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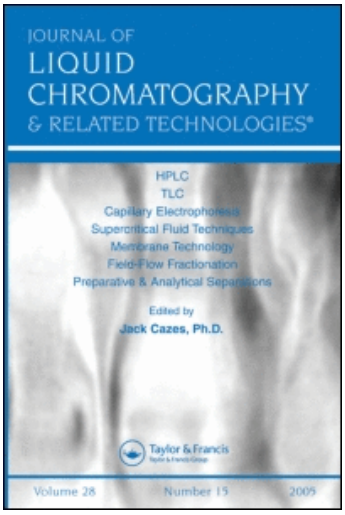

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Stability of ^{13}C-Urea/PEG Capsules by LC-APCI-MS

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ABSTRACT

The applicability of liquid chromatography-atmospheric-pressure chemical-ionization-mass spectrometry (LC-APCI-MS) for the determination of ^{13}C-urea in ^{13}C-urea/PEG capsules has been studied. It is essential to assess the stability of a newly developed low-dose (38 mg) ^{13}C-urea/PEG capsule, which will be used for a ^{13}C-urea breath test (^{13}C-UBT) to detect *Helicobacter pylori* infection. A standard curve was linear over the concentration range 10–1000 μg/mL. Intra- and inter-day variations were less than 2.75% in APCI-MS. The detection limit was 10 pg when selected ion monitoring (SIM) was employed. The content of ^{13}C-urea in capsules was within the acceptable range between 95 and

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105%. Therefore, it was established that ^{13}C-urea/PEG capsules were stable under an accelerated stability condition that was set at $40 \pm 2^{\circ}$C with relative humidity of $75 \pm 5\%$, during six months by using LC-APCI-MS. This research is the first report that describes LC-APCI-MS analysis of ^{13}C-urea capsules.

Key Words: ^{13}C-urea; Stability; *Helicobacter pylori*.

INTRODUCTION

^{13}C-urea breath tests (^{13}C-UBT) are effective tests for the detection of *Helicobacter pylori* infection. *Helicobacter pylori* is a curved Gram-negative rod, which exists in human gastric mucous membrane. *Helicobacter pylori* is recognized as the principal cause of peptic ulcer and a risk factor for gastric adenocarcinoma.[1,2] Currently, invasive and non-invasive methods are available to diagnose *H. pylori* infection. Invasive methods require the collection of biopsy samples via an end endoscopy. However, the endoscopy is unpleasant to the patient, time consuming, and expensive. So, one non-invasive method of detecting *H. pylori*, ^{13}C-UBT, is widely used owing to its safety, accuracy, reliable validation, and the advantage that it can be used in children.

Graham reported ^{13}C-UBT for the first time, which utilizes the high urease activity of *H. pylori*.[3] $^{13}CO_2$ in breath samples are measured by laser assisted ratio analyzer,[4,5] nondispersive infrared spectrometry,[6,7] and gas chromatography-mass selective detection.[8] After the initial report of ^{13}C-UBT, many factors in the procedure have been modified to optimize the test.[9]

Recently, a new formulation, ^{13}C-urea (38 mg)/PEG capsule, was reported to improve sensitivity and stability, reduce the cost of tests by using low doses, and obtain reliable results without the use of a test meal.[10] PEG, in this capsule, was used as a diluent and increased the initial dissolution rates of urea.[10] The purity and stability of ^{13}C in ^{13}C-urea capsule is important, as the administered ^{13}C-urea is converted to $^{13}CO_2$. Then, $^{13}CO_2$ is excreted into respiratory gas and is expressed as a ratio of $^{13}CO_2$ to $^{12}CO_2$ before and after ^{13}C-urea administrations. When *H. pylori* infection is present, the relative amount of $^{13}CO_2$ increases considerably. ^{13}C-urea (38 mg)/PEG capsule is being currently developed for human clinical studies. To do so, the ^{13}C-content and the stability of this capsule should be assessed by rapid and simple analytical methods.

For ^{13}C-urea determination, liquid chromatography-atmospheric-pressure chemical-ionization-mass spectrometry (LC-APCI-MS) is an ideal

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analytical method and is more convenient than GC-MS owing to the simplicity of sample preparation. $^{13}C^{15}N$-urea was determined by GC-MS in serum and spent dialysate after conversion into the trimethylsilylether derivative of 2-hydroxyprimidine.[11] For GC-MS analysis, the complicated procedures are necessary which are tedious and time-consuming. In addition, urea derivatives are usually unstable and decompose during relatively short periods of time.

The aim of this paper is to assess an accelerated stability of capsulated PEG-containing low-dose ^{13}C-urea, by LC-APCI-MS for future clinical studies.

EXPERIMENTAL

Materials and Reagents

HPLC grade acetonitrile and water were purchased from Sigma (St. Louis, MO). Standard ^{12}C-urea and ^{13}C-urea (99 atom %) were from Aldrich (Milwaukee, WI). ^{13}C-urea/PEG 4000 (38/1.9 mg/cap) capsules, Helifinder®, whose manufacturing process holds a Korean patent number 2002-0038271, were provided by Medichems Co. (Kyounggi-Do, Korea). All other chemicals and reagents used were analytical grade.

Instrumentation and Analytical Conditions

HPLC system (model HP 1100) from Hewlett Packard (Agilent, Waldbronn, Germany) consisted of a pump (model G1312A), an auto-injector (model G1313A), and a diode-array detector (model G1315A) that was set at 200 nm. Analytical HPLC column (Xterra™ C_{18}, 3.5 μm in average particle size, 150 × 2.1 mm i.d.) was purchased from Waters (Milford, MA). The mobile phase was a mixture of acetonitrile/water (95 : 5, v/v) with a flow rate of 0.5 mL/min at room temperature. The mobile phase was filtered through a 0.45 μm Whatman nylon membrane filter (Maidstone, UK) and degassed under vacuum before use. Mass spectrometric detection was performed using an ion-trap mass spectrometer equipped with an APCI source in Finnigan LCQ (San Jose, CA). The APCI source was operated under the following conditions: heated capillary temperature 200°C, vaporizer temperature 450°C, and corona discharge current 5.0 μA. The nitrogen sheath gas was set at 70 p.s.i., and the auxiliary gas at 10 units. For MS detection of ^{12}C- and ^{13}C-ureas, selected ion monitoring (SIM) was carried out. As shown in Fig. 1, the protonated molecular ions of ^{12}C- and ^{13}C-ureas were determined by monitoring at m/z 61 and 62, respectively.

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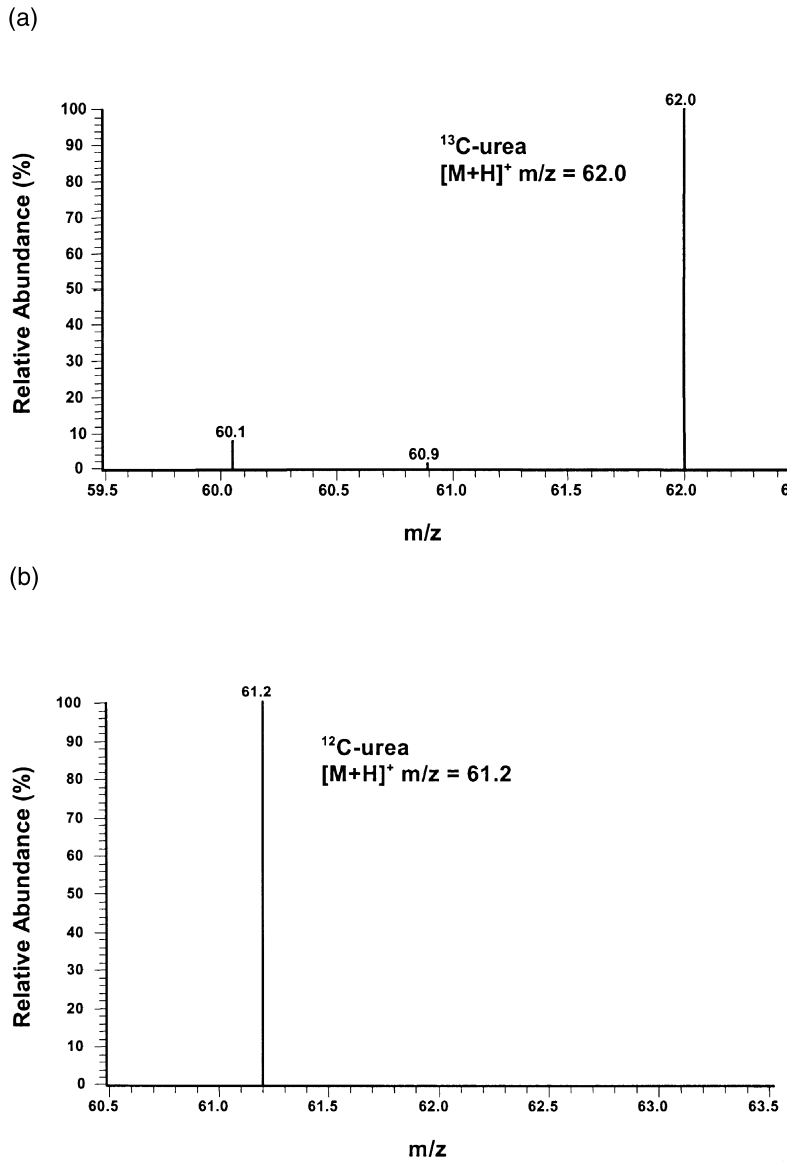

Figure 1. Typical mass spectra. (a) 10 ng of ^{13}C-urea, and (b) 10 ng of ^{12}C-urea were introduced into APCI-MS with SIM mode. Analytical conditions are described in instrumentation section.

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Preparation of Samples

For an accelerated stability assessment, ^{13}C-urea/PEG capsules were stored in a Life Test Chamber to keep constant temperature and humidity. Capsules from three different lots (lot A, B, and C) were pooled at 0, 1, 2, 4, and 6 months time points after storing at $40 \pm 2^{\circ}$C with relative humidity of $75 \pm 5\%$, according to The Korean Pharmacy Law.[12] At each time point, 20 capsules from one lot were opened and mixed well. The amount of powder that was equivalent to 20 mg of ^{13}C-urea was taken and dissolved in 50 mL acetonitrile to prepare a stock solution (400 μg ^{13}C-urea/mL). Appropriate serial dilutions of a stock solution with acetonitile were made. All samples were fleshly prepared on the day of analysis and passed through a 0.45 μm syringe filter before injection. Two microliter of sample was injected into the HPLC.

Validation

The peak area of selected ions was calculated and used to construct calibration curves of peak area ratio against ^{13}C-urea concentration. Slope, intercept, and regression coefficient of the calibration curves were determined. In order to determine the intra- and inter-day precision and accuracy of the assay, quality control samples of fixed concentration (125, 250, and 800 μg/mL, shown in Table 1) were prepared. The intra-day data were collected from the analysis of six replicates of three concentrations (125, 250, and 800 μg/mL) of QC samples on the same day and inter-day data were collected from the analysis of six replicates of three concentrations of QC samples on six separate days. The limit of detection was defined by the concentration of ^{13}C-urea giving a signal to noise ratio of 3 : 1.

Table 1. Intra- and inter-day assay variances for determination of ^{13}C-urea in ^{13}C-urea/PEG capsules by HPLC-APCI-MS.

	Intra-day assay ($n = 6$)			Inter-day assay ($n = 6$)		
^{13}C-urea (μg/mL)	Measured (mean ± SD) (μg/mL)	RSD (%)	Accuracy (%)	Measured (mean ± SD) (μg/mL)	RSD (%)	Accuracy (%)
125	126.3 ± 1.71	1.35	101.0	128.5 ± 3.54	2.75	102.8
250	248.1 ± 1.92	0.77	99.2	252.4 ± 4.11	1.62	100.9
800	802.1 ± 2.91	0.36	100.3	807.1 ± 10.23	1.26	100.9

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RESULTS AND DISCUSSION

Chromatography

Liquid chromatography-atmosphere-pressure chemical-ionization-mass spectrometry was employed for the quantitative assay of ^{13}C-urea in human serum, which combines the mild separation conditions of HPLC with the excellent specificity of mass spectrometry.[13] Furthermore, SIM was used as acquisition mode in order to increase the detector sensitivity of the measurement. It was necessary to employ SIM mode to determine ^{13}C- and ^{12}C-ureas. ^{13}C- and ^{12}C-ureas were characterized by protonated molecular ions at m/z 62 and 61, respectively.

The concentration of ^{13}C-urea was calculated by the following equation:

$$^{13}\text{C-urea}(\%) = \frac{A_{62} \times 100}{A_{61} + A_{62} + A_{63}}$$

A_{61}, A_{62}, and A_{63} are the peak areas of protanated molecular ion peaks of ^{12}C-, ^{13}C-, and ^{14}C-ureas, respectively. The natural abundance of ^{14}C in ^{14}C-urea is very low, thus, A_{63} value was negligible.

Linearity and Detection Limit

The linear regression equation (peak area-concentration) was calculated according to eight concentrations and three replicates per concentration. Linearity was established over the concentration range 10–1000 μg/mL for APCI-MS. Linear regression analysis of detector response vs. ^{13}C-urea concentration yielded the regression curve:

Detector response of HPLC-APCI-MS = 20,035 × ^{13}C-urea concentration (μg/mL)

$$r^2 = 0.9777$$

The intercept was not significantly different from zero. This indicated a definite linear relationship between ^{13}C-urea concentration and each detector response.

The limit of detection was defined by the concentration of ^{13}C-urea giving a signal to noise ratio of 3 : 1. As shown in Fig. 2, the detection limit of ^{13}C-urea was determined as 10 pg by SIM mode, while full scan mode gave 50 pg of detection limit. When both scan methods were compared, SIM gave five times higher sensitivity than that of full scan mode.

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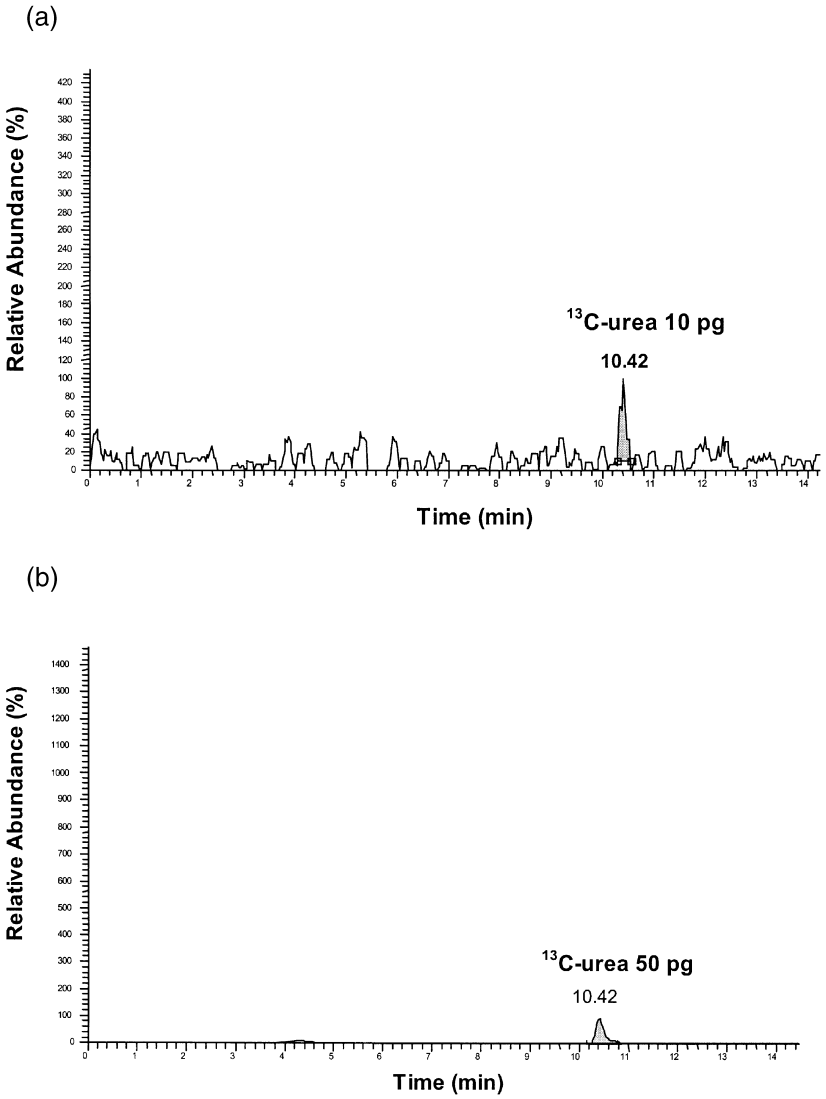

Figure 2. Detection limit of ^{13}C-urea. (a) 10 pg and (b) 50 pg of ^{13}C-urea was introduced into APCI-MS with SIM mode. Analytical conditions are described in instrumentation section.

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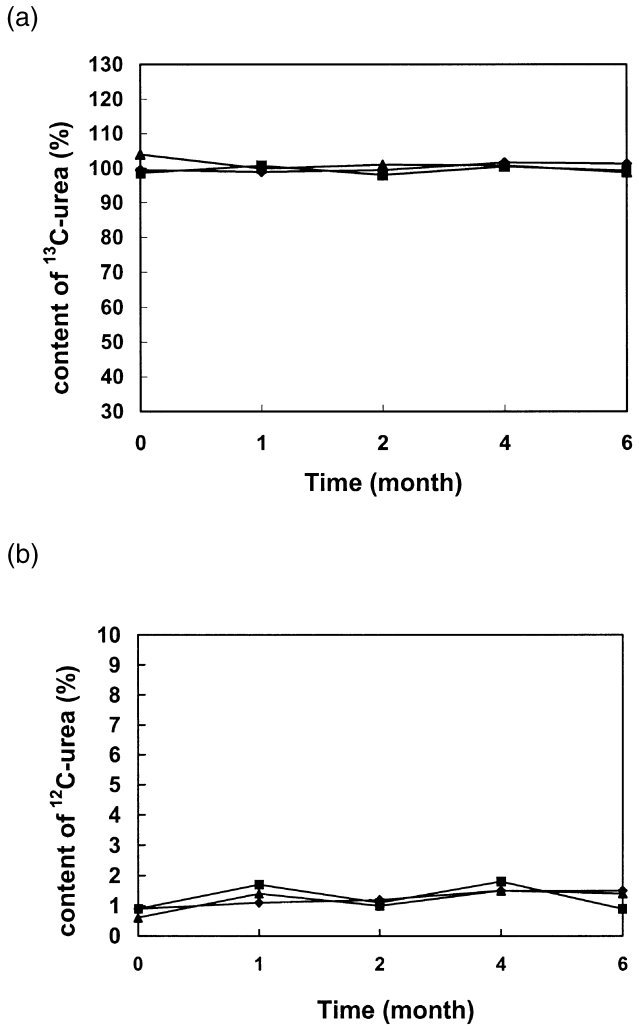

Figure 3. Stability diagrams. ^{13}C-urea/PEG capsules were stored at $40 \pm 2^{\circ}$C with relative humidity of $75 \pm 5\%$ during six months. Contents of (a) ^{13}C-urea, and (b) ^{12}C-urea in ^{13}C-urea/PEG capsules were shown. Filled diamond, square, and triangle symbols represent lot A, B, and C, respectively. Data were taken from Table 2.

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Table 2. Contents of ^{13}C- and ^{12}C-ureas under an accelerated stability condition ($40 \pm 2^{\circ}$C with relative humidity of $75 \pm 5\%$ during six months time period).

	Content of ^{13}C-urea (%) (mean ± SD, $n = 3$)			Content of ^{12}C-urea (%) (mean ± SD, $n = 3$)		
Months	Lot A	Lot B	Lot C	Lot A	Lot B	Lot C
0	99.5 ± 0.55	98.6 ± 0.52	104.0 ± 1.21	0.9 ± 0.38	0.9 ± 0.37	0.6 ± 0.53
1	98.9 ± 3.19	100.7 ± 1.08	99.9 ± 1.05	1.1 ± 0.49	1.7 ± 0.75	1.4 ± 1.25
2	99.5 ± 0.87	98.1 ± 0.29	101.0 ± 1.00	1.2 ± 0.76	1.1 ± 0.71	1.0 ± 0.35
4	101.7 ± 0.29	100.5 ± 0.00	100.8 ± 0.29	1.5 ± 0.66	1.8 ± 0.41	1.5 ± 0.45
6	101.3 ± 0.29	99.3 ± 0.29	98.8 ± 0.29	1.5 ± 0.26	0.9 ± 0.82	1.4 ± 1.25

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Accuracy and Precision

The intra- and inter-day precision and accuracy of the assay were presented in Table 1. The intra-day and inter-day variations of LC-APCI-MS had coefficients that ranged from 0.36 to 1.35% and from 1.26 to 2.75%, respectively. The precision and accuracy were found to be within the acceptable limits.

Stability of ^{13}C-Urea

The accelerated stability of ^{13}C-urea/PEG capsules was monitored at $40 \pm 2^{\circ}$C with relative humidity of $75 \pm 5\%$ during six months according to The Korean Pharmacy Law.[12] As shown in Fig. 3, the content of ^{13}C-urea in capsules was constant without any detectable degradation. The content of ^{13}C-urea (%) as a major compound was between 95 and 105%, and the content of ^{12}C-urea (%) as a related compound was less than 5%. According to The Korean Pharmacy Law,[12] both contents were within the acceptable ranges (Table 2 and Fig. 3). According to the results of stability experiments, a new ^{13}C-urea/PEG formulation is stable enough to be used for ^{13}C-UBT for *H. pylori* infection.

CONCLUSIONS

Liquid chromatography-atmospheric-pressure chemical-ionization-mass spectrometry using a mobile phase of acetonitrile/water (95 : 5, v/v) with an analytical C_{18} column has been validated to determine and quantitate ^{13}C-urea in ^{13}C-urea/PEG capsules. ^{13}C-urea/PEG capsules were stable under an accelerated stability condition that was set at $40 \pm 2^{\circ}$C with relative humidity of $75 \pm 5\%$ during six months without any detectable degradation. This method has proven to be simple, rapid, reliable, sensitive, and may also be suitable for determination of newer generations of ^{13}C-urea formulation in the future. Currently, ^{13}C-urea/PEG capsules are on trial for the ^{13}C-urea breath test to diagnose *H. pylori* in humans.

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