A Method for Quantification of Tetrahydroglucocorticoid Glucuronides in Human Urine by LC/MS/MS with Isotope-coded Derivatization

Takaaki MATSUMOTO,* Wataru YAMAZAKI,* Akihiro JO,* Shoujiro OGAWA,† Kuniko MITAMURA,** Shigeo IKEGAWA,** and Tatsuya HIGASHI††

*Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan
**Faculty of Pharmaceutical Sciences, Kindai University, 3-4-1 Kowakae, Higashi-Osaka, Osaka 577-8502, Japan
***Genmai Koso Co., Ltd., 12-1-1-1 Kita, Sapporo, Hokkaido 001-0012, Japan

To whom correspondence should be addressed.
E-mail: higashi@rs.tus.ac.jp

The determination of urinary tetrahydroglucocorticoid (THGC) glucuronides might prove helpful in the diagnosis, pathophysiological analysis and assessment of the therapeutic efficacy of the diseases caused by abnormal cortisol secretion. We developed and validated a method for the determination of the THGC glucuronides in human urine using liquid chromatography/electrospray ionization (ESI)-tandem mass spectrometry not requiring enzymatic hydrolysis. The method employed a derivatization using an ESI-enhancing reagent for carboxylic acids, 1-[(4-dimethylaminophenyl)carbonyl]piperazine (DAPPZ), and its isotopologue, 3H4-DAPPZ. The deproteinized urine samples were derivatized with DAPPZ. The 3H4-DAPPZ-derivatized standards of known amounts were then added to the DAPPZ-derivatized urine samples and served as the internal standards. The DAPPZ-derivatization enhanced the assay sensitivity and reduced the sample volume, and the use of 3H4-DAPPZ significantly improved the assay accuracy. The developed method enabled the separate quantification and profiling of the urinary THGC glucuronides and had a satisfactory application for the real sample analysis.

Keywords Tetrahydroglucocorticoid glucuronides, isotope-coded derivatization, 3H4/3H0-1-[(4-dimethylaminophenyl)carbonyl]piperazine, LC/ESI-MS/MS, urine

(Received April 27, 2018; Accepted June 1, 2018; Advance Publication Released Online by J-STAGE June 8, 2018)

Introduction

Cortisol, the main glucocorticoid in humans, plays a crucial role in many physiological processes, including metabolism (glycogenolysis, glycogen synthesis, lipolysis and protein catabolism), immune response and anti-inflammatory. Abnormal cortisol secretion leads to life-threatening conditions, such as Addison’s syndrome and Cushing’s disease. Cortisone, which is converted from cortisol by the action of 11β-hydroxysteroid dehydrogenase type 2, is the hormonally-inactive form. Cortisol and cortisone undergo extensive phase I and phase II biotransformations, which enhance their hydrophilicity in order to be easily excreted into urine. The most common phase I biotransformation of the glucocorticoids is the reduction of their α,β-unsaturated carbonyl systems to form the 3α,5β-tetrahydro-metabolites [tetrahydrocortisol (THF) and tetrahydrocortisone (THE)] or 3α,5α-tetrahydro-metabolites [allotetrahydrocortisol (ATHF) and allotetrahydrocortisone (ATHE)]. These metabolites are collectively described as tetrahydroglucocorticoids (THGCs). The most common phase II biotransformation (conjugation reaction) of the THGCs in humans is glucuronidation at the C-3 and C-21 positions, therefore, most of the THGCs are excreted into urine as the 3- or 21-monoglucuronides (3G or 21G, respectively) (Fig. 1a). The alterations in the urinary levels of the THGCs are observed under some clinical conditions; i.e., apparent mineralocorticoid excess syndrome, polycystic ovary syndrome and depression in addition to Addison’s syndrome and Cushing’s disease. The quantification of urinary THGCs is of help in the diagnosis, pathophysiological analysis and assessment of the therapeutic efficacy for these syndromes and diseases.

The THGC glucuronides in urine have been indirectly determined using gas chromatography/mass spectrometry (MS)† and more recently, liquid chromatography (LC)/tandem MS (MS/MS) after enzymatic deconjugation. However, the enzymatic deconjugation approaches have major problems; i.e., the incomplete hydrolysis (mainly due to a urine matrix effect) and loss of information about the type and position of the conjugation. Therefore, a method not requiring the enzymatic deconjugation is optimal for the quantification and profiling of the urinary THGC glucuronides. Based on this background, Ikegawa et al. developed a direct method for the quantification of the THGC glucuronides in human urine by LC/electrospray ionization (ESI)-MS/MS.
An internal standard (IS) is indispensable for the accurate and precise quantification by LC/ESI-MS/MS, because ESI efficiency can vary between two single runs due to a matrix effect, instrument drift and other technical problems even for the same analyte or same sample. A stable isotope-labeled analogue (isotopologue) of the analyte is suitable as the IS in the stable isotope dilution method, but it is practically impossible to provide stable isotope-labeled analogues for all of the THGC glucuronides. In the method of Ikegawa et al., 2H5-THE-3G, which was enzymatically synthesized from 2H5-THE with microsomal glucuronyltransferase, was used as the IS for all the examined THGC glucuronides. However, the enzyme-assisted synthetic approach also has the following problems. 1) Only a few stable isotope-labeled genins (unconjugated THGCs), which are used as starting materials, are available, and furthermore, costly. 2) Positional isomers (3G and 21G) were formed in the enzyme-assisted synthesis, therefore, a preparative HPLC step was needed to obtain the high purity IS. Although satisfactory precision and accuracy were demonstrated in a previous study, 2 only 2H5-THE-3G as the IS for all the THGC glucuronides, especially for those eluted far from the IS, was not always a good idea.

The stable isotope-coded derivatization (ICD)-based method can be an alternative to the stable isotope dilution method and correct the run-to-run ionization differences, including the matrix effect, for precise analysis. In the ICD-based method, a stable isotope-coded moiety is introduced to the multiple analytes by the derivatization and the resulting derivatives work as the ISs for the respective analytes. Specifically, the urine sample is derivatized with the H-coded reagent, while the known amounts of the THGC glucuronic acid standards are separately derivatized with the 2H-coded reagent. These two derivatized samples were mixed and injected together into the LC/ESI-MS/MS instrument. The matrix effect and ionization process for the isotopic pairs of the derivatives are expected to be identical, because they will elute at almost the same time in a single run.

We developed 1\-[(4-dimethylaminophenyl)carbonyl]piperazine (DAPPZ) as a derivatization reagent for the sensitive LC/ESI-MS/MS analysis of carboxylic acids (Fig. 1b). DAPPZ rapidly and quantitatively reacts with various carboxylic acids, such as fatty acids, bile acids, and also steroid glucuronides (unpublished data) under mild conditions. A pair of DAPPZ and its isotopologue (2H4-DAPPZ) is expected to work well as the ICD reagents for the quantification of the urinary THGC glucuronides.

Given this background, in this study, we developed and validated a method for the quantification of eight THGC glucuronides (Fig. 1a) in human urine by LC/ESI-MS/MS.
combined with the ICD. The applicability of the method was evaluated using healthy subjects’ urine samples and these results are also described.

**Experimental**

**Materials and reagents**

Authentic standards of the THGC glucuronides (cortisol-derived metabolites; THF-3G, THF-21G, ATHF-3G and cortisone-derived metabolites; THE-3G, THE-21G, ATHE-3G and ATHE-21G) were the same as used in a previous study. The THGC glucuronides were dissolved in methanol (100 μg/mL), then subsequent dilutions were carried out with methanol to prepare the standard solutions. DAPPZ and 2H4-DAPPZ were synthesized in our laboratories. All other reagents and solvents were of analytical grade or LC/MS grade.

**LC/ESI-MS/MS**

LC/ESI-MS/MS was performed using a Shimadzu LCMS-8030 triple quadrupole mass spectrometer connected to a Shimadzu LC-20AD chromatograph (Kyoto, Japan). A TSKgel ODS 100V column (3 μm, 150 × 2.0 mm i.d., Tosoh, Tokyo, Japan) was used at the flow rate of 0.2 mL/min and at 40°C. The mobile phase of acetonitrile–10 mM ammonium formate (3:7, v/v) was used (isocratic elution) for the urine sample analysis. The derivatized THGC glucuronides were analyzed in the positive-ion mode. The MS/MS conditions were as follows: interface voltage, 4.5 kV; Q1 pre-rod bias voltage, –20 V; Q3 pre-rod bias voltage, –11 V (for cortisol-derivatized metabolites) or –16 V (for cortisone-derivatized metabolites); nebulizer gas (N2) flow rate, 3 L/min; drying gas (N2) flow rate, 15 L/min; desolvation line temperature, 250°C; heat block temperature, 400°C; collision energy, 49 eV (for cortisol-derivatized metabolites) or 46 eV (for cortisone-derivatized metabolites) and collision gas (Ar), 230 kPa. The SRM transitions (precursor and product ions) were m/z 758.4 → 148.1 (DAPPZ-derivatized cortisol-derivatized metabolites), m/z 762.5 → 152.1 (H3-DAPPZ-derivatized cortisol-derivatized metabolites), m/z 756.3 → 148.1 (DAPPZ-derivatized cortisone-derivatized metabolites) and m/z 760.4 → 152.1 (H3-DAPPZ-derivatized cortisone-derivatized metabolites). LabSolutions software (Ver. 5.53 SP3, Shimadzu) was used for the system control and data processing.

**Effect of derivatization for detection response**

The effect of the DAPPZ-derivatization for the detection response was evaluated based on the amounts of intact or derivatized THGC glucuronides per injection giving a signal-to-noise ratio (S/N) of 5. Four THGC glucuronides (cortisol-derivatized metabolites; THF-3G, THF-21G, ATHF-3G and ATHF-21G) were examined as the model compounds. The S/N value was manually calculated by division of the peak height of the intact or derivatized THGC glucuronides by the noise level around the peak. The four THGC glucuronides (100 pg each) were derivatized with DAPPZ and the resulting derivatives were dissolved in the mobile phase (acetonitrile–10 mM ammonium formate (3:7, v/v; 100 μL)), then subjected to the LC/ESI-MS/MS. By stepwise decreasing the injection volume of the resulting solution, the amounts giving a S/N of 5 were determined. The amounts giving a S/N of 5 for the intact THGC glucuronides were determined using a 1.0-ng/mL solution in the same way. The intact THGC glucuronides were analyzed in the negative-ion mode. The MS/MS conditions were as follows: interface voltage, 4.5 kV; Q1 pre-rod bias voltage, 20 V; Q3 pre-rod bias voltage, 16 V; collision energy, –53 eV; nebulizer gas flow rate, 3 L/min; drying gas flow rate, 15 L/min; desolvation line temperature, 250°C; heat block temperature, 400°C and collision gas, 230 kPa. The SRM transitions were m/z 541.3 [M–H] → 85.1. The mobile phases were selected so that the retention times (tR) of the intact THGC glucuronides were almost the same as those of the DAPPZ derivatives; the mix ratio (v/v) of acetonitrile and 10 mM ammonium formate was 3:17 for THF-3G, 2:9 for THF-21G and ATHF-21G, and 3:19 for ATHF-3G.

**Collection and pretreatment of the urine sample**

Urine samples were collected from healthy male subjects and stored at −18°C until used. The subjects understood the purpose and significance of this experiment and donated their urine after signing an agreement.

After thawing, the urine samples were centrifuged at 1000g for 5 min to precipitate the solids. The supernatant (10 μL) was added to acetonitrile (90 μL), vortex-mixed for 30 s and centrifuged at 1000g for 5 min. An aliquot (50 μL) of the resulting supernatant was evaporated and the residue was derivatized with DAPPZ. The mixture of standard THF-3G (200 ng), THF-21G (300 ng), ATHF-3G (150 ng), THE-3G (400 ng), THE-21G (100 ng), ATHE-3G (20 ng) and ATHE-21G (20 ng) were derivatized with H3-DAPPZ, and one-twentieth of the mixed derivatives was added to the DAPPZ-derivatized urine sample. The solvent was evaporated from the mixture, and the residue was dissolved in the mobile phase (60 μL), 15 μL of which was injected into the LC/ESI-MS/MS.

**Derivatization**

To the standard THGC glucuronides or the pretreated urine sample, solutions of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) in ethanol (10 mM, 40 μL) and DAPPZ (or H3-DAPPZ) in ethanol (1 mg/mL, 40 μL) were successively added, and the mixture was stored at room temperature for 5 min.

**Calibration curves**

The THGC glucuronide-free urine sample was prepared by the absorptive removal of the glucuronides with charcoal; the urine (15 mL) was stirred with the charcoal (1.5 g, Norit®, Nacalai Tesque, Kyoto) for 15 h, then the charcoal was removed by centrifugation at 2000g for 10 min. The calibration curves were constructed with seven points for each THGC glucuronide (Table S1, Supporting Information). The THGC glucuronide-free urine sample (10 μL) was added to acetonitrile (90 μL) containing the standards of THF-3G (0.80, 2.0, 8.0, 20, 40, 100 or 400 ng, corresponding to 0.080, 0.20, 0.80, 2.0, 4.0, 10 or 40 μg/mL, respectively), THF-21G (1.2, 3.0, 12, 30, 60, 150 or 600 ng, corresponding to 0.12, 0.30, 1.2, 3.0, 6.0, 15 or 60 μg/mL, respectively), mixture of ATHF-3G and ATHF-21G (59:1) (0.60, 1.5, 6.0, 15, 30, 75 or 300 ng, corresponding to 0.060, 0.15, 0.60, 1.5, 3.0, 7.5 or 30 μg/mL, respectively), THE-3G (1.6, 4.0, 16, 40, 80, 200 or 800 ng, corresponding to 0.16, 0.40, 1.6, 4.0, 8.0, 20 or 80 μg/mL, respectively), THE-21G (0.40, 1.0, 4.0, 10, 20, 50 or 200 ng, corresponding to 0.040, 0.10, 0.40, 1.0, 2.0, 5.0 or 20 μg/mL, respectively), ATHE-3G (0.080, 0.20, 0.80, 2.0, 4.0, 10 or 40 ng, corresponding to 0.0080, 0.020, 0.080, 0.20, 0.40, 1.0 or 4.0 μg/mL, respectively) and ATHE-21G (0.080, 0.20, 0.80, 2.0, 4.0, 10 or 40 ng, corresponding to 0.0080, 0.020, 0.080, 0.20, 0.40, 1.0 or 4.0 μg/mL, respectively), then the resulting samples were pretreated and derivatized with DAPPZ as previously described. The standards of THF-3G (200 ng), THF-21G (300 ng), ATHF-3G (150 ng), THE-3G (400 ng), THE-21G (100 ng), ATHE-3G...
(20 ng) and ATHE-21G (20 ng) were derivatized with $^3$H$_2$-DAPPZ, and one-twentieth of the derivatives was added to the DAPPZ-derivatized urine sample. The solvent was evaporated from the mixture, and the residue was dissolved in the mobile phase (60 μL), 15 μL of which was injected into the LC/ESI-MS/MS. The peak area ratio (DAPPZ derivative/THGC-DAPPZ derivative) ($y$) was plotted versus the concentration of each THGC glucuronide (μg/mL) ($x$) with a weighting of 1/$x$ to construct the calibration curve.

Assay precision and accuracy

The intra- ($n = 5$) and inter-assay ($n = 5$) precisions were assessed by the repeated measurement of two urine samples (A and B) on one day and over five days, respectively. The precision was determined as the relative standard deviation (RSD, %).

The assay accuracy was examined using urine samples C and D. The urine samples (10 μL) were added to acetonitrile (90 μL) containing the THGC glucuronides whose amounts are shown in Table S3 (Supporting Information), then pretreated in the same way as previously described (spiked sample). The $^3$H$_2$-DAPPZ-derivatized THGC glucuronides were added to the samples as previously described. The assay accuracy was defined as $F(F_0 + X) \times 100$ (%), where $F$ is the concentration of the THGC glucuronides in the spiked sample, $F_0$ is the concentration of the THGC glucuronides in the intact sample and $X$ is the spiked concentration.

Matrix effect

The matrix effect was examined in a post-extraction addition experiment. Standard sample: a mixture of THF-3G (20 ng) and ATHE-21G (20 ng) was derivatized with DAPPZ or $^3$H$_2$-DAPPZ and dissolved in the mobile phase (60 μL). Matrix sample: the urine sample (10 μL) was added to acetonitrile (90 μL), vortex-mixed and centrifuged as previously described. The supernatant (50 μL) was transferred to another tube and evaporated. The above standard sample (DAPPZ derivatives or $^3$H$_2$-DAPPZ derivatives) was added to this residue (urine matrix) to prepare the matrix sample, and finally dissolved in the mobile phase (60 μL). The ratios of the peak areas of the DAPPZ- or $^3$H$_2$-DAPPZ-derivatized THF-3G and ATHE-21G in the matrix sample to those in the standard sample were determined as the matrix effect.

Results and Discussion

ESI-MS/MS behavior of DAPPZ- and $^3$H$_2$-DAPPZ-derivatized THGC glucuronides

DAPPZ and $^3$H$_2$-DAPPZ have a promising characteristic as ICD reagents. Because the reagents possess a high proton-affinitive moiety, the $N,N$-dimethylamino (calculated proton-affinity: 941 kJ/mol),$^{19}$ the resulting derivatives will be highly responsive in the positive ESI-MS. The reagents rapidly and quantitatively react with various carboxylic acids,$^{16,17}$ including steroid glucuronides, in the presence of a condensation agent (DMT-MM) to form amide derivatives (Fig. 1b). Moreover, the dimethylamino group is the right moiety to introduce $^3$H atoms by reductive amination using $^3$H-acetaldehyde.$^{19}$

The DAPPZ-derivatized THGC glucuronides gave intense protonated molecules ([M+H]$^+$) at $m/z$ 758 or 756 for the cortisol- or cortisone-derived metabolites, respectively, as the base peaks in the positive ESI-MS. These protonated molecules provided the characteristic product ion ([C$_9$H$_{10}$NO]$^+$) at $m/z$ 148, which was formed by cleavage of the amide bond in the DAPPZ moiety, by collision induced dissociation (Fig. 1b). This fragmentation pattern was quite identical to those observed in the DAPPZ derivatives of fatty acids$^{18}$ and bile acids.$^{17}$ Based on these results, the selected reaction monitoring (SRM) mode using the transition of [M+H]$^+$ (m/z 758 or 756) → [C$_9$H$_{10}$NO]$^+$ (m/z 148) was employed for the detection and quantification of the urinary THGC glucuronides as the DAPPZ derivatives.

The isotopic purity of $^3$H$_2$-DAPPZ was determined by LC/ESI-MS (selected ion monitoring) after it was reacted with THF-3G. The peak area of the $^3$H$_2$-form (m/z 762.5) was 98.5% of the total, that of the $^3$H$_1$-form was only 1.5%, and the $^3$H$_0$-, $^3$H$_2$-, and $^3$H$_3$-forms were not detected at all. These results demonstrated that the isotopic purity of $^3$H$_2$-DAPPZ was greater than 98.5% and sufficient for use.

The $^3$H$_2$-DAPPZ-derivatized THGC glucuronides showed a similar fragmentation pattern ([M+H]$^+$ → [C$_9$H$_{10}$NO]$^+$) during MS/MS. When an equal amount of THF-3G was derivatized with DAPPZ or $^3$H$_2$DAPPZ, then the resulting derivatives were mixed and subjected to LC/ESI-MS/MS. As a result, the peak areas of both derivatives were almost equal, which demonstrated that the two DAPPZ isotopologues equally reacted with the glucuronide and the ESI-efficiencies of the DAPPZ and $^3$H$_2$-DAPPZ derivatives were almost equal.

Effect of derivatization for detection response

The amounts giving peaks with a S/N of 5 were 13 fmol on the column for all the examined DAPPZ derivatives (Table 1); there was no significant difference in the detection response between the stereoisomers (SαSβ) or positional isomers (3G/21G). On the other hand, those of the intact glucuronides measured in the negative-ion mode ([M–H]$^-$ → m/z 85.1) were 65, 200, 110 and 150 fmol for THF-3G, THF-21G, ATHF-3G and ATHF-21G, respectively. The detection responses of the DAPPZ-derivatized THGC glucuronides were 5 - 16-fold higher than the intact glucuronides. The mobile phase with the higher organic solvent content was used for the DAPPZ derivatives. Such a mobile phase is suitable for the generation of charged droplets by electrospray and produces a higher ESI-MS response.$^{20}$ Thus, the DAPPZ-derivatization enhanced the detectability of the glucuronides, which was of help in reducing the sample volume. The previous method required a large volume sample (50 μL) and the purification step using solid-phase extraction cartridge.$^{2}$ The reduction of the sample volume was conductive to the reduction of the matrix effect as will be discussed later.

| Table 1 Detection responses of intact and DAPPZ-derivatized THGC glucuronides |
|----------------|-------|---------|--------|----------|
| Mobile phase | $t_R$ min | S/N = 5 fmol | Increasing response |
| THF-3G (intact) | 3.17 | 16.5 | 65 | 1 |
| THF-3G-DAPPZ | 3.7 | 17.1 | 13 | 5 |
| THF-21G (intact) | 2.9 | 27.0 | 200 | 1 |
| THF-21G-DAPPZ | 3.7 | 25.2 | 13 | 16 |
| ATHF-3G (intact) | 3.19 | 23.5 | 110 | 1 |
| ATHF-3G-DAPPZ | 3.7 | 23.3 | 13 | 9 |
| ATHF-21G (intact) | 2.9 | 24.2 | 150 | 1 |
| ATHF-21G-DAPPZ | 3.7 | 23.3 | 13 | 11 |

a. Mixture of acetonitrile and 10 mM ammonium formate (v/v).
b. The injection amounts producing a peak with a S/N of 5.
c. The detection responses of intact glucuronides are taken as 1.

ANALYTICAL SCIENCES SEPTEMBER 2018, VOL. 34
Separation of DAPPZ-derivatized THGC glucuronides

We examined several LC columns [TSKgel ODS-100V (5 μm, 150 × 2.0 mm i.d.), J’spher ODS-H80 (4 μm, 150 × 2.0 mm i.d., YMC) and YMC Pack Pro C18 RS (4 μm, 150 × 2.0 mm i.d., YMC)] including ones packed with superficially porous particles [Ascentis Express C18 (5 μm, 150 × 2.0 mm i.d., Sigma-Aldrich) and Sun Shell C18 (2.6 μm, 100 × 2.1 mm i.d., ChromaNik Technologies)] and mobile phases [combination of two organic modifiers (methanol and acetonitrile) and two additives (ammonium formate and formic acid)] to obtain the better separation and peak shapes of the DAPPZ derivatives. We found that the following condition was optimal; the TSKgel ODS-100V (5 μm, 150 × 2.0 mm i.d.) column was used with the mobile phase of acetonitrile –10 mM ammonium formate (3:7, v/v). However, even under this condition, the DAPPZ derivatives of ATHF-3G and ATHF-21G co-eluted and therefore, these two glucuronides were quantified by adding them together as the ATHF-G. Although we cannot explain why ATHF-3G and ATHF-21G co-eluted as the DAPPZ derivatives, the inability of separate quantitation of these two positional isomers is a drawback of our new method.

Pretreatment of urine

Our method required only deproteinization for the urine pretreatment. Chromatograms of the urine sample collected from a healthy subject are shown in Fig. 2. The sample was analyzed after spiking the 2H₄-DAPPZ-derivatized THGC glucuronide standards. By using a diversion valve, the LC eluent entered the mass spectrometer from 15 to 30 min after injection of the sample. As previously described, the derivatized ATHF-3G and ATHF-21G were not separated, therefore, seven peaks of the derivatized THGC glucuronides were observed. The 2H₄-DAPPZ derivatives always eluted slightly earlier (0.2 – 0.4 min) than the DAPPZ derivatives during the reversed
phase LC. It is noted that the \textsuperscript{3}H-coded derivatives generally have weaker hydrophobic interactions with the stationary phase than their H-coded counterparts and elute earlier during the reversed-phase LC.\textsuperscript{21} This deuterium isotope effect had a slightly undesirable effect on the quantification of the THGC glucuronides. These data proved that the use of the \textsuperscript{2}H\textsubscript{4}-DAPPZ derivatization method proposed here is advantageous over \textsuperscript{1}H-coded derivatives and \textsuperscript{1}H-coded derivatives.

\section*{Method validation}

The calibration curves showed a satisfactory linearity with the determination coefficients ($r^2$) of greater than 0.997 for all the glucuronides (Table S1, Supporting Information). As previously mentioned, the derivatized ATHF-3G and ATHF-21G were not chromatographically separated in spite of our efforts. Therefore, ATHF-3G and ATHF-21G were mixed with the ratio of 59:1 and the calibration curve was made for ATHF-G. The mix ratio (59:1) was determined on the basis of the literature.\textsuperscript{2} The reproducible calibration curves were obtained as the RSD of the slopes of five curves constructed using five different urine samples were below 7\% (Table S1, Supporting Information). As shown in Table 2, with the ICD technique, the quantification accuracy was significantly enhanced at the lowest calibration points (lower limits of quantification). When the peak areas (absolute values, $y$) were plotted versus the concentrations of the THGC glucuronides ($\mu$g/mL, $x$) for the absolute calibration method (without the ICD technique), high relative errors (REs) of the back-calculated concentrations were found; the REs were over –10\% for most of the glucuronides. On the other hand, the REs of the ICD-based method were within ±10\% for all the glucuronides. These data proved that the use of the \textsuperscript{3}H\textsubscript{2}-DAPPZ derivatives worked well in enhancing the quantification accuracy at the low concentration levels.

To examine the matrix effect, the post-extraction addition experiments were performed. The responses of the DAPPZ-derivatized THF-3G and ATHE-21G of the matrix samples were 102.9 ± 2.6 and 103.8 ± 3.3\% of those of the standard samples, respectively. The responses of the \textsuperscript{3}H\textsubscript{2}-DAPPZ derivatives in the matrix samples were 103.8 ± 1.3 and 101.4 ± 1.4\% of those of the standard samples for THF-3G and ATHE-21G, respectively. Thus, the matrix effect was negligible. As previously described, the proposed method requires only a 10-\muL urine sample, which produced a minimal matrix effect result.

\section*{Determination of THGC glucuronides in the urine of healthy subjects}

To prove the applicability of the ICD-based method for the urine sample analyses, the determination of the THGC glucuronides in the urine of healthy male subjects was performed. Although the absolute concentrations of the THGC glucuronides in the urine are different from sample to sample, the percent of each THGC glucuronide to the sum of eight THGC glucuronides (the relative concentrations) showed little

\begin{table}[h]
\centering
\caption{Accuracy at the lowest calibration points (lower limits of quantification)}
\begin{tabular}{|c|c|c|c|c|}
\hline
Nominal concentration/\mu g mL\textsuperscript{-1} & ICD method & Absolute calibration method \\
\hline
THF-3G 0.080 & 0.075 ± 0.008 & –6.3 & 0.070 ± 0.011 & –12.5 \\
THF-21G 0.12 & 0.13 ± 0.01 & 8.3 & 0.11 ± 0.01 & –8.3 \\
ATHF-G 0.060 & 0.062 ± 0.007 & 3.3 & 0.050 ± 0.007 & –16.6 \\
THE-3G 0.16 & 0.15 ± 0.02 & –6.3 & 0.13 ± 0.02 & –18.8 \\
THE-21G 0.040 & 0.038 ± 0.004 & –5.0 & 0.036 ± 0.006 & –10.0 \\
ATHE-3G 0.0080 & 0.0073 ± 0.0007 & –8.8 & 0.0069 ± 0.0006 & –13.8 \\
ATHE-21G 0.0080 & 0.0079 ± 0.0008 & –1.3 & 0.0072 ± 0.0010 & –10.0 \\
\hline
\end{tabular}
\begin{flushleft}
\textsuperscript{a} Mean ± SD ($n = 5$). b. RE (relative error) = (back-calculated concentration – nominal concentration)/nominal concentration × 100 \%.
\end{flushleft}
\end{table}

\begin{table}[h]
\centering
\caption{Concentrations of THGC glucuronides in urine of five healthy male subjects}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Subject} & \textbf{THF-3G} & \textbf{THF-21G} & \textbf{ATHF-G} & \textbf{THE-3G} & \textbf{THE-21G} & \textbf{ATHE-3G} & \textbf{ATHE-21G} \\
\hline
No. 1 & \textbf{Concentration/\mu g mL\textsuperscript{-1}} & 2.43 & 6.02 & 8.86 & 9.23 & 8.54 & 0.44 & 0.60 \\
No. 2 & \textbf{Percent\textsuperscript{b}} & 6.7 & 16.7 & 24.5 & 25.6 & 23.6 & 1.2 & 1.7 \\
No. 3 & \textbf{Concentration/\mu g mL\textsuperscript{-1}} & 9.8 & 15.6 & 18.3 & 37.0 & 17.2 & 1.3 & 0.7 \\
No. 4 & \textbf{Percent\textsuperscript{b}} & 12.9 & 17.2 & 22.4 & 26.3 & 18.6 & 1.6 & 0.9 \\
No. 5 & \textbf{Concentration/\mu g mL\textsuperscript{-1}} & 3.62 & 8.13 & 9.60 & 11.07 & 7.41 & 0.80 & 0.46 \\
\hline
\textbf{Mean ± SD} & \textbf{Concentration/\mu g mL\textsuperscript{-1}} & 2.55 & 4.83 ± 0.45 & 8.24 & 9.35 ± 0.38 & 7.43 & 0.45 ± 0.21 & 0.33 ± 0.19 \\
\hline
\end{tabular}
\begin{flushleft}
a. Sum of ATHF-3G and ATHF-21G. b. The percentage of each THGC glucuronide to the sum of eight THGC glucuronides.
\end{flushleft}
\end{table}
difference between the samples (Table 3). Both the concentrations and percentages determined by our new ICD-based method were generally consistent with the previous results. The sum concentration of THF-3G and -21G was lower than that of THE-3G and -21G. In contrast, the sum concentration of ATHF-3G and -21G was significantly higher (about 10-fold) than that of ATHE-3G and -21G. Interestingly, the concentration of THF-21G was higher than that of its positional isomer (3G), whereas the concentration of THE-21G was lower than that of THE-3G. The differences in the main conjugation position between the tetrahydro-metabolites may be due to the substrate specificity of the glucuronyltransferase. Thus, the developed method could be applied without difficulties due to the substrate specificity of the glucuronyltransferase.

Conclusions

In this study, we developed and validated an LC/ESI-MS/MS method for the simultaneous quantification of the THGC glucuronides in human urine. The method employed the ICD to overcome the difficulties that previous methods had; i.e., the limited availability of the ISs, the required enzymatic deconjugation step and the impossible separate quantification of the positional isomeric glucuronides. The ICD using the paired reagents, DAPPZ and \( ^{2}H_{4}\)-DAPPZ, enabled the sensitive, accurate and separate quantification of the urine THGC glucuronides. In the pilot study using the healthy subjects’ urine, the method enabled the quantitative profiling of the THGC glucuronides as intended. This well-characterized method will prove helpful in the diagnosis, pathophysiological analysis and assessment of the therapeutic efficacy of the diseases caused by abnormal cortisol secretion.

Acknowledgements

This study was supported in part by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant No. 17K08250.

Supporting Information

Calibration curves, assay precision, assay accuracy and stability data are available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References