation tests, thin-layer chromatography, and nuclear magnetic resonance analysis performed by the manufacturers confirmed the chemical purity (>98% L-[1-^{13}C]phenylalanine). Chiral high-performance liquid chromatography (HPLC) performed by the manufacturers on lots B, C, and D showed enantiomeric purity of these three lots (actual measurements, <1% D-isomer). Such data were not available for lot A. The enantiomeric purity of lots A, B, C, and D was reanalyzed in our laboratory using a chiral column attached to a GC-MS instrument.

L-[1-^{13}C]lysine (99% 1-^{13}C) was obtained from Cambridge Isotope

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Submitted June 30, 1998; accepted December 28, 1998.

Supported by Medical Research Council Grant No. MT-10321 and in part by a MRC studentship (P.B.D.).

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0026-0495/99/4806-0010$10.00/0

Laboratories. Enrichment and chemical purity were verified by GC-MS. Chiral HPLC performed by the manufacturer showed enantiomeric purity (minimum 98% L-isomer, actual measurement of 1.6% D-isomer). Because the presence of 1.6% D-isomer was suspected to perturb urinary lysine enrichment, a second tracer was obtained from Mass Trace (Woburn, MA). Isomeric purity of the second tracer was determined by Mass Trace using a chiral column attached to a GC-MS instrument (D-isomer < 0.2% and no detectable amount of D-[1-^{13}C]lysine, qualitative results).

Subjects

Eighteen healthy, growing, low–birth weight infants recruited from the transitional care nursery at Women's College Hospital participated in the neonatal study, which was part of a larger investigation examining the metabolism of phenylalanine and threonine in preterm infants fed their mother's milk or formula with varying whey to casein ratios.[4] The mean birth weight was 1,720 g, gestational age 32 weeks, weight at study 1,971 g, and postnatal age at study 21 days. Informed written consent was obtained from one or both parents. The protocol was approved by the Human Subject Review Committee of The Hospital for Sick Children and the Research Ethics Board of Women's College Hospital.

Seven healthy adult females (mean age, 26.3 years; weight, 56.0 kg; height, 162.1 cm) participated as outpatients of the Clinical Investigation Unit at The Hospital for Sick Children (Toronto, Ontario, Canada). The subjects were participants in a larger study in which a minimally invasive infusion model was developed to study amino acid kinetics in adults.[5] Written informed consent was obtained for the study, which was approved by the University of Toronto Human Experimentation Committee and the Human Subjects Review Committee of The Hospital for Sick Children.

Experimental Protocols

Neonatal study. The experimental procedures and formulas for the neonatal study are described in detail elsewhere.[4] Briefly, 12 formula-fed infants were randomly assigned to receive one of three study formulas (Wyeth-Ayerst Laboratories, Philadelphia, PA) that differed only in the whey to casein ratio (60:40, 40:60, and 20:80, respectively). An additional six preterm infants fed their mother's expressed milk (preterm milk) participated in the study. Formula-fed infants and those fed preterm milk were nourished via nasogastric tube or by bottle every 3 hours. Following 3 days of feedings, a phenylalanine oral primed equal-dose tracer infusion study, which included a 6-hour baseline period and an 18-hour tracer administration period, was performed on each infant. Each infant received an oral priming dose of L-[1-^{13}C]phenylalanine (15 μmol/kg). Equal oral doses of 45 μmol/kg were administered every 3 hours directly into the feeding tube or bottle nipple at the beginning of each of six feedings over the 18-hour period. Urine was collected by a condom urine collector at baseline and every 3 hours during the oral tracer infusion. Breath samples were collected with a ventilated hood prior to tracer administration and then during the last 2 hours of the 18-hour oral tracer administration period.[4] No blood samples were taken from the infants during the phenylalanine infusion because of ethical constraints.

Adult study. The adult subjects stayed in a temperature-controlled metabolic facility at The Hospital for Sick Children during the oral primed equal-dose tracer infusion studies. L-[1-^{13}C]lysine (1.6% D-isomer) was administered to five subjects in experiment 1. To evaluate and account for the impact of D-lysine in the tracer observed during experiment 1, two subjects received L-[1-^{13}C]lysine (<0.2% D-isomer) during a second experiment. Each subject received an oral priming dose of [1-^{13}C]lysine (21.89 μmol/kg) at time 0. Eight equal oral doses (4.79 μmol/kg) of the isotope were administered every 30 minutes, beginning 15 minutes after the priming dose. Isotope administration involved swallowing the prime or equal infusion dose, followed by water to rinse the tube that contained the isotope.

In experiment 1, eight hourly meals were consumed beginning at time −240 minutes, and each meal represented one twelfth of the subjects' total daily energy requirement. The experimental diet has been reported in detail elsewhere.[10] Urine was collected hourly, and blood samples were taken at minutes 160, 200, and 240. The procedures for measuring the CO_2 production rate (VCO_2) and for collecting breath and blood samples were performed as described previously.[11]

The second experiment was conducted using L-[1-^{13}C]lysine (<0.2% D-isomer) to determine whether urine and plasma enrichments of [^{13}C]lysine were equal with only trace amounts of D-lysine present in the tracer. The first oral tracer administration study was performed on one subject and involved a 4-hour baseline period and a 4-hour oral primed equal-dose tracer infusion during which hourly meals were consumed. The second oral tracer administration study was performed on a subject in the fasted state and involved a 4-hour oral primed equal-dose tracer infusion. Three paired urine and blood samples were collected at 30-minute intervals in the hour preceding the start of isotope administration. Four paired urine and blood samples were collected during the final 120 minutes of the oral tracer administration protocol as described for the first experiment.

Analytical Methods

Nonchiral GC-MS analysis of amino acids was performed as follows. Phenylalanine and tyrosine in 250 μL urine and lysine in 500 μL urine were derivatized to their *N*-heptofluorobutyryl *O*-isobutyl esters[12] and *N*-heptafluorobutyryl *N*-propyl esters,[13] respectively, before the measurement of isotopic enrichment that did not differentiate between D- and L-amino acids. Derivatized phenylalanine and tyrosine were separated on a gas chromatograph (model 5840A; Hewlett-Packard, Mississauga, Ontario, Canada) fitted with a 25-m × 0.20-mm ID × 0.33-μm film thickness fused silica capillary column (Ultra 2; Hewlett-Packard). Derivatized lysine was separated on a gas chromatograph (Hewlett-Packard model 5890 Series II) fitted with a 30-m × 0.32-mm × 1.0-μm fused silica capillary column (Hp5). Both gas chromatographs were coupled directly to quadropole mass spectrometers under conditions of negative chemical ionization and selective ion monitoring. Selected ion chromatograms were obtained by monitoring *m/z* 397 and 398 for phenylalanine and 431 and 432 for tyrosine (first product of phenylalanine metabolism), and 560 and 561 for lysine, corresponding to the unenriched (M) and enriched (M + 1) peak, respectively. Ion peak ratios were calculated as the mole ratio percent and plotted for each subject.

The isotopic enrichment of ^{13}C in breath CO_2 was measured on a dual-inlet isotope-ratio mass spectrometer (VG Micromass 602D; Cheshire, UK) using techniques described previously.[14] CO_2 enrichment from baseline samples and from samples taken during the last 2 hours of tracer infusion are expressed as the atoms percent excess (APE) $^{13}CO_2$ over a reference standard of compressed CO_2 gas.

Isotopic steady state in the metabolic pool was indicated by plateaus in urinary [^{13}C]phenylalanine, [^{13}C]tyrosine, and [^{13}C]lysine, plasma [^{13}C]lysine, and breath $^{13}CO_2$ enrichment. Enrichment due to the tracer at isotopic steady state, either in molecules percent excess (MPE) for urinary and plasma amino acids or in APE for breath CO_2, was calculated according to standard equations.[15]

Chiral GC-MS analysis of amino acids was performed as follows. The enantiomeric composition of [^{13}C]phenylalanine, contained in tracer lots A, B, C, and D of L-[1-^{13}C]phenylalanine and in the 18 individual urine samples collected from each infant during the last 3 hours of tracer infusion (at plateau), was determined by GC-MS analysis of the *N*-heptafluorobutyryl *N*-propyl ester derivative[13] with a 0.2-μm film thickness × 0.32-mm × 25-m Heliflex chirasil-val capillary column (Alltech Associates, Deerfield, IL). Selective ion monitoring for both L-phenylalanine and D-phenylalanine was per-

formed at *m/z* 383 and 384 corresponding to the unenriched (M) and enriched (M + 1) ion peaks, respectively. Isotopic enrichment of D- and L-isomers contained in tracers was expressed as molecules percent (MP). Isotopic enrichment due to tracer of L-[1-^{13}C]phenylalanine of single urine samples taken at plateau from individual infants was calculated according to a standard equation and expressed as MPE.[15]

The percent D-phenylalanine and percent D-lysine were calculated as the sum of the unenriched (M) and enriched (M + 1) peaks of the D-amino acid divided by the total unenriched (M) and enriched (M + 1) peaks of both the D- and L-amino acids. The percent D-[^{13}C]phenylalanine was calculated as the enriched (M + 1) peak of the D-phenylalanine divided by the total unenriched (M) and enriched (M + 1) peaks of both D- and L-phenylalanine. The presence of D-[^{13}C]lysine in tracer, a plateau urine sample and a plateau plasma sample from one subject who received L-[1-^{13}C]lysine (1.6% D-isomer), was determined qualitatively with a chiral column attached to a GC-MS instrument under conditions of electron-impact single-ion monitoring (Mass Trace).

Statistical Analysis

Results are expressed as the mean ± SD. Differences in urinary enrichment of [^{13}C]phenylalanine and [^{13}C]tyrosine and in breath CO_2 enrichment between the two phenylalanine tracer groups were analyzed by unpaired Student *t* tests for equal or unequal variances, as appropriate. Equality of variance was tested by an F test for two-group comparisons. The differences between urinary [^{13}C]phenylalanine enrichment (determined by GC-MS) and urinary L-[^{13}C]phenylalanine enrichment (determined by chiral column attached to GC-MS) and between plasma and urine lysine enrichment were compared by paired Student *t* test. The mean [^{13}C]lysine plasma urine enrichment ratio was calculated for each tracer group, and the difference from 1 was evaluated by *t* test. Results were considered statistically significant at a *P* value of .05 or less. Statistical analyses were conducted using SAS software (Version 6.03; SAS Institute, Cary, NC).

RESULTS

The mean isotopic enrichments (MPE) of urinary [^{13}C]phenylalanine and [^{13}C]tyrosine and of breath $^{13}CO_2$ in preterm infants who received lot A of L-[1-^{13}C]phenylalanine and in those who received lots B, C, and D of L-[1-^{13}C]phenylalanine are shown in Table 1. Tracer lot A was found to contain 0.4% D-[^{13}C]phenylalanine. There was no detectable enrichment of D-[^{13}C]phenylalanine in lots B, C, and D. The D-phenylalanine content of lot A was 0.45% due to the presence of a small amount of unlabeled D-phenylalanine. Lots B, C, and D each contained 0.1% D-phenylalanine, which was entirely unlabeled. The mean enrichment of urinary [^{13}C]phenylalanine in infants who received lot A was almost threefold ($P < .0001$) the level in infants who received tracer lots B, C, and D. Isotopic steady state in urinary [^{13}C]phenylalanine was attained in 16 infants by 9 hours following the start of oral tracer infusion and was maintained to the end of the study at 18 hours. The coefficient of variation (mean ± SD) for urinary [^{13}C]phenylalanine at baseline and plateau was 0.4% ± 0.3% (n = 18) and 2.7% ± 1.7% (n = 16), respectively. Steady state was not achieved in two infants who received tracer lot A and were fed preterm milk. Isotopic enrichment of urinary [^{13}C]tyrosine (first metabolite of phenylalanine) and of breath $^{13}CO_2$ attained isotopic steady state in all subjects. The mean enrichments of urinary [^{13}C]tyrosine and breath $^{13}CO_2$ at isotopic steady state were similar among infants irrespective of tracer lot and of its D-[^{13}C]phenylalanine content (Table 1).

Analysis of the enantiomeric composition of urinary [^{13}C]phenylalanine showed the striking presence of an enriched (M + 1) peak of D-[^{13}C]phenylalanine in the urine of infants receiving tracer lot A. A single-ion chromatograph depicting the enriched and unenriched ion peaks for both the L- and D-isomers of phenylalanine in the urine of an infant who received tracer lot A that contained 0.4% D-[^{13}C]phenylalanine is shown in Fig 1. Urinary D-phenylalanine was 97 MP enriched and accounted for 10% to 30% (20.5% ± 7%) of total phenylalanine in the urine of infants who received lot A. In contrast, the isotopic enrichment of D-phenylalanine in the urine of infants receiving lots B, C, and D was at the level of natural abundance (17 MP), and the amount of D-phenylalanine did not exceed 8% of total urinary phenylalanine.

The isotopic enrichment of [^{13}C]phenylalanine (MPE) determined by conventional GC-MS analysis (which includes both the L- and D-isomers) and the isotopic enrichment of L-[^{13}C]phenylalanine (MPE) determined by chiral GC-MS analysis of single urine samples obtained at plateau from infants who received lot A and from those who received lots B, C, and D are

Table 1. Isotopic Enrichment Due to Tracer of Urinary [^{13}C]Phenylalanine and [^{13}C]Tyrosine and of Breath $^{13}CO_2$ in Infants Receiving Tracer Lot A and Lots B, C, and D of L-[1-^{13}C]Phenylalanine

L-[1-^{13}C] Phenylalanine	Urinary Enrichment (MPE) [1-^{13}C] Phenylalanine	Urinary Enrichment (MPE) [1-^{13}C] Tyrosine	Breath Enrichment of $^{13}CO_2$ (APE)*
Lot A (n = 7)	28.6 ± 7.1†	2.6 ± 0.3	6.2 ± 2.6§
Lots B, C, and D (n = 11)	10.2 ± 1.5‡	2.6 ± 0.8	6.7 ± 1.6

NOTE. Lot A contained 0.4% D-[^{13}C]phenylalanine and lots B, C, and D contained undetectable D-[^{13}C]phenylalanine.

*×1,000.

†Isotopic steady state was not achieved in urinary [^{13}C]phenylalanine for 2 subjects.

‡Significantly different *v* lot A ($P < .0001$) by Student's *t* test.

§n = 6; 1 outlier with a low level of enrichment (0.9 APE) due to transient tyrosinemia of the newborn was removed from the analysis.

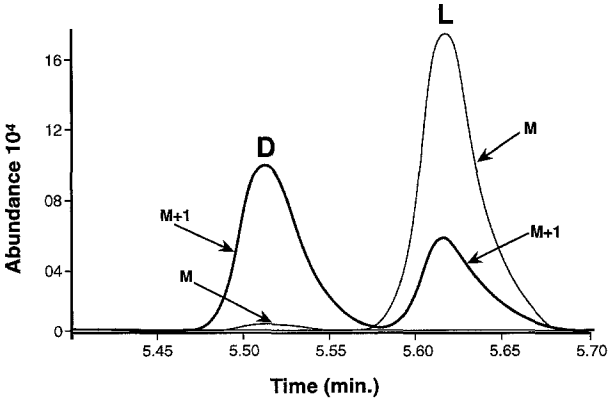

Fig 1. Selected ion monitoring (SIM) chromatogram of the *N*-heptafluorobutyryl *N*-propyl ester derivative of phenylalanine in the urine of an infant who received lot A of L-[1-^{13}C]phenylalanine (0.4% D-[^{13}C]phenylalanine). GC-MS analysis was conducted under negative chemical ionization conditions using a chiral column. The enriched (M + 1) and unenriched (M) peaks of both the D- and L-isomers are shown. D-Phenylalanine was 97% enriched and L-phenylalanine was 31% enriched. D-[1-^{13}C]phenylalanine accounted for 66% of total [^{13}C]phenylalanine enrichment in the urine sample.

shown in Table 2. The mean isotopic enrichment of urinary [13C]phenylalanine determined by GC-MS was more variable and significantly higher ($P < .0001$) in infants receiving lot A versus those who received lots B, C, and D. In contrast, the mean isotopic enrichment of the L-isomer of [13C]phenylalanine in urine of infants receiving lot A was similar to that of infants receiving lots B, C, and D. Among infants who received tracer lots B, C, and D, there was no significant difference between the mean isotopic enrichment of urinary [13C]phenylalanine determined by conventional GC-MS and urinary L[13C]phenylalanine determined by chiral column GC-MS.

The mean isotopic enrichment of [13C]lysine in plasma and urine (MPE) due to the lysine tracer in adult subjects is shown in Table 3. At isotopic steady state, the mean urinary [13C]lysine enrichment in subjects who received L-[1-13C]lysine containing 1.6% D-lysine was approximately 3.7-fold the level in plasma ($P = .002$). The enrichment ratio of plasma to urine [13C]lysine at isotopic steady state was significantly different from 1 ($P < .0001$). Nonetheless, isotopic steady state was achieved in breath $^{13}CO_2$ in all subjects (data not shown). The mean urine [13C]lysine enrichment in two subjects who received L-[1-13C]lysine (<0.2% D-lysine) was similar to the level in plasma ($P = .24$) and the plasma to urine ratio did not differ significantly from 1 ($P = .27$).

Table 2. Isotopic Enrichment Due to Tracer of [13C]Phenylalanine and L-[13C]Phenylalanine of Single Urine Samples Taken at Plateau From Individual Infants Receiving Lot A and Lots B, C, and D of L-[1-13C]Phenylalanine and Fed Formula With a Whey to Casein Ratio of 60:40, 40:60, or 20:80 or Their Mother's Milk (preterm milk)

Infant No.	Diet	Urinary Enrichment (MPE) [13C] Phenylalanine*	Urinary Enrichment (MPE) L-[13C] Phenylalaninet
Infants receiving lot A of L-[1-13C]phenylalanine (0.4% D-[13C]phenylalanine)			
1	20:80	29.8	9.4
2	20:80	17.8	7.1
3	40:60	26.9	10.1
4	40:60	26.4	10.2
5	60:40	28.4	9.1
6	Preterm milk	40.7	11.3
7	Preterm milk	32.4	11.4
Mean ± SD		28.9 ± 6.9‡	9.8 ± 1.5
Infants receiving lots B, C, and D of L-[1-13C]phenylalanine (undetectable D-[13C]phenylalanine)			
8	20:80	6.6	5.2
9	20:80	6.9	8.5
10	40:60	11.7	12.2
11	40:60	13.3	13.3
12	60:40	9.2	11.0
13	60:40	10.3	9.8
14	60:40	8.8	8.3
15	Preterm milk	14.7	13.3
16	Preterm milk	10.2	9.5
17	Preterm milk	9.7	8.9
18	Preterm milk	8.2	7.4
Mean ± SD		10.0 ± 2.5	9.8 ± 2.5

*Determined by conventional GC-MS.

†Determined by chiral column attached to GC-MS.

‡Significantly different ($P < .0001$) *v* measurement of L-[13C]phenylalanine obtained by chiral column attached to GC-MS and *v* infants who received lots B, C, and D of L-[13C]phenylalanine containing undetected D-[13C]phenylalanine.

Table 3. Isotopic Enrichment of Plasma and Urinary [13C]Lysine at Plateau in Subjects Receiving L-[1-13C]Lysine (1.6% D-lysine) and L-[1-13C]Lysine (<0.2% D-lysine)

Parameter	Tracer: L-[1-13C]Lysine (1.6% D-lysine) (n = 5)	Tracer: L-[1-13C]Lysine (<0.2% D-lysine) (n = 2)
Plasma enrichment of [13C]lysine (MPE)*	11.1 ± 1.6	8.7 ± 0.3
Urine enrichment of [13C]lysine (MPE)*	40.8 ± 9.2†	8.1 ± 0.1
Plasma:urine enrichment ratio	0.27 ± 0.04‡	1.07 ± 0.03

*Calculated from Bross et al.[5]

†Significantly different ($P = .02$) *v* plasma enrichment.

‡Significantly different from 1 ($P < .0001$).

Qualitative analysis of the enantiomeric composition of the lysine tracer and one sample each of urine and plasma obtained at isotopic steady state from one adult subject who received L-[1-13C]lysine (1.6% D-isomer) revealed a significantly enriched (M + 1) peak of D-[13C]lysine in urine.[5] The plasma sample contained negligible amounts of the D-[13C]isomer. The chiral column attached to GC-MS could not be applied quantitatively to study the enantiomeric composition of lysine in urine, plasma, and tracer. Lysine resolution by chiral column is poorer than for other amino acids because the elution of lysine requires a high column temperature that approaches the maximum temperature stability of the chiral column.[16]

DISCUSSION

This investigation was aimed at determining the reason for the higher-than-expected urinary amino acid enrichment observed during the course of two recent studies at our laboratory. This study demonstrates that urinary [13C]phenylalanine and [13C]lysine enrichments, determined by conventional GC-MS analysis in subjects receiving orally administered stable isotope tracers, are distorted by the presence of D-[13C]phenylalanine and D-[13C]lysine that originated as contaminants of the tracers. An attempt was made to correct and control for this problem to be able to reliably substitute urine collection for blood sampling in future studies of amino acid metabolism.

Amino acid enrichment in urine is assumed to reflect that in arterialized blood. Arterial blood is delivered to the nephron, where plasma amino acids are freely filtered through the glomerulus. The amino acids are then almost entirely reabsorbed by stereospecific active-transport carriers in the proximal renal tubules, except for small amounts that are lost in the urine.[17,18]

In our investigation, the amounts of D-[13C]phenylalanine relative to L-[13C]phenylalanine and of D-[13C]lysine relative to L-[13C]lysine infused were small (0.4% and ≤1.6%, respectively) and led to the marked perturbation in urinary amino acid enrichments. Discrimination by the amino acid transport carriers during the process of renal tubular reabsorption against

labeled D-amino acids would explain the higher enrichment of [^{13}C]lysine in urine compared with plasma in the adults and the higher-than-expected enrichment of urinary [^{13}C]phenylalanine in the infants who received a particular lot of L-[1-^{13}C]phenylalanine.

Waterlow previously reported increased radioactivity in amino acid extracts made from urine versus plasma in humans[8] and rats[9] in studies that involved constant infusion of L-[U-^{14}C]lysine. However, no direct evidence for the presence of D-lysine was presented, and the possibility remains that the small amount of highly radioactive non–L-lysine material in urine arose from metabolites of the L-lysine tracer.[19]

In the present study, direct analysis of the D-[^{13}C]phenylalanine content of tracer and of urine showed that the elevated urinary enrichment was entirely explained by the presence of D-[^{13}C]phenylalanine that must have originated from lot A containing 0.4% D-[^{13}C]phenylalanine. At the end of the 18-hour infusion with lot A of L-[1-^{13}C]phenylalanine, the contribution of D-[^{13}C]phenylalanine to total [^{13}C]phenylalanine enrichment in urine was as high as 63%. A failure to recognize the presence of the D-isomers in urine would have resulted in substantial underestimation of flux and oxidation, since flux is inversely proportional to isotope enrichment.

The isotopic enrichment of [^{13}C]tyrosine in urine and of breath CO_2 in neonates was not affected by the D-[^{13}C]phenylalanine content of the tracer. D-isomers of amino acids as such are generally not used for protein synthesis. The metabolic availability of D-amino acids depends on the extent to which D-amino acids can be inverted to the L-form.[20] The bioavailability of D-phenylalanine in human adults has been studied by the nitrogen balance technique.[21] The results of these studies were interpreted as showing that D-phenylalanine can be inverted to L-phenylalanine and become bioavailable to human adults.[20] A possible mechanism for this conversion in the human is the demonstration by Nagata et al[22] of D-amino oxidase in the mouse kidney, which catalyzes the oxidative deamination of neutral free D-amino acids to their ketoacid analogs. The ketoacid formed would then be transaminated to produce the L-amino acid. D-Lysine cannot be converted to the L-form and therefore cannot be utilized by humans or other animals.[20] The bioavailability of D-phenylalanine has not been studied in infants, to our knowledge.

The problem of D-isomer contamination of L-phenylalanine was conveniently overcome with chiral column and GC-MS methodology. However, this method was not suitable for determining quantitatively the contribution of D-lysine to the total lysine enrichment in urine. The presence of D-[^{13}C]lysine in the urine sample from one adult who received L-[1-^{13}C]lysine (1.6% D-isomer) was nevertheless demonstrated.

Oral infusion of L-[1-^{13}C]lysine (1.6% D-isomer) produced isotopic steady states in breath $^{13}CO_2$ and plasma [^{13}C]lysine; however, urinary and plasma isotopic enrichments were significantly different, with urinary enrichment being 3.7-fold the level in plasma. Baseline data showed comparable enrichment of [^{13}C]lysine in urine and plasma before isotope administration.[5] If ^{13}C isotopes were biologically discriminated against by transport mechanisms in the kidney, they would be preferentially retained or eliminated over the long term. This would result in baseline urine samples showing higher or lower enrichment compared with plasma samples, which is not the case.[2,5-7] If plasma and urinary enrichments are similar before an isotopic infusion, then the enrichment in urine should also be reflective of the enrichment in the plasma pool during an infusion of amino acid tracers, provided there is no isotopic effect due to the tracer. Therefore, the difference in plasma and urine enrichment during infusion of [1-^{13}C]lysine could be attributed entirely to the presence of D-[1-^{13}C]lysine in the lysine tracer that was administered, and not to an isotopic effect of the [1-^{13}C]lysine tracer itself.

A substantial accumulation of radioactively labeled D-tyrosine in the blood and the intracellular space within muscle was reported in a previous study.[19] However, the tracer, L-[side-chain2,3-^{3}H]tyrosine, was highly contaminated with 30% D-isomer. In our investigation, the lysine tracer (1.6% D-lysine) did not appear to accumulate significantly in the plasma.

An isotope effect in the process of reabsorption in the kidney was proposed by Zello et al[7] to explain the higher urinary versus plasma enrichment in adults after intravenous infusion of L-[ring-$^{2}H_5$]phenylalanine tracer. The nature of the deuterated ring labeling of the phenylalanine molecule was proposed to have affected the amino acid's reabsorption by active-transport carriers, which would invalidate its use as a biological tracer. The D-isomer content of this tracer was reported to be within the specifications of the manufacturer. Given the results of our study, we now recognize that a small amount of labeled D-isomer originating from the tracer may have caused the higher urinary versus plasma enrichment observed by Zello et al.[7]

A D-isomer tracer effect might also explain the results of Krempf et al,[23] who reported higher phenylalanine flux rates for [ring-$^{2}H_5$]phenylalanine versus [^{15}N] and L-[1-^{13}C]phenylalanine tracers in adult men after intragrastric infusion of the three tracers. The investigators suggested that proton exchange involving [ring-$^{2}H_5$]phenylalanine might explain the difference in fluxes. Zello et al[7] proposed an alternate explanation for these findings: discrimination against the deuterated isotope by the active-transport carriers in the lumen of the small intestine resulted in less isotope entering the systemic circulation and thus the higher flux observed. However, the discrimination may be against D-isomer originating from the deuterated ring phenylalanine tracer rather than the molecular nature of the [ring-$^{2}H_5$]phenylalanine per se. This area deserves further study.

This investigation has revealed problems associated with the use of amino acid tracers contaminated with D-isomers in amounts currently within the specifications of the manufacturer (<2%). Based on the results of this study, an amino acid tracer with a detectable amount of D-[^{13}C]isomer cannot be recommended for any study in which urine will be used to represent the arterial plasma amino acid pool enrichment. Alternatively, chiral column methodology could be applied to correct for a D-isomer problem, except in studies that use lysine as a tracer.

ACKNOWLEDGMENT

The authors thank Dr A. Ajami (Mass Trace) for analyzing the samples contaminated with D-[^{13}C]lysine, and Cambridge Isotope Laboratories for their responsiveness to the D-[^{13}C]lysine problem. We thank Mahroukh Rafii and Lawrence Fisher for technical assistance.

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