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Simple and rapid quantitative assay of ^{13}C-labelled urea in human serum using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Abstract

A simple and rapid quantitative method for ^{13}C-labelled urea ([^{13}C]urea) in human serum was developed by using high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (HPLC–APCI-MS). This method is used to establish and normalize the [^{13}C]urea breath test, which is considered as an effective diagnostic method for *Helicobacter pylori* infection. HPLC–APCI-MS, involving a simple pretreatment process such as diluting serum with water, was shown to be able to discriminate the extrinsic [^{13}C]urea from intrinsic urea present at high concentration in serum. In addition, a ^{13}C nuclear magnetic resonance spectroscopic quantitative method for [^{13}C]urea in human urine is also described. The precision and accuracy of measured concentrations in these two methods were found to be within the acceptable limit. An application of these methods to investigate the pharmacokinetic profile of orally administered [^{13}C]urea in human serum and urine is also presented.

Keywords: Urea; *Helicobacter pylori*; [^{13}C]Urea breath test

1. Introduction

It was found that *Helicobacter pylori* (HP) exists in human gastric mucous membrane [1], and causes serious gastric and duodenal ulcers [2]. Marshall et al. reported that relapse of these diseases can be avoided by eradicating HP from the human body [3]. Unfortunately, no reliable diagnosis method for HP infection has been established to date. Recently, the [^{13}C]urea breath test (^{13}C-UBT), however, proved to be effective for evaluating HP infection [4–7]. This method, developed by Graham et al. [4], utilizes the fact that administered ^{13}C-labelled urea is converted to ^{13}C-labelled carbon dioxide and excreted into respiratory gas by means of HP's high urease activity. ^{13}C-UBT is expected to be a new diagnostic method, since it can simply and conveniently provide information on HP infection without using painful endoscopic method for patients. In order to establish and normalize the ^{13}C-UBT, it is necessary to investigate the pharmacokinetic profile and human tolerance of ^{13}C-labelled urea as a diagnostic drug.

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In this sense, development of a specific quantitative assay method for urea in human serum and urine is essential.

A number of works on quantitative analysis of urea in serum or urine has been performed using semi-micro Kjeldhal method [8], colorimetric method [9], ammonia-selective electrode [10,11], immobilized urease column [12], high-performance liquid chromatography (HPLC) [13] and nuclear magnetic resonance spectroscopy [14]. However, all these methods are time-consuming due to their complex sample preparation, and they lack of specificity and/or are not very sensitive due to the characteristics of detection methods. Recently, mass spectrometry (MS) combined with a separation system, eg., gas chromatography (GC), has been used to qualify and quantify drugs and their metabolites in body fluids. As to urea, ^{15}N- or $^{13}C^{15}N_2$-labelled urea in serum has been determined by means of GC–MS in order to clarify pharmacokinetic and metabolic properties [15–20]. GC–MS provides an extremely high specificity and sensitivity in qualitative and quantitative analysis of urea, but is not suitable for the rapid analysis required in clinical applications, because urea requires complicated sample derivatization treatment in order to be analyzed by GC–MS. A typical sample derivatization method [15] includes two steps: urea is first converted into a 2-hydroxypyrimidine derivative, followed by trimethylsilylation or trifluoroacylation. Furthermore, the urea derivatives are unstable and decompose during relatively short periods of time, and cause peak tailing in the chromatography [15,20]. Complicated and time-consuming sample preparation is a major disadvantage of GC–MS in regard to rapidity of analysis.

Atmospheric pressure chemical ionization (APCI)-MS [21] has been recognized as a powerful detector for HPLC, and has been applied to quantitative analysis of biologically active compounds [22,23]. HPLC–APCI-MS is superior to GC–MS in terms of direct qualitative and quantitative analysis of urea in addition to simplicity of sample preparation. HPLC–APCI-MS seemed to be an ideal analytical method for urea determination. This study was intended to develop a simple and rapid analytical method to identify and quantify [^{13}C]urea in human serum using HPLC–APCI-MS, and to show its applicability to human clinical study.

2. Experimental

2.1. Materials and reagents

HPLC-grade methanol, reagent grade [^{12}C]urea, uracil, acetamide and chromium(III) nitrate nonahydrate were commercially obtained from Wako Pure Chemical (Osaka, Japan). [^{13}C]Urea (99 atom-%) was purchased from ICON (NJ, USA). Deuterium oxide (99.95 atom-%) and physiological saline were the product of Merck (Darmstadt, Germany) and Otsuka Pharmaceuticals (Tokushima, Japan), respectively. Distilled water was purified using Nano-Pure II system (Barnstead, MA, USA). Human serum samples were purchased from Wako Pure Chemical, Ortho Diagnostics systems (Tokyo, Japan) and Nissui Pharmaceuticals (Tokyo, Japan). Human urine was collected from six healthy Japanese male volunteers (aged 23 to 29 years). All the other chemicals used were reagent grade.

2.2. Instrumentation and analytical conditions

2.2.1. HPLC–APCI-MS

The HPLC system consisted of a pump (Model 600-MS, Waters, MA, USA) and an autoinjector (Model 717, Waters). A Sumipax ODS C-212 column (15 cm×6.0 mm I.D., 5 μm in average particle diameter; Sumika Chemical Analysis Service, Osaka, Japan) was used. The mobile phase was methanol–water (15:85, v/v). The chromatography was performed at room temperature with the flow-rate of 1.0 ml/min. Mass spectrometric detection was carried out using a triple-stage quadrupole mass spectrometer (Model TSQ 700, Finnigan MAT, CA, USA) equipped with an APCI source. The APCI source was operated under the following conditions: heated capillary temperature 250°C, vaporizer temperature 400°C and corona discharge current 5.0 μA. The nitrogen sheath gas was set at 60 p.s.i. and the auxiliary gas at 10 units. Selected ion monitoring

(SIM) was employed for MS detection of the analytes. [^{12}C]Urea, [^{13}C]urea and uracil were determined by monitoring the protonated molecular ions at m/z 61, 62 and 113, respectively.

2.2.2. ^{13}C Nuclear magnetic resonance spectroscopy (^{13}C NMR)

^{13}C NMR spectra were obtained in deuterium oxide solution using a Bruker Model AC-300P (Karlsruhe, Germany), using sodium [1,2-$^{2}D_4$]trimethylsilyl propionate as an external standard. The measurements were carried out at the temperature of 298 K. The observed frequency, pulse width and pulse repetition time were set at 75.5 MHz, 2.8 μs and 2.0 s, respectively. [^{13}C]Urea and acetamide (internal standard) were determined by monitoring the signal at $\delta_{C=O}$ 165.2 ppm and $\delta_{C=O}$ 179.8 ppm, respectively.

2.3. Sample preparation

Serum (125 μl) was collected and diluted 200 times with water. Then, 50 μl of the internal standard solution of uracil (40 μg/ml) was added to 1 ml of diluted serum. Aliquots of this solution (10 μl) were injected onto the HPLC–APCI-MS system.

Urine samples were first passed through a filter (pore size 0.45 μm), and a 0.4-ml aliquot was then sampled into a NMR tube. A 100-μl volume of the internal standard (acetamide 500 mg/ml) in deuterium oxide containing chromium(III) nitrate nonahydrate (5 mg/ml) was added. This solution was subjected to NMR measurement.

2.4. Preparation of calibration curves

2.4.1. HPLC–APCI-MS

Calibration curves were individually prepared for every volunteer, using the serum sample collected before administration of [^{13}C]urea. Spiked serum samples at 0, 200, 600 and 1000 μg/ml for [^{12}C]urea, and at 0, 2.0, 6.0 and 12.0 μg/ml for [^{13}C]urea were prepared. Calibration curves were obtained by plotting peak-area ratio of [^{13}C] or [^{12}C]urea to the internal standard (uracil) against concentration of urea with the same nuclear species.

2.4.2. ^{13}C-NMR

For ^{13}C NMR determination of [^{13}C]urea levels in urine, the calibration curves were prepared using physiological saline instead of urine. Standard samples containing to 0, 50, 200, 500, 1000 and 2000 μg/ml of [^{13}C]urea were prepared by spiking physiological saline with [^{13}C]urea. These samples were subjected to the sample preparation procedure described above. Calibration curves were obtained by plotting peak-area ratios of [^{13}C]urea to the internal standard (acetamide) against [^{13}C] urea concentrations.

2.5. Phase I study

Three healthy male volunteers received 100, 200 or 300 mg/person of [^{13}C]urea. Testing drug was in a powder state containing 100 mg of [^{13}C]urea per package. The volunteers were administered [^{13}C]urea orally in 100 ml of water in the morning. They had fasted from 21:00 the day prior to administration and continued to fast until 3 h after administration. A meal was given to them at 3 and 12 h after administration. Blood and urine samples were collected just before administration (0 h) and at 0.5, 1.0, 2.0, 3.0, 6.0, 12.0, and 24 h after administration. The total amount of urine collected during 0–3, 3–6, 6–12 and 12–24 h was measured. Serum and urine samples were kept frozen until analysis.

3. Results and discussion

3.1. [^{13}C]urea in serum

3.1.1. HPLC–APCI-MS

Fig. 1 shows the mass spectra of [^{12}C]urea, [^{13}C]urea and uracil obtained by injecting 100 ng of each sample via flow injection. The protonated-molecular ion $[M+H]^+$ was observed in neutral mobile phase containing no electrolytes like ammonium acetate. Urea gave relatively simple mass spectrum pattern, and produced no product ion peak under

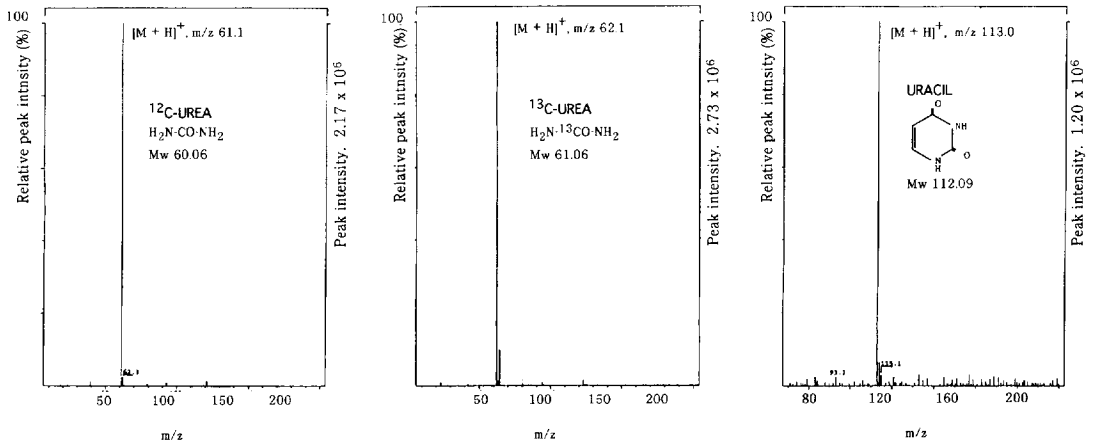


Fig. 1. Mass spectra of urea and uracil (internal standard). A 100-ng amount of each compound was introduced into APCI-MS via flow injection. Analytical conditions are described in Section 2.

collision induced dissociation (MS–MS) condition. Consequently, we employed selected ion monitoring (SIM) of the $[M+H]^+$ ion for the quantitation of urea.

There have been several problems for chromatographic analysis of urea in biological fluids. The high polarity of urea made it difficult to separate from biological constituents in reversed-phase chromatography. Therefore, specificity of urea determination was insufficient by HPLC only. HPLC–APCI-MS, which combines the mild separation conditions of HPLC with the excellent specificity of mass spectrometry, was considered to be able to evaluate the serum urea without influence of biological constituents. In addition, urea often gave a tailing peak in chromatography when a neutral mobile phase was used. We selected a SUMIPAX ODS C-212 column because it showed the best performance in methanol–water (15:85, v/v) mobile phase, compared with other analytical columns tested.

Stable isotope-labelled compounds have been successfully employed as internal standard in HPLC–MS analyses. Although $[^2H_4]$- or $[^{13}C\text{-}^{15}N_2]$-urea were considered to be the best candidates as the internal standard, urea analog compounds, having an amide moiety in their molecules were tested because of their commercial availability and freedom from urea contamination. In the present work, since urea was directly introduced onto HPLC–MS system without purification, it was not necessary to take the recovery of the internal standard during sample preparation into account. Acetamide, acrylamide and uracil were examined because these compounds, like urea, have an amide structure in their molecules and gave suitable retention times under the HPLC conditions used. The peak intensity of acetamide and acrylamide, however, fluctuated significantly, and the calibration curves showed poor linearity. Only uracil showed good peak stability, resulting in good linearity of the calibration curve. Consequently, it was considered that uracil was the best choice. In addition, it was confirmed that no interfering peak was observed at the elution time of uracil for 6 batches of blank serum.

Fig. 2 shows a typical mass chromatogram for serum urea. Based on the natural abundance of ^{13}C, ^{17}O, ^{15}N and ^2H (deuterium) [24], it is calculated that natural urea ($[^{12}C]$urea) includes $[^{13}C]$urea to the extent of about 1%. The combination of other isotopes such as $[^{17}O]$-, $[^{15}N]$-, and $[^2H]$-urea gives the same mass number as $[^{13}C]$urea (m/z 62). There are two and four sites of isotopic substitution for ^{15}N and deuterium in urea, respectively. Therefore, it is considered that the observed $[^{13}C]$urea peak is the sum of those from extrinsic $[^{13}C]$urea and ions giving the same m/z number, involved in $[^{12}C]$urea, such as $[^{13}C]$, $[^{15}N]$, $[^{17}O]$ and $[^2H]$ urea. As shown in Table 1, the content of natural isotopic urea is

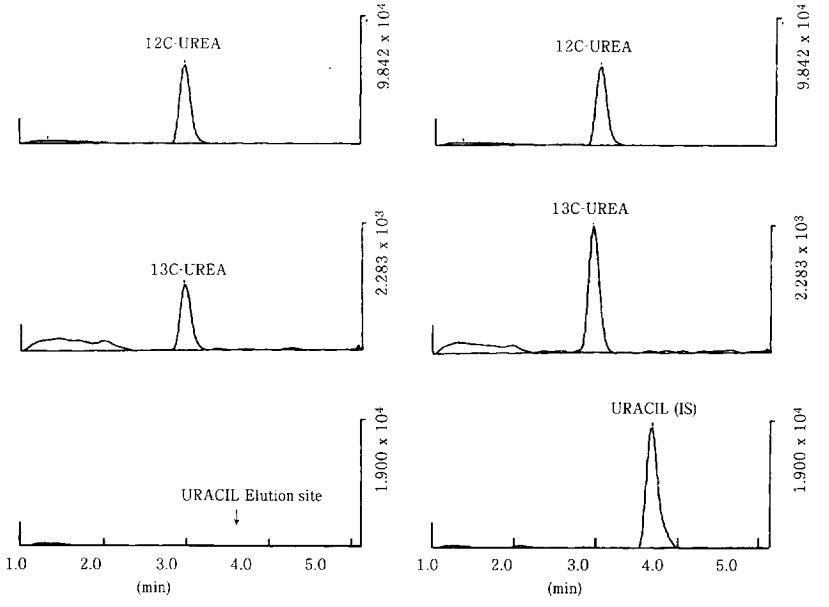


Fig. 2. Typical mass chromatogram of urea spiked in serum. (I) Blank serum ([^{12}C]urea and [^{13}C]urea peaks correspond to intrinsic urea), (II) Serum spiked with 6.0 μg/ml [^{13}C]urea and 400 μg/ml uracil. Horizontal: retention time, vertical: peak intensity. Analytical conditions are described in Section 2.

1.946% relative to [^{12}C]urea. Then, extrinsic [^{13}C]urea concentration can be directly determined using the following equation.

Extrinsic [^{13}C]urea concentration =

(observed [^{13}C]urea concentration)

$-$ ([^{12}C]urea concentration) $\times$ 1.946/100.

Table 1
Calculated percentage of other isotopic urea relative to the [^{12}C]urea

Compound	Relative abundance (%)
[^{12}C]Urea	100
[^{13}C]Urea	1.113
[^{17}O]Urea	0.0381
[^{15}N]Urea	0.7348
[^{2}H]Urea	0.0600
Total	1.946

These values were obtained based on the isotopic abundance described in Ref. [24].

From the mass chromatogram of blank serum (Fig. 2-I), the peak-area ratio percentage of [^{13}C]urea to [^{12}C]urea was found to be 1.99%. This value was in good agreement with the theoretically predicted one. Extrinsic [^{13}C]urea concentration was calculated by using the above equation in measuring clinical samples.

3.1.2. Calibration curves

Generally, intrinsic serum urea concentration is different from person to person. In addition, the level fluctuates within the same person depending on physiological conditions. To avoid the influence of these variations, a calibration curve prepared from physiological saline instead of serum was used. It was predicted that the physiological saline spiked calibration curve would be applicable for all serum samples, if it exhibited exactly the same slope value as the serum spiked curve. Fig. 3 shows the comparison of the physiological saline spiked calibration curve with the serum spiked one. As is evident, the slopes were different: the slope of physiological

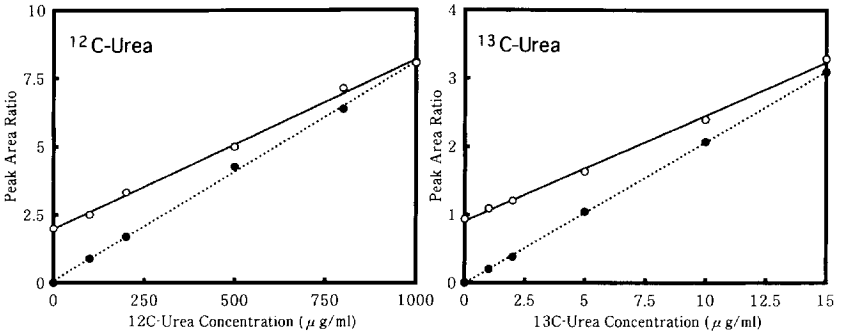


Fig. 3. Comparison of calibration curve of [^{12}C]- and [^{13}C]urea spiked in serum and physiological saline made by means of HPLC–APCI-MS. (○) Calibration curve of urea spiked in serum. (●) Calibration curve of urea spiked in physiological saline. Vertical: peak-area ratio ([^{12}C]- or [^{13}C]urea/uracil); horizontal: urea concentration.

saline spiked calibration curve is more steep than that of serum spiked one. Since the samples were directly introduced into the mass spectrometer without any purification process, it was considered that loss of urea during sample preparation is negligible. This result suggested that the ionization process of urea would be affected by the presence of unknown constituents in serum. Thus, it was considered that the physiological saline spiked calibration curve is unsuitable for use in determining serum urea levels.

Some commercially available serum preparations were subsequently examined to determine if they give the same slope, when the calibration curves were compared with each other. Fig. 4 shows that the slope differs significantly with respect to the serum batch. These results can be explained as follows. In APCI-MS, ions are produced by gas-phase molecule reactions in which electric charge is transferred from reagent ion molecules to urea. The ionization process of urea is influenced by the existence of other constituents in serum, such as proteins and inorganic ions that ionize more readily than urea. This matrix effect is different in each serum batch qualitatively and/or quantitatively. Based on these considerations, and assuming that the matrix effect on urea ionization is constant within the serum samples from the same human, it was decided to prepare an exclusive calibration curve for each volunteer using the serum sample collected before administration.

The peak response of [^{12}C]urea was linear up to an injected amount of at least 100 ng, corresponding to 1000 μg/ml serum concentration. In order to determine the calibration range, ten sets of calibration curves were prepared from ten different blank sera. The average intrinsic [^{13}C]urea concentration was found to be 7.0 μg/ml serum calculated from the intercept of each calibration curve. The average concentration of serum samples spiked with [^{13}C]urea at 2.0 μg/ml was measured to be 9.1 μg/ml using each calibration curve. This observed

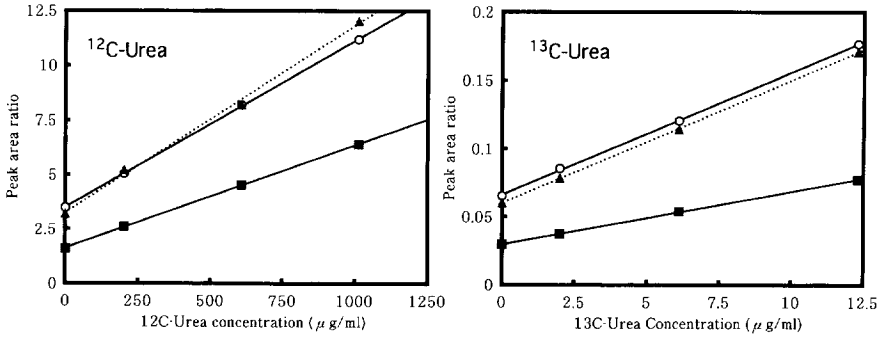


Fig. 4. Calibration curves of urea prepared from 3 serum batches. (○) Batch I; (▲) batch II; (■) batch III.

value was in good agreement with the calculated one, 9.0 μg/ml, and was statistically different from the average intrinsic urea concentration when examined by using a Student's *t*-test at 1% level. Consequently, the level of 2.0 μg/ml was regarded as the lowest limit of quantitation for [^{13}C]urea. Also, the lowest limit of quantitation for [^{12}C]urea was determined to be 200 μg/ml, in a similar manner.

3.1.3. Validation study

In the inter-day validation study, three replicated trials were performed on three different days. One batch of the control serum sample commercially obtained from three different suppliers was used in each trial. In the intra-day validation study, one batch of the control serum sample was used. In order to evaluate inter- and intra-day variations, 5 serum samples spiked at each of prescribed concentration of [^{12}C]- or [^{13}C]urea were prepared. The results are shown in Table 2. Acceptable accuracy and precision were demonstrated for both [^{12}C]- and [^{13}C]urea. Thus, this method enables us to obtain both intrinsic and extrinsic concentration of [^{13}C]urea as well as [^{12}C]urea in serum samples.

Table 2
Intra-day and inter-day variations of [^{12}C]urea and [^{13}C]urea in human serum

Concentration added (μg/ml)	*n*	Concentration found (mean±S.D.) (μg/ml)	R.S.D. (%)	Accuracy[a] (mean) (%)
[^{12}C]Urea				
Intra-day assay[b]				
202.4	5	201.8±14.1	7.0	99.7
607.3	5	646.5±34.5	5.3	106.5
1012.2	5	1110.7±16.6	1.5	109.7
Inter-day assay				
202.4	15	215.4±15.5	7.2	106.4
607.3	15	609.4±38.9	6.4	100.3
1012.2	15	1009.3±78.7	7.8	99.7
[^{13}C]Urea				
Intra-day assay[b]				
2.0	5	2.1±0.2	9.1	104.7
6.1	5	6.5±0.3	5.1	105.9
12.3	5	13.0±0.5	4.0	105.8
Inter-day assay				
2.0	15	2.1±0.1	6.4	105.4
6.1	15	6.2±0.4	6.6	101.3
12.3	15	12.7±1.0	8.0	102.9

[a] Accuracy (%)=(concentration found/concentrationd added)×100.
[b] Intra-day variation was obtained on the first experimental day.

3.2. [^{13}C]urea in urine

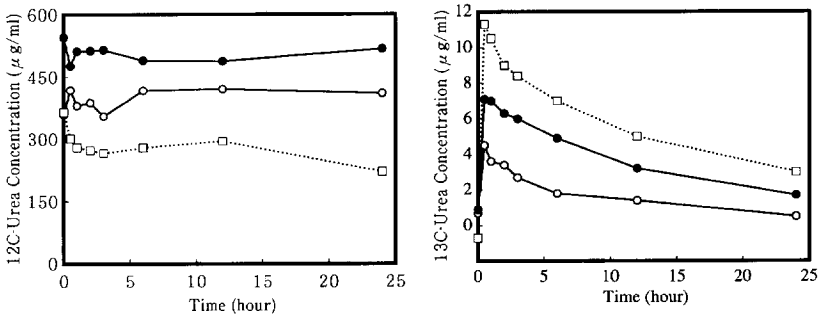
3.2.1. ^{13}C Nuclear magnetic resonance spectroscopy

It was found that acetamide is suitable as an internal standard in terms of its high solubility in deuterium oxide and signal separation; urea $\delta_{C=0}$ 165.2 ppm, acetamide $\delta_{C=0}$ 179.8 ppm. Although a lot of acetamide is required to obtain a significant signal due to the low natural isotopic abundance of ^{13}C atom, non-labelled acetamide was used as the internal standard. It was decided to add a relaxation agent, chromium (III) nitrate nonahydrate, including paramagnetic metallic ions in the molecule in order to shorten the relaxation time [25,26]. The optimum concentration of the relaxation agent giving the best resolution was examined and found to be 1 mg/ml. It was confirmed that the signal of acetamide was not affected by the presence of the relaxation agent.

The physiological saline-spiked calibration curve was found to be applicable in determining [^{13}C]urea instead of urine spiked one. In contrast to the results of HPLC–APCI-MS, the slope of urine spiked calibration curve was approximately equal to the physiological saline spiked one. This result permitted direct determination of [^{13}C]urea using physiological saline spiked calibration curve. The lowest limit of quantitation of this method was set at 50 μg/ml level. The inter- and intra-day variations of [^{13}C] urea were also evaluated at concentrations ranging from 50 to 2000 μg/ml (data are not shown). Acceptable accuracy and precision were also obtained for [^{13}C]urea in human urine.

3.3. Clinical application

Fig. 5 shows [^{12}C]- and [^{13}C]urea concentration profiles for each volunteer, given 100, 200 or 300 mg of [^{13}C]urea per person. Fig. 5a shows that the intrinsic serum urea concentrations of these volunteers were not the same and fluctuated with time, regardless of administered [^{13}C]urea amount. In



a) ^{12}C-Urea b) ^{13}C-Urea

Fig. 5. Serum concentration profiles of [^{12}C]- and [^{13}C]urea determined by HPLC–APCI-MS. Administered 100 (○), 200 (●) and 300 (□) mg of [^{13}C]urea orally, per person.

contrast, as to the extrinsic [^{13}C]urea, a clear time course change of the concentration with a distinct dose-dependency was observed (Fig. 5b). The serum [^{13}C]urea concentration was increased to the maximum at 30 min after administration, and decreased gradually thereafter.

From the results of ^{13}C NMR, the average total excreted amounts of [^{13}C]urea were found to be 412 mg, 550 mg and 504 mg for volunteers given 100, 200 or 300 mg/person of [^{13}C]urea, respectively (data are not shown). These values were larger than the predicted ones (150–300 mg), calculated from daily total excreted amount and natural isotopic abundance of carbon atom. The results for clinical investigation will be discussed elsewhere in detail [27].

The pharmacokinetic behavior of [^{13}C]urea was revealed by using HPLC–APCI-MS and ^{13}C NMR. These quantitative methods will provide a contribution to the establishment and normalization of the ^{13}C-UBT.

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