Gas Chromatography/Mass Spectrometric Determination of [^{15}N]Urea in Plasma and Application to Urea Metabolism Study

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The study of urea metabolism in humans necessitates sensitive methods, so that very low isotopic enrichments of plasma or urinary urea can be measured. A stable derivative of urea, suitable for the measurement of ^{15}N or ^{13}C enrichments of urea using gas chromatography/mass spectrometry, is described. The trimethylsiloxypyrimidine could be used to measure [$^{15}N_2$]- and [$^{15}N_1$]urea enrichments as low as 0.5% with a coefficient of variation below 5%. Determination of a lower enrichment of [$^{15}N_2$]urea required the use of 2-trifluoroacetoxypyrimidine, which had a background of 0.4% at the (M + 2) ion. The coefficient of variation in determining this background was 1.6%; this means a low enrichment, such as 0.1%, could be determined with confidence. This technique was applied to the study of urea metabolism in humans and dogs.

The quantitation of urea turnover rate in humans has generally been done by isotope tracer dilution techniques (*1–3*). In these experiments, the nonradioactive tracer, [$^{15}N_2$]urea, is administered to the subject by either single bolus injection or constant infusion. Serial sampling of plasma or urine and the determination of [^{15}N]urea enrichments in these samples provide data for the calculation of turnover rate. Traditionally, the measurement of [^{15}N]urea enrichments has been accomplished by using an isotope ratio mass spectrometer after converting urea to nitrogen (*1–3*). Such methods have the disadvantages of being time-consuming and requiring large sample size. The preparation of sample for ratio mass spectrometric measurement requires tedious operation of a vacuum line; this operation usually takes a couple of days. The amount of urea needed for a ratio mass spectrometric measurement would require the use of 0.5 mL of plasma as a minimum. In fact, such a sample size requirement precludes the study of urea metabolism from serial plasma sampling. Up to now, the majority of urea turnover rate measurement has been done by serial urine sampling (*1–3*). In a study design, the collection of serial plasma samples for [^{15}N]urea measurements is easier to perform and the result is more accurate for a kinetic calculation than those done on serial urine samples. Besides, the complete collection of urine from a small infant is sometimes an impossible task.

Since the only feasible way of quantitizing urea turnover in small infants is by serial blood sampling, we have developed a micromethod for the determination of [^{15}N]urea enrichment in plasma. This method utilized a gas chromatograph/mass spectrometer and used only 100 μL of plasma (20 μg of urea).

EXPERIMENTAL SECTION

Reagents. Analytical grade ion exchange resins, AG 50W-X8 and AG 1-X8, were purchased from Bio-Rad Laboratories, Richmond, CA. Malonaldehyde bis(dimethylacetal) was obtained from Aldrich Chemical Co., Milwaukee, WI. [$^{15}N_2$]Urea (99 atom % ^{15}N) was purchased from Merck and Co., Inc./Isotopes, Quebec, Canada; [$^{15}N_1$]urea (99 atom % ^{15}N) and L-[^{15}N]alanine (99 atom % ^{15}N) were from KOR Inc., Cambridge, MA.

Isolation of Urea from Plasma. A modification of the procedure of Kreisberg et al. (*4*) was used. Plasma (100 μL) was deproteinized with the addition of 0.32 M barium hydroxide (50 μL) and 0.32 M zinc sulfate (50 μL). After centrifugation, the supernatant (100 μL) was chromatographed on a microcolumn (Quik-Sep radioassay column, Isolab, Akron, OH) packed with ion exchange resins: AG 50W-X8 (H^+, 1.6 cm) in the bottom and AG 1-X8 (formate, 1.6 cm) on the top. The column was eluted with water, the first 5 mL fraction contained glucose. Urea was eluted in the second 5 mL fraction.

Derivatization. The urea fraction was evaporated to dryness in a Vortex-Evaporter (Buchler, Fort Lee, NJ) at 40 °C. To the dried residue was added 200 μL of 6 N hydrochloric acid and 10 μL of freshly prepared 5% malonaldehyde bis(dimethylacetal) solution in water. After incubation for 2 h at room temperature, the solution was dried with a stream of air. The 2-hydroxypyrimidine formed was then converted to trifluoroacetyl or trimethylsilyl derivative. For the preparation of trifluoroacetyl derivative, the residue was mixed with 10 μL of 20% trifluoroacetic anhydride in ethyl acetate, followed by the addition of 10 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) after 0.5 h. The mixture was kept at room temperature for 10 min before analyzing with GC/MS. Trimethylsilyl derivative was prepared by the addition of 10 μL of BSTFA to the dried residue and incubated at room temperature for 10 min.

Gas Chromatography/Mass Spectrometry. A Hewlett-Packard 5985A gas chromatograph/mass spectrometer was used. The glass column ($^1/_8$ in. × 6 ft) was packed with 10% OV-17 on 100/120 mesh gas chrom Q (Applied Science, College Station, PA). The column temperature was kept at 100 °C (TFA derivative) or 120 °C (Me_3Si derivative). Helium was used as carrier gas at a flow rate of 30 mL/min. The injection port and jet separator temperatures were 200 °C, while the ion source temperature was 150 °C. Electron impact (70 eV) ionization was used. For the analysis of the 2-hydroxypyrimidine–TFA derivative of [$^{15}N_2$]urea, the ions m/z 192 vs. 194 (or m/z 123 vs. 125) were monitored with a dwell time of 50 and 500 ms, respectively. Ions, m/z 153 vs. 155, were monitored for the 2-hydroxypyrimidine–Me_3Si derivative. The quantification of the enrichment of [$^{15}N_1$]urea was obtained by monitoring m/z 153 vs. 154 of the 2-hydroxypyrimidine–Me_3Si derivative. Standard solutions with known ^{15}N enrichment were analyzed everyday to correct for the instrumental variation. The peak area percentage of the two ions monitored was used to construct standard curve. The ^{15}N enrichments of the unknowns were determined by comparing the peak area percentage with the standard curve constructed.

Urea Production Rate Determination. Human subjects were infused with [$^{15}N_2$]urea according to the prime-constant infusion technique for 4–6 h. The priming dose was 2 mg/kg and the infusion rate was 0.20 (mg/kg)/h. With this technique, a steady state of urea enrichment was usually obtained between 120 and 180 min after the start of infusion. The production rate was calculated by tracer dilution according to the equation

$$P = [(1/E) - 1]I \quad (1)$$

where P = production rate, (mg/kg)/h, E = [$^{15}N_2$]urea enrichment of plasma urea, derived from standard curve assuming [$^{15}N_2$]urea tracer was 100% enriched, and I = infusion rate of [$^{15}N_2$]urea, (mg/kg)/h.

Quantitation of Contribution of Alanine Amino Nitrogen to Urea in the Dog. Dogs were infused with L-[^{15}N]alanine (99% ^{15}N) with a loading dose of 4 mg/kg and an infusion rate of 40 (μg/kg)/min for 5 h. The plasma enrichment of [^{15}N]urea was then determined by the present technique. A steady-state enrichment of plasma L-[^{15}N]alanine and [$^{15}N_1$]urea were usually reached within 3 h after the start of infusion. The percentage

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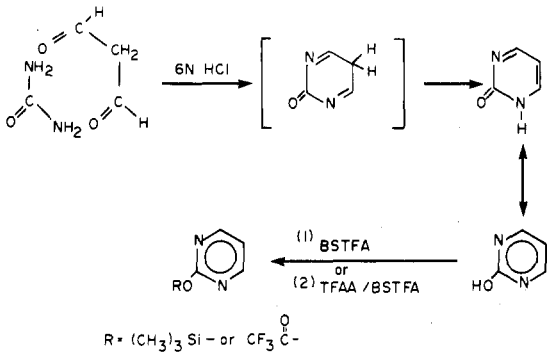

Figure 1. Reaction sequence for the preparation of trimethylsilylated or trifluoroacetylated 2-hydroxypyrimidine derivative of urea.

of urea nitrogen derived from alanine was calculated by the following equation:

$$\% \text{ urea N from alanine} = (E_{\text{urea}}/E_{\text{alanine}}) \times 100 \qquad (2)$$

where E is the ^{15}N enrichment of urea and alanine at steady state.

RESULTS AND DISCUSSION

The conversion of urea into a volatile derivative suitable for gas chromatographic or gas chromatograph/mass spectrometric analysis has been the subject of several studies in the past years (*5*, *6*). However, a satisfactory derivative which is easy to prepare and stable and with symmetric chromatographic peaks has not been reported thus far. The bis(trifluoroacetyl) derivative used by Mee (*5*) is unstable and produces chromatographic peaks with severe tailing. The barbiturate derivative used by Bjorkhem et al. (*6*) gives symmetric peaks, but it is difficult to prepare. Besides the low and variable yield, the mass spectral fragmentation pattern of the barbiturate derivative is also less than ideal. Not only is there extensive fragmentation of the barbiturate derivative but also a minor fragment was used for analysis. The bis(trimethylsilyl) derivative, used by us previously (*7*) and described recently by Wolfe (*8*) also has the same disadvantages as bis(trifluoroacetyl) derivative. Although the di-Me_3Si derivative has better chromatographic properties, it is very unstable both chemically and chromatographically. It is often decomposed within an hour and hence the yields vary between different samples. Worst of all, the decomposition of this derivative in injection port or column of a gas chromatograph usually produced a "memory effect" and thus interfered with analysis of another sample with different isotopic enrichment. In addition, the high background of the (m + 2) peak of di-Me_3Si urea (8%) makes the detection of low enrichment of [$^{15}N_2$]urea difficult.

The 2-hydroxypyrimidine derivative, which has been used by Hamberg et al. (*9*) for the analysis of malonaldehyde in biological fluids has all the desirable gas chromatographic and mass spectrometric properties. A substantial modification was made on this procedure for the analysis of urea.

Preparation of 2-Hydroxypyrimidine from Urea. The reaction is depicted in Figure 1. For the quantitative conversion of urea to 2-hydroxypyrimidine, large excess of malonaldehyde cannot be used. The excess malonaldehyde undergoes self-condensation to colored polymers which reduce the yield of 2-hydroxypyrimidine and interfere with the subsequent analysis with GC/MS. We found that 10 μL of 5% malonaldehyde aqueous solution added to 200 μL of 6 N hydrochloric acid was the optimal condition required to convert urea (obtained from 100 μL of plasma, approximately 20 μg) into 2-hydroxypyrimidine quantitatively with negligible formation of the colored polymer. The amount of malonaldehyde in the reaction mixture was approximately 10 times the molar equivalent of urea. The reaction was completed within 2 h.

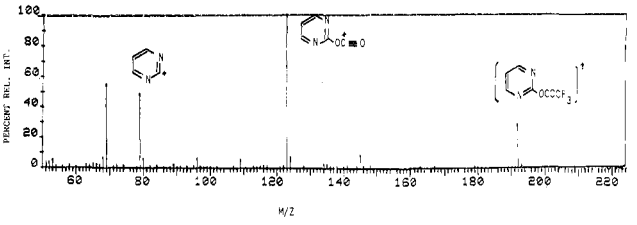

Figure 2. Electron impact ionization (70 eV) mass spectrum of 2-trifluoroacetoxypyrimidine. The spectrum was obtained through a gas chromatographic inlet. Ion source temperature was 150 °C.

Derivatization of 2-Hydroxypyrimidine. 2-Hydroxypyrimidine was converted to a volatile derivative for GC/MS analysis. The ideal derivative should be stable, giving few mass fragments, and should have low background at (M + 2) peak for a successful isotope dilution analysis of [$^{15}N_2$]urea. Theoretically, the best candidate would be 2-methoxypyrimidine or 2-acetoxypyrimidine. Unfortunately, the attempted preparation of these two derivatives failed under numerous conditions.

The 2-hydroxypyrimidine can be converted readily into trimethylsilyl derivative (*9*). It has m/z 153 (M − 15) as major fragment ion upon 70 eV electron impact ionization. This derivative was most readily prepared with the addition of bis(trimethylsilyl)trifluoroacetamide (BSTFA). The use of acidic or basic silylation reagents, such as (trimethylsilyl)-diethylamine, trimethylchlorosilane, or any other silylation reagent containing pyridine as solvent resulted in a reaction mixture, which was unstable upon gas chromatographic analysis. The acid or base catalyzed the decomposition of 2-trimethylsiloxypyrimidine to 2-hydroxypyrimidine, in injection port or column. The 2-hydroxypyrimidine formed in the column or injection port was then partially reconverted back to 2-trimethylsiloxypyrimidine upon subsequent injection of another sample solution and thus resulted in "memory" effect. In contrast, the trimethylsilyl derivative obtained from neutral silylation reagents, such as BSTFA, bis(trimethylsilyl)acetamide, or *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, was stable chromatographically. No "memory" effect was observed by alternate injection of samples containing 0% and 6% of [$^{15}N_2$]urea. The 2-trimethylsiloxypyrimidine derivative is suitable for analysis of both [$^{15}N_2$]urea and [$^{15}N_1$]urea down to the enrichment of 0.5% with an average coefficient of variation of 4.2% (n = 11). However, the high background of (M + 1) and (M + 2) peaks (10% and 3.5%, respectively), even though lower than the di-Me_3Si derivative, made the reliable detection of lower enrichment difficult.

The trifluoroacetyl derivative of 2-hydroxypyrimidine, which can be easily prepared by reacting 2-hydroxypyrimidine with trifluoroacetic anhydride in ethyl acetate, has a background of only 0.4% at (M + 2) ion. The coefficient of variation of determining the background of (M + 2) ion of unlabeled urea was 1.6%; this indicated that as low as 0.1% enrichment of [$^{15}N_2$]urea can be measured with confidence, using this derivative. The 70 eV EI mass spectrum of 2-trifluoroacetoxypyrimidine is shown in Figure 2.

The quantitation of enrichment can be obtained by either monitoring m/z 123 or 192. The 2-trifluoroacetoxypyrimidine prepared from trifluoroacetic anhydride alone was unstable upon gas chromatographic analysis. Approximately half of this derivative was decomposed upon injection and resulting in "memory" effect on subsequent injection of samples, as described previously for Me_3Si derivative. However, this phenomenon was corrected when an excess of BSTFA was added to the reaction mixture. The function of BSTFA to stabilize the 2-trifluoroacetoxypyrimidine derivative is probably 2-fold: it serves to protect the labile 2-trifluoroacetoxypyrimidine against the hydrolysis of moisture; in addition,

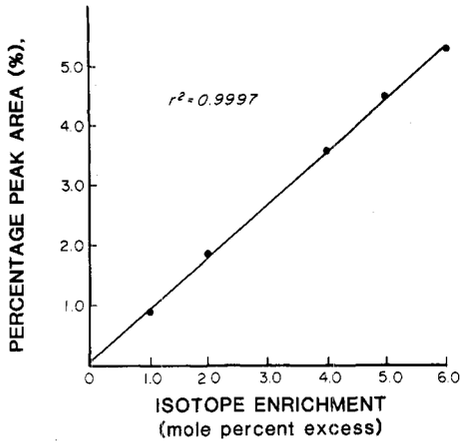

Figure 3. A representative standard curve for [$^{15}N_2$]urea enrichment analysis with GC/MS/SIM technique. Urea was derivatized as 2-trimethylsiloxypyrimidine derivative. The percentage of peak area was obtained by dividing the area of *m/z* 155 peak with the total area of *m/z* 153 and 155 peaks.

it serves to neutralize the trifluoroacetic acid produced during derivatization. The acid is known to catalyze the decomposition of 2-trimethylsiloxypyrimidine as discussed previously. No trans reaction was observed even after standing overnight at 5 °C. The analysis of the reaction mixture with GC/MS–selected ion monitoring failed to detect the presence of 2-trimethylsiloxypyrimidine in the reaction mixture of 2-trifluoroacetoxypyrimidine.

Gas Chromatography/Mass Spectrometry. The retention time of urea derivatives was 2–3 min, when the 2-trimethylsiloxypyrimidine was analyzed at 120 °C or 2-trifluoroacetoxypyrimidine was analyzed at 100 °C with a helium flow rate of 30 mL/min and a 6 ft 10% OV-17 column. A representative standard curve of the analysis is shown in Figure 3.

Choice of Derivatives. 2-Trifluoroacetoxypyrimidine has the advantage of lower background at $(M + 2)^+$ and hence is suitable for low enrichment analysis. The disadvantage of this derivative comparing with 2-trimethylsiloxypyrimidine is that it is less stable even with the protection of BSTFA. Besides, the trifluoroacetic anhydride is such a powerful acid and solvent, it has to be purified constantly (by repeated distillation) to eliminate the interference peak resulting from decomposition or contamination. For the determination of [$^{15}N_1$]urea and [$^{15}N_2$]urea enrichments above 0.5%, the more stable and easier to prepare 2-trimethylsiloxypyrimidine derivative is preferred. Only the extremely low level enrichment warrants the extra effort of reagent purification for the preparation of 2-trifluoroacetoxypyrimidine.

Since both 2-trimethylsiloxypyrimidine and 2-trifluoroacetoxypyrimidine have high background at (M + 1) ion, 10% and 6% respectively, the determination of the enrichment of [$^{15}N_1$]urea below 0.5% is unreliable.

Urea Production Rate Measurement in Humans. By use of the prime-constant infusion technique, the urea production rate was measured in human adults and infants (*10–12*). A representative study is shown in Figure 4. A steady state of urea enrichment is usually reached between 120 and 180 min. Data show that urea production rate is attenuated during pregnancy (9.85 ± 3.58 (mg/kg)/h (antepartum) vs. 15.9 ± 4.43 (mg/kg/h) (postpartum)) (*10, 11*). Similar studies have been done in the newborn infants (*12*).

If recycling of urea occurred, the labeled urea would be recycled back as [$^{15}N_1$]urea since the chance of two [$^{15}N_1$]-ammonia combined to form [$^{15}N_2$]urea is very remote. However, recycling of urea was not detectable in our studies, when *m/z* 153 vs. 154 were monitored.

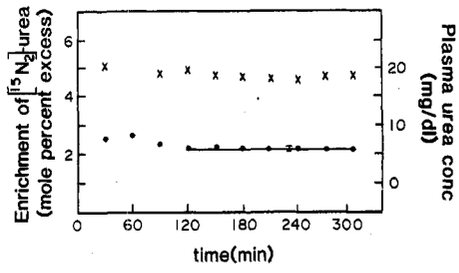

Figure 4. Plasma urea concentration (X) and [$^{15}N_2$]urea enrichment (O) of a pregnant women after a prime-constant infusion of [$^{15}N_2$]urea. The steady state of isotopic enrichment was reached 120 min after the start of the infusion.

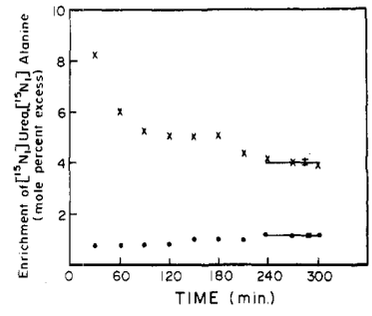

Figure 5. Plasma enrichment of [$^{15}N_1$]alanine (X) and [$^{15}N_1$]urea (O) of a dog after a prime-constant infusion of [$^{15}N_1$]alanine. The isotopic enrichments at steady state are expressed as mean ± standard deviation. The enrichment of alanine was determined as a propionyl ester acetate derivative on a GC/MS.

Incorporation of Nitrogen from L-[^{15}N]Alanine into [^{15}N]Urea in Dogs. The incorporation of alanine nitrogen into urea was studied by the prime-constant infusion of L-[^{15}N]alanine into dogs, and the [$^{15}N_1$]urea enrichment of the urea pool was determined by the present method. A representative experiment is shown in Figure 5. In a steady state, the percentage of urea nitrogen from alanine was calculated to be 29% in this particular example. The details of these investigations will be published separately.

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