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Determination of ^{13}C and ^{15}N enrichments of urea in plasma by gas chromatography–combustion isotope ratio mass spectrometry and gas chromatography–mass spectrometry using the 2-methoxypyrimidine derivative

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Abstract

We describe a GC–MS and GC-c-IRMS method for the determination of labeled urea tracer enrichments in plasma as a result of combined ^{13}C- and $^{15}N_2$-urea infusion experiments in piglets. Urea was converted into 2-methoxypyrimidine, a stable derivative, suited for analyses by both GC–MS and GC-c-IRMS. Using calibration curves for the respective working ranges (^{13}C-urea: 0–1% APE; $^{15}N_2$-urea: 0–7% MPE) enrichments were established in single point measurements; for $^{15}N_2$-urea as values±0.15% MPE (95% confidence interval); for ^{13}C-urea as values±0.02% APE (95% confidence interval). $^{15}N_1$-urea enrichments were determined by measurement of the same sample with GC-c-IRMS and GC–MS. Subtraction of the ^{13}C specific GC-c-IRMS data from the nondiscriminating GC–MS data for the sum of ^{13}C- and $^{15}N_1$-urea resulted in $^{15}N_1$-urea enrichments±0.15% MPE (95% confidence interval). Application of the method in a combined ^{13}C-urea bolus and $^{15}N_2$-urea primed constant infusion experiment in piglet was demonstrated.

Keywords: Derivatization, GC; Urea

1. Introduction

Urea tracer experiments can help to assess metabolic responses to physiologic stress, nutritional status, diet, and pharmacological interventions. The application of urea tracers, labeled with stable isotopes, allow determination of e.g. endogenous urea synthesis rates [e.g. 1–4], enterohepatic recycling of urea nitrogen [e.g. 5–7], protein synthesis/breakdown [e.g. 4] or the metabolic fate of ^{15}N-labelled amino acids [e.g. 8].

Numerous assays have been described for the analysis of stable isotope labeled urea enrichments in body fluids using gas chromatography–mass spectrometry (GC–MS) [e.g. 9–16] and several assays have been described using gas chromatography–(combustion)-isotope ratio mass spectrometry (GC–(c)-IRMS) [e.g. 15,17,18] or high-performance liquid

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chromatography–atmospheric pressure chemical ionization mass spectrometry (HPLC–APCI-MS) [e.g. 19]. The extent of sample preparation can vary with the MS method of choice. For example ^{13}C-urea was determined indirectly by the ^{13}C-enrichment of generated CO_2 from plasma samples treated with urease [17]; for the analysis with HPLC–APCI-MS samples were only diluted before direct injection on the MS system [19].

Especially, the accurate measurement of urea kinetics (in human) metabolism [e.g. 2,3,5,15,20–25] put high demands on the analytical method. The measurement of low enrichments and small changes in isotopic ratios necessitates the use of an analytical method suited for GC–IRMS and preferably for GC-c-IRMS. In the latter case it should be possible to measure labeled ^{13}C-urea enrichments (plasma, urine) directly. In multiple tracer studies, it would be preferable to use the same derivatized sample for GC–MS analysis as well: in the GC–MS analysis the urea concentration or the ^{15}N (and/or ^{18}O) label can then be measured simultaneously.

For the determination of ^{13}C-urea enrichment by GC-c-IRMS it would be best to add a minimum of carbon atoms by derivatization. For GC–MS analysis the best derivative would be a stable molecule giving little fragmentation and with a low natural abundance in the mass trace for the labeled tracer. For that purpose, 2-methoxypyrimidine is a good candidate. In the past the attempted preparation of this derivative has failed under numerous conditions [9]. In this paper, however, we describe a successful method for its preparation and its combined use in analyses with GC–MS and GC-c-IRMS in a dual urea tracer experiment in an animal model.

2. Experimental

2.1. In vivo experiment

One female cross-bred (Landrace×Yorkshire) piglet (body mass 10 kg) was studied under sedation with diazepam in the fasting and fed state, as previously described [26]. The animal had been instrumented with a venous catheter, positioned in a jugular vein, and an arterial catheter. At $t=0$ min a single oral bolus of 0.25 mmol ^{13}C-urea was administered. During the infusion, a tracer amount of $NaH^{13}CO_3$ was given as an intravenous bolus at $t=0$ (to prime the bicarbonate pool), and immediately thereafter an intravenous primed continuous infusion of $^{15}N_2$-urea (prime: 135 μmol/kg, constant infusion rate 15 μmol/kg/h) was given over 10 h. Arterialised blood samples of 2 ml were taken at $t=-5$, -3, -1 min (baseline samples) and at regular intervals of 30 min from $t=0$ min. Blood samples were collected in heparinized tubes (BD vacutainer Systems, Plymouth, UK) and after sampling immediately put on ice, plasma was separated and frozen at −20 °C. The experimental protocol had been approved by the Animal Experiment Commission of Utrecht University.

2.2. Instrumentation

2.2.1. Combustion-isotope ratio mass spectrometry

c-IRMS was performed on a VG Optima Isochrom Isotope ratio mass spectrometer (VG Micromass, Manchester, UK). The system, in detail described previously [27], was optimized for the determination of the $^{13}CO_2/^{12}CO_2$ ratio of the combustion products of 2-methoxypyrimidine. The combustion oven was kept at 850 °C, the cryogenic trap at −100 °C. Ion currents were measured continuously for m/z 44, m/z 45, m/z 46.

2.2.2. Gas chromatography

The instrument was equipped with a HP 5890A gas chromatograph (series II, Hewlett-Packard, Palo Alto, CA, USA). Samples were injected using a heated split/splitless injector ($T=290$ °C). A fused-silica WCOT, CP-WAX 51 AMIN column of 25 m×0.25 mm I.D.×0.39 O.D.×0.20 μm film thickness (Chrompack International, Middelburg, The Netherlands) was used for analyses of urea in plasma and standards with helium as a carrier gas (100 kPa). Typically, 1 μl of sample was injected splitlessly. The GC oven was programmed to increase from 40 °C (2 min) to 120 °C at a rate of 7 °C/min and to increase from 120 to 275 °C at a rate of 30 °C/min where its temperature was kept constant for 5 min. Under these conditions the retention time of 2-methoxypyrimidine was approximately 14.5 min.

The flame ionization detector (FID) was at a constant temperature of 340 °C. The GC column was connected, via a splitter union, to the combustion furnace via a heated interface at 350 °C.

2.2.3. Gas chromatography–mass spectrometry

For analyses with GC–MS we used two instruments: (i) Kratos Concept 1 H magnetic sector MS with EB geometry (Kratos Analytical, Manchester, UK), equipped with a Shimadzu GC model 14A (Shimadzu, Kyoto, Japan), (ii) Hewlett-Packard 5989B quadrupole MS equipped with a Hewlett-Packard 5890 series II GC (Hewlett-Packard, Avondale, PA, USA). Positive ion chemical ionization was used with ammonia as reaction gas at optimized pressure. Source temperature was 200 °C. The analytes were monitored for their $[M+H]^+$ ions at m/z 111.1, m/z 112.1 and m/z 113.1. The GC conditions were as described for the GC-c-IRMS system.

2.3. Derivatization

For isotopic analysis urea was converted into the 2-methoxypyrimidine derivative. Plasma samples were deproteinized with ethanol (96%): 500 μl of plasma was mixed with 5 ml of ethanol, vortexed (2 min) and after centrifugation (3600 g/4000 rpm, 2 min) the upper layer was evaporated to dryness (40 °C, stream of N_2). A 400-μl volume of malondialdehydbis(dimethylacetal) (MDBMA) (5% in water) and 640 μl of hydrochloric acid (37%) were added. After vortexing and centrifugation samples were kept at room temperature for 1 h followed by evaporation to dryness (40 °C, stream of N_2). The residue was dissolved in 400 μl of methanol (99.5%) and 400 μl of diazomethane–ether was added. Samples were vortexed (2 min), centrifuged (3600 g/4000 rpm, 1 min) and the upper layer was purified over a 3-ml silica column (Varian, Harbor City, CA, USA). The analytes were eluted with four times 400 μl of methanol. Ether was subsequently removed by a mild vacuum evaporation for 30 min. After extraction with hexane (99%) the end volume was reduced to 100 μl. Samples were stored at 4 °C. Depending whether GC-c-IRMS or GC–MS was performed the sample was concentrated or diluted.

2.4. Chemicals

Hydrochloric acid (36–38%) was purchased from J.T. Baker (Deventer, The Netherlands); hexane, methanol, urea, from Merck (Darmstadt, Germany); ethylacetate from Vel (Leuven, Belgium). Malondialdehydbis(dimethylacetal) from Merck-Schuchardt (97%, Darmstadt, Germany). $^{15}N_2$-urea (98%) was obtained from Cambridge Isotope Labs. (Andover, MA, USA), ^{13}C-urea (99%) from Campro Scientific (Veenendaal, The Netherlands).

2.5. Preparation of standards

Labeled urea enriched standards were prepared by (i) weighing an amount of labeled urea; (ii) addition of a weighed amount of unlabelled urea to obtain the required enrichment. The combined urea was dissolved completely in (heated) water. Thereafter, the mixture was freeze-dried and the resulting powder was stored at room temperature under nitrogen. The procedure was repeated to obtain independent standards with different enrichments of ^{13}C-urea (0%, 0.0487%, 0.0979%, 0.2272%, 0.4656% APE) and $^{15}N_2$-urea (0%, 0.970%, 2.764%, 4.364%, 7.041% MPE). Standards were analyzed at urea concentrations typical for plasma samples.

2.6. Statistics

For GC-c-IRMS enrichments were expressed as atom percentage excess (APE, %). For GC–MS enrichments were expressed as mole percentage excess (MPE, %), see also Appendix A. Data are expressed as mean±standard deviation (SD). Calibration curves were analyzed using linear regression (least squares).

3. Results and discussion

3.1. Method

Based upon Hamberg et al. [28] urea was converted with malonaldehyde into 2-hydroxy-

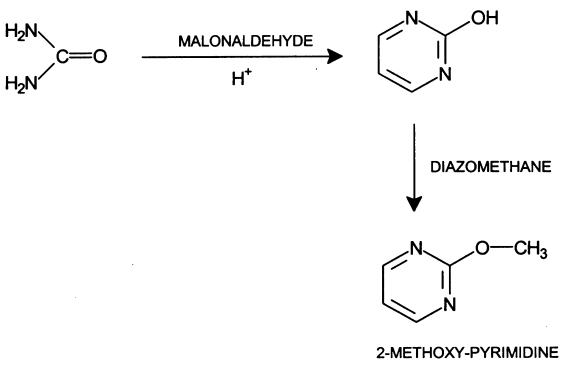

Fig. 1. Schematic for the conversion of urea into 2-methoxypyrimidine.

pyrimidine; subsequent reaction with diazomethane resulted in 2-methoxypyrimidine (Fig. 1). The product was a stable molecule which was well suited for MS analysis with GC as inlet system.

In GC-c-IRMS the eluting 2-methoxypyrimidine peak was introduced in the combustion oven and the resulting CO_2 was analyzed for its $^{13}CO_2/^{12}CO_2$ ratio (m/z 44–46). From these data ^{13}C-urea enrichment could be calculated; in this setting ^{15}N-label could not be observed.

In GC–MS 2-methoxypyrimidine could be ionized easily by positive chemical ionization producing the $[M+H]^+$ ion without major fragmentation. For quantitation the area under the curve in the ion chromatograms was used; m/z 111 for unlabeled 2-methoxypyrimidine; m/z 112 for [^{13}C]- and [$^{15}N_1$]-labeled analogues; m/z 113 for [$^{15}N_2$]-2-methoxypyrimidine; representative ion chromatograms of the analysis of a piglet plasma are shown in Fig. 2.

In the combined $^{15}N_2$-urea and ^{13}C-urea tracer experiment, the low resolution GC–MS measurement did not discriminate between $^{15}N_1$-urea and ^{13}C-urea. The area under the curve for m/z 112 resulted from the sum of both analogous. The same sample injected on GC-c-IRMS, however, excluded detection of the ^{15}N analogue. Combination of the results of both analyses gave the abundances of $^{15}N_1$-urea and ^{13}C-urea separately.

Although the described derivatization method is

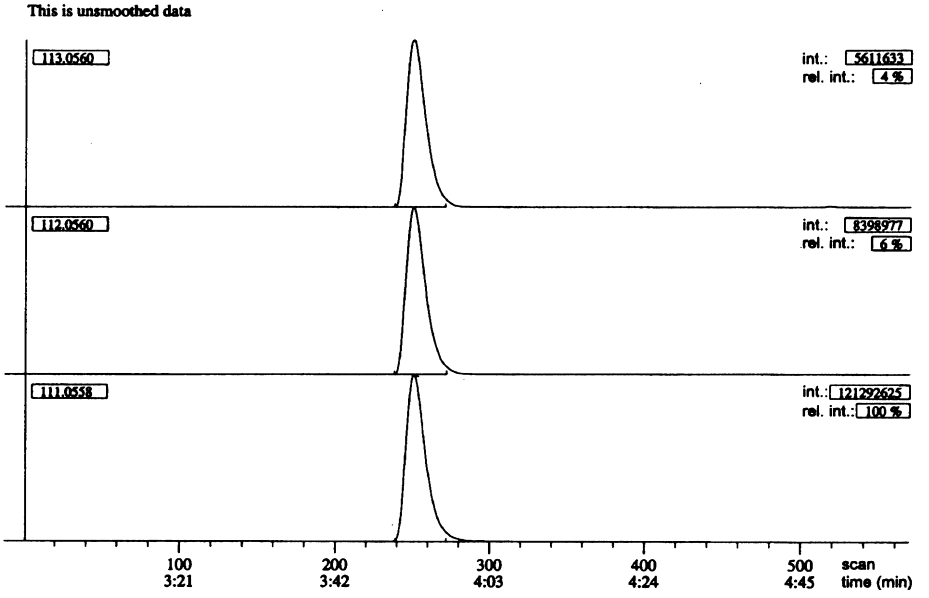

Fig. 2. Selected ion chromatograms (normalized peaks) in the GC–MS analysis of derivatized urea in plasma for m/z 111 (unlabeled urea), m/z 112 (Σ $^{15}N_1$-urea,^{13}C-urea), m/z 113 ($^{15}N_2$-urea).

also very well suited for the accurate measurement of urea concentrations in either plasma or urine, we have focused on its use in the measurement of low enrichments in plasma urea as a result of (combined) tracer experiments.

3.2. Linearity

The $^{15}N_2$-urea calibration curve as shown in Fig. 3 was typical of its use in the practice of GC–MS analyses, i.e. single point measurements of standards (calculated enrichments based on weighed amounts of labeled and unlabeled urea) vs. measured ratios for the respective areas under curve of selected ion chromatograms.

Good linear relationships were obtained in the respective working ranges for calculated enrichment (MPE) vs. measured enrichments (MPE) on 4 separate days over a period of 6 months; typically for GC–MS ($^{15}N_2$-urea): $r = 0.9989 \pm 0.0003$, slope = 0.95 ± 0.03 (enrichment range 0–7% MPE); for GC-c-IRMS (^{13}C-urea): $r = 1.0000 \pm 0.0001$, slope = 0.999 ± 0.003 (enrichment range 0–1% APE).

3.3. Accuracy and precision

The accuracy of the applied method was assessed with a set of standard mixtures by determining the differences in APE values obtained from GC-c-IRMS measurements and calculated from weighed amounts of urea at normal abundance and ^{13}C-urea (99% APE). Calibration was based upon urea of known natural abundance. The results (mean ± SD) of series of separate, single point measurements are summarized in Table 1.

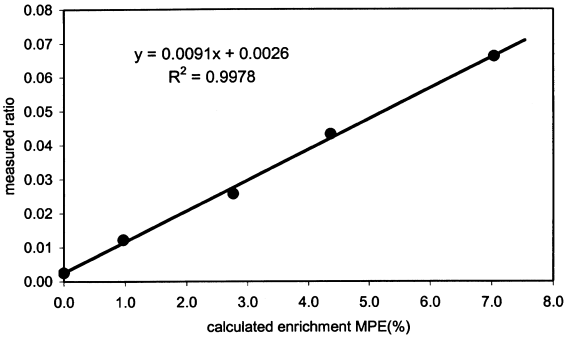

Fig. 3. Typical calibration curve for $^{15}N_2$-urea enrichments (single point measurements): calculated enrichment based on weighed amounts of (un)labeled urea vs. measured ratio of area under the curve for m/z 113 and m/z 111.

For the GC-c-IRMS analyses the comparison of calculated vs. measured showed a mean difference of 0.002% APE (in the range 0.05–0.3% APE). This implicated that even low enrichments could accurately be determined ±0.002% APE.

For the GC–MS analyses the accuracy was determined in a similar way. Comparison of the calculated enrichment (based on weighed amounts) and measured enrichment showed a mean difference between calculated vs. measured enrichment of 0.00 ± 0.14% MPE. This implicated that enrichments in the range of 0–7% MPE could accurately be determined within margins of ±0.15% MPE.

Based on the measurement of quality control samples we determined that the within day precision ($n = 3$) for GC-c-IRMS analyses was at least ±0.002% APE; the between day precision ($n = 10$, 1-year period) was at least ±0.01% APE

For GC–MS analyses the within day precision ($n = 3$) was ±0.03% MPE; the between day precision ($n = 11$, 1-year period) was ±0.15% MPE

In general, it meant, that ^{13}C-urea enrichments based on single point measurements with GC-c-IRMS could be obtained as values −0.02 to 0.02% APE (95% confidence interval); $^{15}N_2$-urea urea enrichments with GC–MS as values −0.15 to 0.15% MPE (95% confidence interval). Since the uncertainty in GC-c-IRMS values was much lower than in GC–MS values, calculated values for $^{15}N_1$-urea enrichments (i.e. GC–MS enrichment in trace m/z 112 minus the measured GC-c-IRMS value for ^{13}C-urea enrichment) could be obtained as values −0.15 to 0.15% MPE (95% confidence interval).

The $^{15}N_2$-urea used as tracer contained a carbon atom with an $^{13}C/^{12}C$ ratio which was slightly different from the $^{13}C/^{12}C$ ratio in unlabeled urea of natural origin. It meant that if a sample was composed of natural urea and 5% MPE of $^{15}N_2$-urea of tracer origin, the resulting CO_2 from the combustion process contained a ^{13}C-enrichment of 0.0006%

Table 1
Precisions characteristic for the GC–MS and GC-c-IRMS analyses of labeled urea tracers in standards and plasma samples

MPE(%)±SD[#]	n	GC-c-IRMS		GC–MS	
		MPE(%)±SD	n	MPE(%)±SD	n
^{13}C-urea					
Standard					
0.0000±–		0.0004±0.0020	3	0.00±0.02	3
0.0487±0.0002	3	0.0481±0.0014	3	Not measured	
0.0979±0.0003	3	0.0965±0.0021	3	0.086±0.02	3
Sample					
a		0.753±0.002	3	0.78±0.15	4
b		0.212±0.002	3	0.26±0.13	4
$^{15}N_2$-urea					
Standard					
0.000±–		N.D.		0.06±0.08	3
0.970±0.003	3	N.D.		1.06±0.09	3
2.764±0.009	3	N.D.		2.62±0.08	3
4.364±0.006	3	N.D.		4.55±0.11	3
7.041±0.006	3	N.D.		6.97±0.07	3
Sample					
c		N.D.		0.02±0.04	4
d		N.D.		4.19±0.11	3
e		N.D.		5.34±0.14	3

a, b = plasma samples at $t = 30$ and 600 min, respectively, after a single bolus of ^{13}C-urea; c,d,e = plasma samples at $t = 0$, 120 and 480 min, respectively, from a $^{15}N_2$-urea primed, continuous infusion experiment.
[#], SD of weighing; N.D., not determined.

APE. In practice the contribution of $^{15}N_2$-urea to the ^{13}C-enrichment measured for the ^{13}C-urea tracer was much lower than the precision of the whole method; correction for this contribution did not affect the outcome significantly.

4. Application in an experimental stable isotope study

The method was applied in simultaneous ^{13}C-urea bolus and a $^{15}N_2$-urea primed constant infusion study in an experimental piglet model. Typical enrichment curves during a combined bolus and primed constant infusion in a piglet are shown in Fig. 4. The signal-to-noise ratio (S/N) for the ions with the lowest intensity were always >100. For handling convenience we used arterial plasma samples of 500 μl. However, the signal-to-noise ratios and the size of the end volumes, containing the derivatized compounds, allows for miniaturization of the method.

The results of studies concerning the biological validation and methodological pitfalls of measurement of urea production using the oral ^{13}C-urea bolus method in a larger group of experimental animals will be published elsewhere [29].

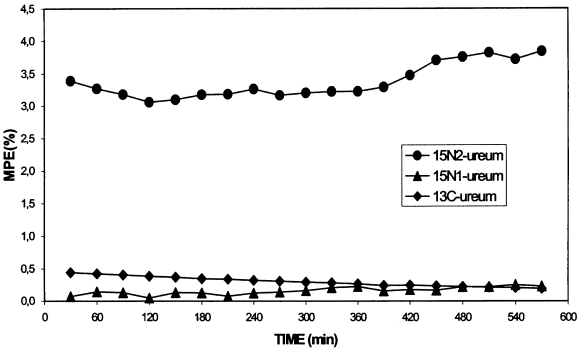

Fig. 4. Enrichments of ^{13}C-urea, $^{15}N_1$-urea and $^{15}N_2$-urea in plasma during a combined bolus ^{13}C-urea and primed continuous infusion $^{15}N_2$-urea tracer experiment.

5. Conclusion

We described a simple analytical method for the conversion of urea in plasma into 2-methoxy-pyrimidine, a derivative well suited for both GC–MS and GC-c-IRMS analyses. Deconvolution of GC–MS and GC-c-IRMS measurements resulted in the enrichments for $^{15}N_1$-urea, ^{13}C-urea and $^{15}N_2$-urea separately. Overall, ^{13}C-urea enrichments based on single point measurements with GC-c-IRMS could be obtained as values ±0.02% APE (95% confidence interval; range 0–1% APE); $^{15}N_2$-urea urea enrichments with GC–MS could be obtained as values ±0.15% MPE (95% confidence interval, range 0–7% MPE). $^{15}N_1$-urea enrichments with GC–MS (corrected for ^{13}C-urea contribution by GC-c-IRMS) could be obtained as values ±0.15% MPE (95% confidence interval, range 0–1% MPE). The method was developed for combined tracer experiments with a bolus of ^{13}C-urea and a primed constant infusion of $^{15}N_2$-urea.

Appendix A

In kinetic modelling it can be more convenient to express enrichment as tracer:tracee ratio (TTR, %) instead of mole percent excess (MPE, %). MPE (%) and TTR (%) are related as follows: R_s = isotopic ratio sample R_b = isotopic ratio background (or natural abundance)

$$\text{MPE}\ (\%) = [R_s - R_b]/(1 + [R_s - R_b]) \cdot 100 \qquad (A.1)$$

$$\text{TTR}\ (\%) = [R_s - R_b] \cdot 100 \qquad (A.2)$$

and thus:

$$\text{MPE}\ (\%) = [\text{TTR}\ (\%)/100]/[1 + \text{TTR}\ (\%)/100] \cdot 100 \qquad (A.3)$$

$$\text{TTR}\ (\%) = [\text{MPE}\ (\%)/100]/[1 - \text{MPE}\ (\%)/100] \cdot 100 \qquad (A.4)$$

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