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Analysis of acetaminophen glucuronide conjugate accompanied by adduct ion production by liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry

Motoko Ohta, Nozomi Kawakami, Susumu Yamato, Kenji Shimada *

Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, 5-13-2 Kamishin'ei-cho, Niigata 950-2081, Japan

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

Liquid chromatography-mass spectrometry (LC-MS) is an effective method for the analysis of polar compounds. A coupling of LC-MS, which is used under conventional conditions, and atmospheric-pressure chemical ionization (APCI), which applies mild ionization for the analysis of water-soluble drug conjugates, would offer a very convenient method. The APCI method is effective for ionizing low- and medium-polarized compounds, but not for highly polarized compounds. In this study, we have tried derivatization of carboxyl group of glucuronic acid, to which direct ionization is difficult to apply under the APCI method, was conducted using glucuronides. Methyl ester derivatives were found to be effectively ionized. Furthermore, acetaminophen glucuronide conjugate was investigated in detail. Methyl ester derivatives of acetaminophen glucuronide conjugate (ACEG) were detected at m/z 373 as O_2 adduct ion $[M+O_2]^-$ in the negative mode, and *p*-nitrophenyl β-D-glucuronide (PNPG) demonstrated ionization behaviors very similar to ACEG. Quantitation of ACEG was examined using PNPG as an internal standard, and satisfactory results were obtained for the recovery test and quantification.
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1. Introduction

In order to understand pathophysiology through the administration of drugs, establishment of a reliable method of analyzing drug conjugates excreted in the urine is difficult to

* Corresponding author. Tel.: +81-25-268-1172; fax: +81-25-268-1177
E-mail address: shimada@niigata-pharm.ac.jp (K. Shimada).

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apply. Sulfates and glucuronides do not carry an atomic group that would allow detection with a high degree of sensitivity. Moreover, because conjugates are generally unstable, many studies have found that it would be extremely difficult to analyze with high sensitivity such information of conjugation as location of conjugation and bonding characteristics, without applying hydrolysis to the drugs.

In general, the method of measuring aglycon, which is obtained by enzymatic or chemical hydrolysis, is utilized for analysis of conjugates. However, in many cases, hydrolysis does not proceed quantitatively due to different location or type of conjugation. In order to solve such problems, the use of LC-MS or MS-MS for the analysis of drug conjugates has been receiving attention.

Electrospray ionization-mass spectrometry (ESI-MS) is an effective method for measuring highly polarized compounds. However, this method often requires the use of microcolumns with a low flow rate. Therefore, ESI-MS is not the proper method for the analysis of glucuronides, since regular LC conditions could not be used.

We have been using the method of producing aglycon through a hydrolysis reaction using acids and oxygen, and measuring aglycon to analyze drug metabolites. However, this method analyzes only aglycon rather than drug metabolites. When aglycon is produced from conjugates through a hydrolysis reaction, side reactions also occur. Since this method measures aglycon, metabolites are not directly measured.

Acetaminophen is used as an antipyretic medication. Rarely reported adverse effects include thrombocytopenia, granulocytopenia, and cyanosis. Adverse effects more commonly reported include nausea, emesis, and poor appetite. Most of the acetaminophen administered undergoes phase-II metabolism and is excreted as glucuronic acid conjugate and glutathione conjugate, which are the major metabolites of acetaminophen [1].

Although two kinds of metabolites of acetaminophen (glutathione conjugate and glucuronide conjugate) are identified in bile of rats using LC-thermospray (TSP)-MS [1], the peaks of mass spectroscopy of positive-ion glucuronide (by protonation, ammonium adducts, sodium adducts) are not large. Strong signals of glucuronides are thus not easily observed using LC-TSP-MS.

2. Experimental

2.1. Reagents

Acetaminophen (ACE), acetaminophen glucuronide conjugate (ACEG), and *p*-nitrophenyl β-D-glucuronide (PNPG) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA), while *p*-tolylsulfonylmethylnitrosamide for the preparation of diazomethane was obtained from Wako Pure Chemicals (Osaka, Japan). High quality products were utilized for the other reagents. Solvents of HPLC-grade purity were used.

2.2. Liquid chromatography-mass spectrometry (LC-MS)

LC-MS was performed using a quadrupole mass spectrometer (Model M-1000, Hitachi, Japan) and a liquid chromatography pump (Model L-6200, Hitachi, Japan), equipped with APCI. Shim-pack CLC ODS (150 × 4.6 mm, i.d., Shimadzu, Japan) was used as a separation column, using methanol–water (9:1,v/v) as the mobile phase. The flow rate was set at 0.6–1.0 ml/min. The following operating conditions were used: vaporization temperature for APCI-MS, 200 °C; desolvation temperature, 399 °C; ionizing voltage, 2000 V. Operating conditions on the positive-ion modes were as follows: drift voltage, 30 V; focus voltage, 120 V. Operating conditions for the negative-ion modes were as follows: drift voltage, −30 V; focus voltage, 140 V. TIC mode was utilized for the mass chromatogram, and SIM mode was utilized for quantitative analysis.

2.3. Preparation of LC-MS sample

Ethereal diazomethane was added into ACEG and PNPG in methanol to produce methyl ester derivatives at room temperature [2]. After removing solvent, materials were dissolved into methanol, and reaction products were then introduced

into LC-APCI-MS. Methyl ester derivative of ACEG was measured at m/z 342 as the $[M+H]^+$ ion in the positive mode followed by at m/z 373 as $[M+O_2]^-$ ion in the negative mode. Methyl ester derivatives of PNPG were measured at m/z 361 as $[M+O_2]^-$ ion in the negative mode.

2.4. Quantitative analysis for ACEG

Half a milliliter of PNPG aqueous solution (1.0 μg/ml) was added to 0.5 ml of ACEG aqueous solution (0.01–50 μg/ml) and mixed well. The resulting mixture was then poured into the pre-treatment cartridge (Waters Corporation, OASIS MCX 1 ml, 30 mg), and adsorbed for 30 min. The cartridge was rinsed with 0.7 ml of 0.01 mol/l hydrochloric acid, and dissolved using 0.7 ml of methanol. For the pre-conditioning of the cartridge column used in solid-phase extraction, the cartridge was rinsed with 1 ml of methanol, and the cartridge column was dried under reduced pressure (10 mmHg). One milliliter of water was then added to equilibrate the cartridge column. Ethereal diazomethane was added to the eluate from the cartridge to form methyl ester derivatives. Solvent was removed using a stream of nitrogen gas, then methyl ester derivatives were dissolved into 0.5 ml of methanol, and 50 μl of the mixture was introduced into the LC-APCI-MS. The ratio of ion intensities (ion intensity of ACEG methyl ester at m/z 373 vs. ion intensity of PNPG methyl ester at m/z 361) was calculated.

2.5. Recovery rate of ACEG

An aqueous solution of PNPG (1.0 μg/ml) was mixed into samples containing 0.4, 2.5, and 10.0 μg/ml of ACEG, and measured the recovery rate of ACEG quantitatively using the method described above.

3. Results and discussion

Brouwer et al. used a C_{18} reversed phase HPLC column to perform quantitative analysis of ACEG in serum, urine, and bile samples, and found that the limit of detection was 1.75 μg/ml [3]. In addition, Alkharfy et al. conducted quantitative analysis of ACEG in human liver microsomes by HPLC, and found that ACEG could be quantitatively and accurately measured over the range of 0.1–25 nmol [4].

Iwabuchi et al. used three types of ionization methods, and examined the efficiency of each method in measuring the metabolites of pravastatin, and the association between sensitivity and polarity. The results showed that using an APCI interface, several nanograms of hydrophobic compounds (log p 2) were detected at high sensitivity. With the TSP method, hydrophobic compounds were detected at higher sensitivity than with the APCI method. With the ESI method, hydrophilic compounds were detected at higher sensitivity: 10–100-fold higher sensitivity than with the TSP and APCI methods [5].

In addition, the detection-oriented derivatization method in LC-MS is reviewed by Shimada et al. [6]. Studies using derivatization methods in LC-MS analysis to increase the efficiency of ionization include the studies by Kusaka et al. that performed analysis of long-chain fatty acids using LC-APCI-MS. They produced the $[M+H]^+$ ion efficiently by generating anilide [7]. Ikeda et al. reported a method to produce the $[M+H]^+$ ion by generating *n*-propylamide from hydroxy or non-hydroxy fatty acids [8].

Furthermore, Shimada et al. produced $[M+H]^+$ or $[M+NH_4]^+$ ions by generating methyl ester derivatives of vitamin D glucuronide conjugates using a mobile phase containing ammonium acetate [9], and also they reported the usefulness of derivatization of vitamin D glucuronides to the adducts with 4-phenyl-1,2,4-triazoline-3,5-dione, the methyl esters and the acetates [10]. Schockcor et al. identified metabolites by coupling MS-MS and nuclear magnetic resonance spectroscopy [11].

However, these are not appropriate methods for detecting ACEG at high sensitivity. Therefore, the present study reduced the polarity of ACEG to enable measurement by APCI-MS, and examined many types of derivatization using HPLC reagents to label carboxyl groups. The results demonstrated that methyl ester derivative of ACEG is efficiently produced by APCI (Fig. 1).

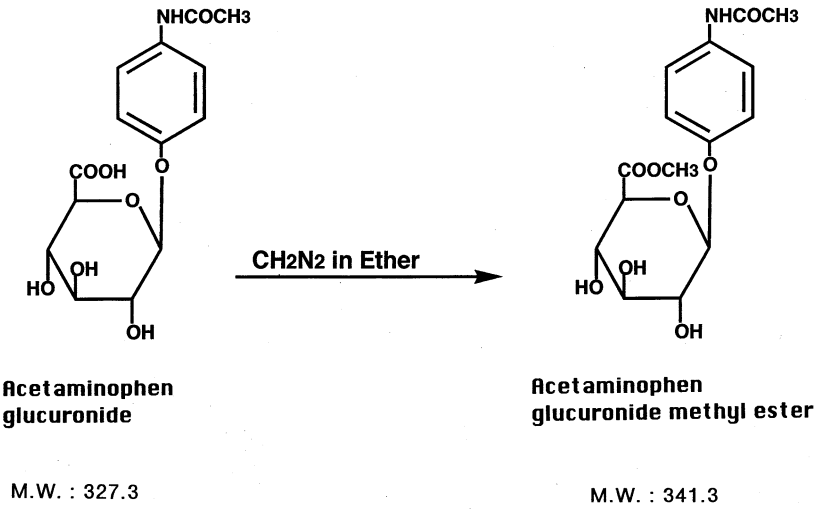

Fig. 1. Methyl esterification of acetaminophen glucuronide with ethereal diazomethane.

When carboxyl group in glucuronic acid is converted to methyl esters in advance through the action of diazomethane, precursor ions of ACEG were observed at *m/z* 342 $[M+H]^+$ in the positive mode, and moreover, appeared strongly at *m/z* 373 in the negative mode (Fig. 2).

As the molecular weight of the methyl ester of ACEG is 341.2, the difference of 32 from *m/z* 373 can be attributed to O_2, so that $[M+O_2]^-$ is observed at *m/z* 373. APCI methods perform ionization at atmospheric pressure. Therefore, in APCI, O_2 in air is taken up using the liquid nebulization, and O_2^- ions are easily produced. This O_2^- ion produces a stable adduct-ion of $[ACEGMe\ O_2].^-$

In addition, ion intensity of ACEG methyl ester in the negative mode was approximately twice as strong as that in the positive mode (Table 1).

Ion intensity could therefore be increased 10–20-fold stronger by O_2^- ions using the APCI-MS method, using an easy reaction for converting ACEG to its methyl esters. The addition of acetic acid or ammonium acetate into the mobile phase was not observed to increase ion intensity.

Evaluation of quantitation using the negative mode demonstrated that ACEG shows a good linear relationship in the range of 0.01–50.0 μg/ml. This could allow measurement by the LC-APCI-MS method under conventional conditions.

For quantitative analysis of drugs using GC-MS or LC-MS, quantitative methods utilizing internal standard compounds need to be established. As PNPG demonstrates an ionization behavior at the carboxymethyl terminal of glucuronide by APCI-MS similar to that of ACEG, it was selected from among the ether type glucuronides. In addition, PNPG was found to be detected at *m/z* 361 as $[M+O_2]^-$, as were ACEG methyl ester, and generates O_2 adduct ion, O_2^-, just as ACEG generates O_2^-.

The retention time of methyl ester of ACEG was 4.8 min, and that of methyl ester of PNPG was 6.5 min: this allows a clear separation, as shown in Fig. 3.

Calibration curves using internal standard compounds demonstrated a good linear relationship in the range of 0.01–50 μg/ml ($r^2 = 0.9971$).

Recovery rates of ACEG were good: 107–111%. Samples containing 0.4, 2.5, and 10.0 μg/ml of ACEG were utilized (Table 2).

A quantitative method was established demonstrating higher sensitivity using PNPG as an internal standard compound, compared with an absolute calibration method.

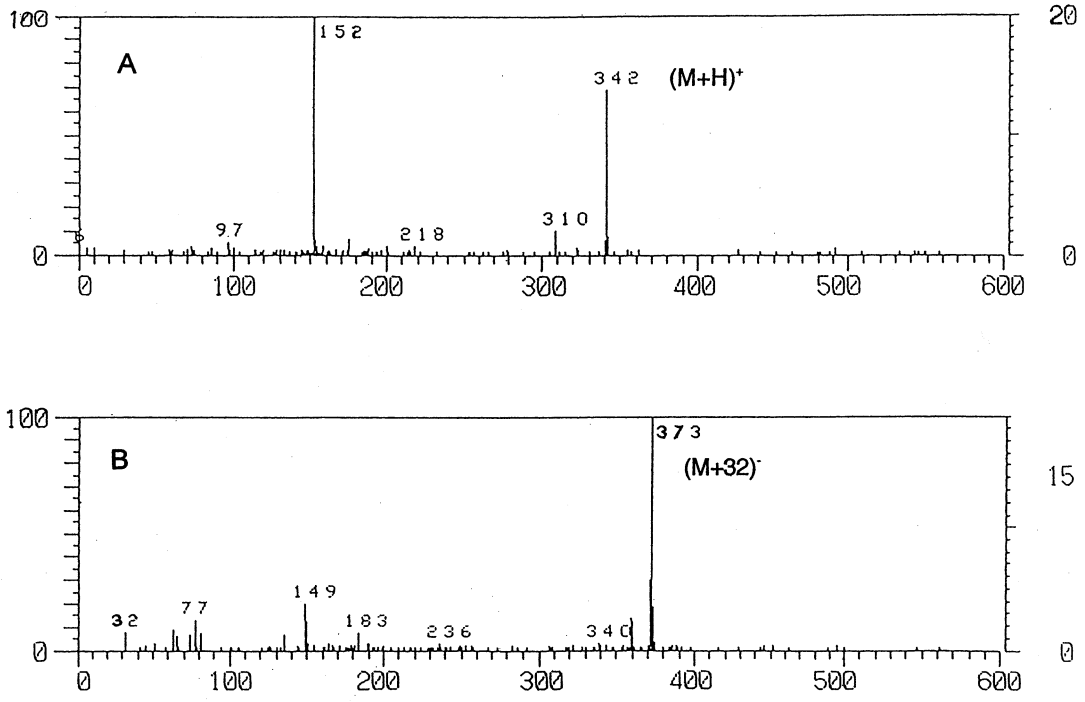

Fig. 2. Mass spectra of acetaminophen glucuronide methyl ester. (A) Positive mode, (B) negative mode.

Table 1
Ion intensity of acetaminophen glucuronide and its methyl ester by LC-MS

	Positive mode	Negative mode
Acetaminophen glucuronide	5[a]	5[b]
Acetaminophen glucuronide methyl ester	52[c]	100[d]

[a] *m/z* 328 $[M+H]^+$.
[b] *m/z* 326 $[M-H]^-$.
[c] *m/z* 342$[M+H]^+$.
[d] *m/z* 373 $[M+O2]^-$.

The detection limit at a signal-to-noise (S/N) ratio of 4 was 5 ng/ml.

4. Conclusion

ACEG, a pharmaceutical and high-polarized glucuronide conjugate, was converted into low-polarized compounds under easily achieved and mild conditions, allowing use of the LC-APCI-MS method.

ACEG was methyl-esterified in methanol solution using diazomethane ethereal solution at room temperature. As a result, the precursor ion of ACEG was observed at *m/z* 342 $[M+H]^+$ in the positive mode, and moreover, O_2 adduct ion appeared strongly at *m/z* 373 in the negative mode. The negative mode demonstrated an ion intensity approximately twice as strong as that in the positive mode. By converting ACEG into ACEG methyl ester, ion intensity could be increased 10–20-fold using the APCI-MS method. Ionization intensity did not increase despite the addition of ammonium acetate into the mobile phase. When quantitation was evaluated using the negative ion mode, ACEG displayed a good linear relationship in the range of 0.05–25.0 μg/ml. This could enable measurement by the LC-APCI-MS method under conventional conditions.

The limitation of detection was 5 ng/ml, with a S/N ratio of 4.

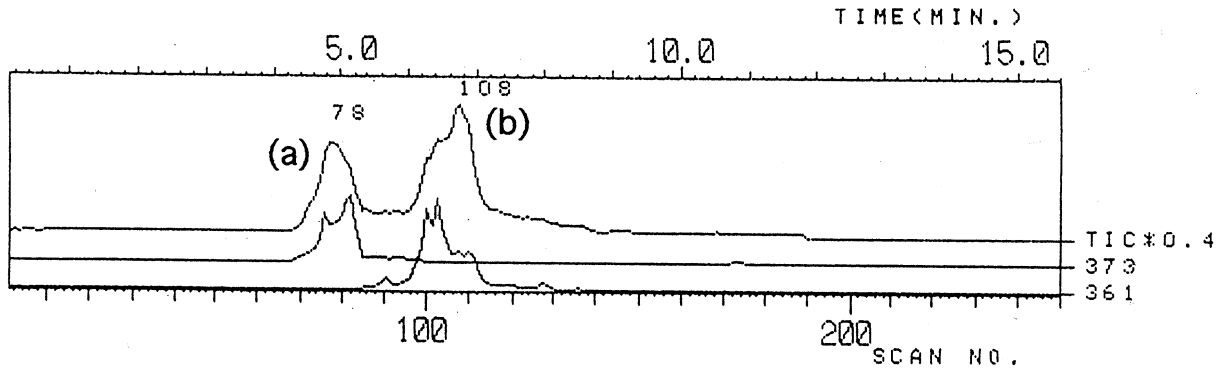

Fig. 3. Mass chromatograms of acetaminophen glucuronide (ACEG) methyl ester and *p*-nitrophenyl glucuronide (PNPG) methyl ester. (a) ACEG methyl ester (*m*/*z* 373), (b) PNPG methyl ester (*m*/*z* 361). Column: shimpack CLC-ODS (150 × 4.6 mm, i.d.); Mobile phase: MeOH:H_2O (9:1, v/v); flow rate: 1.0 ml/min. Mass conditions are described in Section 2.

Table 2
Recovery of acetaminophen glucuronide by LC-MS measurement

Acetaminophen glucuronide (μg/ml)	*p*-Nitrophenyl glucuronide[a] (μg/ml)[b]	Recovery	
		(μg/ml)[b]	(%)[b]
0.4	0.50	0.44±0.06	111±13
2.5	0.50	2.66±0.56	107±21
10.0	0.50	10.8±1.04	108±10

[a] Added as an internal standard.
[b] $n = 4$, $x \pm$S.D.

Easy methylation using diazomethane reduced the polarity of the highly-polarized compound ACEG. Furthermore, the negative mode can be used to produce O_2 adduct ions effectively using selective ionization conditions. Application of these conditions therefore allowed quantitative determination of the polar compound ACEG using the LC-APCI-MS method.

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