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Original Article

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Development and validation of a method for the simultaneous quantification of endogenous steroids metabolized by CYP3A

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ABSTRACT

Cytochrome P450 (CYP) 3A enzymes, the most important phase 1 drug-metabolizing enzymes, are responsible for 50% of the metabolism of clinically used drugs. CYP3A activity varies widely among individuals, which can affect the probability of adverse drug reactions and drug-drug interactions mediated by the induction or inhibition of the enzyme. Hence, it is important to be able to predict CYP3A activity in individuals to reduce the incidence of unexpected drug responses. To specifically and quickly measure CYP3A activity, we developed method based on gas chromatography interfaced with triple-quadrupole mass spectrometry for the quantification of cortisol, cortisone, 6β-hydroxycortisol, and 6β-hydroxycortisone simultaneously in urine and 4β-hydroxycholesterol in plasma. The results were calculated based on charcoal-stripped steroid-free urine and plasma control samples. The accuracy and precision were 93.18% to 110.0% and 1.96% to 5.34%, respectively. This method was then applied to measure endogenous steroids from urine and plasma samples of healthy Korean males and females. The calibration curves of all analytes showed good linearity with a correlation coefficient (r^2) that ranged from 0.9953 to 0.9999. Therefore, this validated method can be used to measure endogenous biomarkers to predict CYP3A activity and might be applicable in the prediction of CYP3A-mediated drug interactions of new drug candidates.

Keywords: Cytochrome P-450 CYP3A; Metabolomics; Biomarkers; Gas Chromatography; Mass Spectrometry

INTRODUCTION

Cytochrome P450 (CYP) 3A enzymes, the most important phase 1 drug-metabolizing enzymes in the liver and intestines, are responsible for 50% of the metabolism of clinically used drugs [1-3]. Although CYP3A is involved in the detoxification of drugs, in many cases, CYP3A leads to adverse drug reactions (ADRs) and drug-drug interactions (DDIs) mediated by the induction or inhibition of the enzyme [4-7]. Thus, it is necessary to reduce ADRs and DDIs by phenotyping CYP3A activity. CYP3A activity exhibits a large variation among

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individuals, owing to the expression of CYP3A4 and CYP3A5 isoforms (dominant CYP3A isoforms) [8], and the activity is regulated by genetic variants, endogenous metabolites, and co-administered drugs [6,7,9]. However, although CYP3A4 and CYP3A5 have several genetic variants, including *CYP3A4*22* and *CYP3A5*3*, they have a minor effect on CYP3A activity, unlike external factors, such as CYP3A inducers and inhibitors, which have a major effect on the activity [4,5,10,11]. Thus, genetic variants alone make it difficult to predict CYP3A activity. Biomarkers other than genetic variants are required to predict individual CYP3A activity and decrease the incidence of unexpected CYP3A-mediated drug reactions and determine the inhibitory or inducible characteristic of a new drug.

Studies have been performed to predict CYP3A activity using experimental and pharmacokinetic modeling [12-14]. However, most studies used probe drugs, such as midazolam, triazolam, and simvastatin. To avoid unnecessary drug exposure, the prediction of the CYP3A activity of an individual using endogenous biomarkers is ideal [1,8,15]. Because CYP3A is involved in the hydroxylation of endogenous steroids, such as cortisol, cortisone, and cholesterol, the metabolic ratio (the ratio of metabolite/steroid) and concentration values have been reported as endogenous biomarkers for CYP3A activity [8,16-19]. Thus, we have continuously evaluated CYP3A activity in diverse populations using the endogenous biomarkers in urine and plasma samples collected using a relatively non-invasive method [8,20-22]. However, the analysis of CYP3A activity requires a lot of time per sample and a large amount of sample (2 mL) [15,22]. Therefore, we sought to develop a new method to specifically and quickly measure CYP3A activity using a small amount of sample [23]. This study validated the use of this fast, efficient, and sensitive method to measure CYP3A activity using gas chromatography interfaced with triple-quadrupole mass spectrometry (GC-MS/MS).

METHODS

Chemicals and reagents

Steroids measured in this study were as follows: cortisol, cortisone, 6β-hydroxycortisol, 6β-hydroxycortisone, and 4β-hydroxycholesterol (**Fig. 1**). All steroids listed above and internal standards, cortisol-9,11,12,12-d_4, cortisone-2,2,4,6,6,9,12,12-d_8, 6β-hydroxycoltisol-6,11,17,21-d_4, 4β-hydroxycholesterol-d_7 were from Sigma-Aldrich (St. Louis, MO, USA), Santa Cruz Biotechnology (Dallas, TX, USA), or Steraloids (Newport, RI, USA). Activated charcoal was from Sigma-Aldrich. The Oasis HLB cartridges were obtained from Waters Corp. (Milford, MA, USA) and 1M ethanolic potassium hydroxide were purchased from Honeywell Fluka™ (Morris Plains, NJ, USA). All organic solvents (in detail, ethyl acetate, hexane…) of analytical or HPLC grade were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). *Escherichia coli* β-glucuronidase was purchased from Roche Diagnostics (Basel, Switzerland). For derivatization, N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE) were purchased from Sigma-Aldrich. Mixed stock solutions of all reference standards were prepared at a concentration of 100 μg/mL in methanol.

Charcoal-stripped control urine and plasma samples

The charcoal-stripped control samples for calibration curve and quality control (QC) were prepared as steroids-free urine and plasma. Pooled urine and plasma samples (40 mL) were mixed with activated charcoal (0.8 g), respectively and these were shaken for overnight at 4°C, followed by centrifugation at 1977 RCF for 15 minutes. The supernatant was filtered at 0.22-

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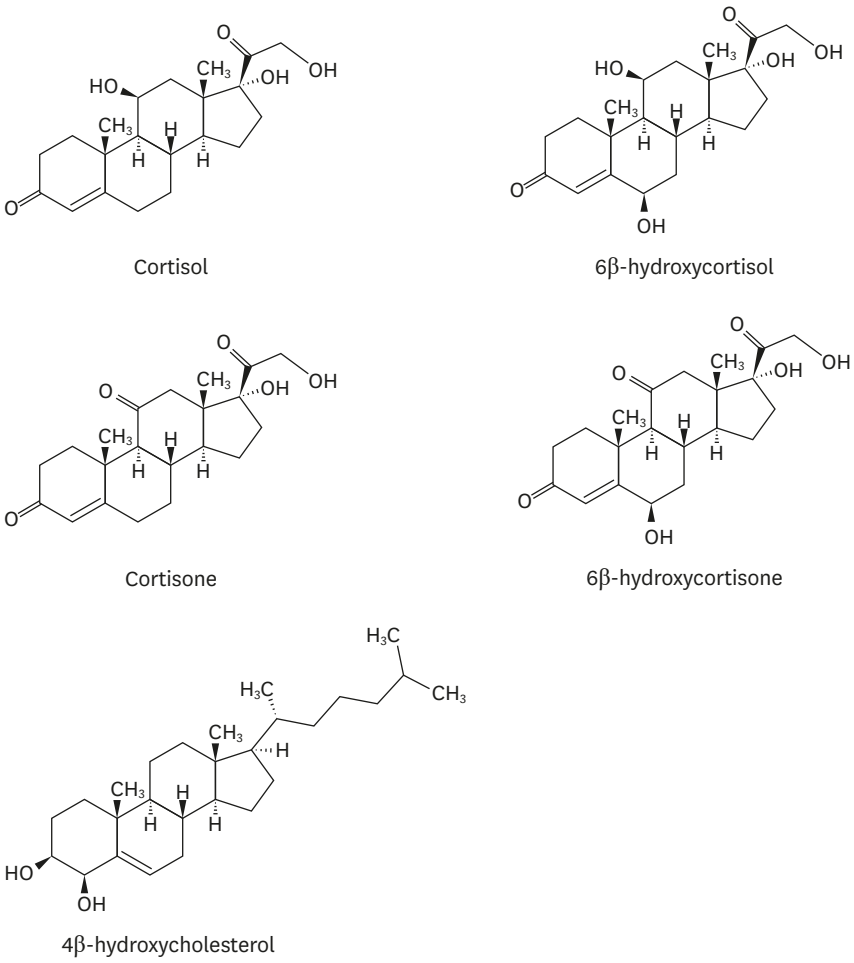

Figure 1. Structures of endogenous steroids.

μm polyethersulfone membrane filter. Purified urine and plasma were transferred to tubes and stored at −80°C until use.

Sample preparation

For urine sample, 1 mL of each samples were spiked with 40 μL of the IS mixtures (cortisol-d_4, cortisone-d_8, and 6β-hydroxycoltisol-d_4 2.5 μg/mL). Subsequently, steroids were extracted from the samples by solid-phase extraction using Oasis HLB cartridges. The eluates were dried with an N_2 evaporator and resuspended in 500 μL of 0.2 M sodium phosphate buffer (pH 7.2) and 25 μL of β-glucuronidase to hydrolyze steroid conjugates (e.g. cortisol glucuronide) into unconjugated form, which is used to accurately calculate metabolic ratios (6β-hydroxycortisol/cortisol, 6β-hydroxycortisone/cortisone). After incubation at 55°C for 1 hour, the solution was extracted with 2.5 mL of ethyl acetate:n-hexane (2:3, v/v) (liquid-liquid extraction). The organic solvents were evaporated using a N_2 evaporator at 37°C and dried in a vacuum desiccator with P_2O_5-KOH for at least 1 hour. Finally, the dried residue was derivatized with 80 μL of mixture of MSTFA, NH_4I, and DTE (200:2:1, v/w/w) at 60°C for 20 minutes. Three microliters of the derivatized sample was injected for GC-MS analysis in the multiple reaction monitoring (MRM) mode.

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For plasma sample, 50 μL of each sample was spiked with 20 μL of 4β-hydroxycholesterol-d_7 (IS, 500 ng/mL) and saponified with 500 μL of 1 M ethanolic potassium hydroxide for 30 minutes at 37°C to cleave ester bonds and make free form of 4β-hydroxycholesterol. After saponification, the samples were added with 300 μL of water and extracted twice with 1 mL of hexane per time. The extracted samples were dried with a N_2 evaporator at 37°C and in a vacuum desiccator with P_2O_5-KOH for at least 1 hour. Finally, the dried residue was derivatized with 80 μL of mixture of MSTFA, NH_4I, and DTE (200:2:1, v/w/w) at 60°C for 20 minutes. Three microliters of the derivatized sample was injected for GC-MS analysis in the MRM mode.

Instrumental conditions

An Agilent 7890B gas chromatograph interfaced with an Agilent 7000 triple-quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA). Electron impact ionization (70 eV) at MRM mode was used, with the ion source temperature at 250°C. Three microliters of each sample were injected in splitless mode at 280°C and separated through a Agilent 19091A-102 ULTRA 1 (25 m × 200 μm × 0.33 μm) column. Grade 5 (99.999% purity) Helium gas was used as a carrier gas, with the constant flow rate of 0.8 mL/min. A temperature gradient was given for sample analysis. The GC oven temperature was maintained at 70°C for 2 minutes, then increased to 320°C at a rate of 20°C/min and held for 5 minutes. For quantitative analysis, the characteristic ions of steroids and internal standard were determined as their trimethylsilylation derivatives in the MRM mode (**Table 1**).

Partial validation

Partial validation constituted evaluation of the accuracy, precision, linearity, stability, and carry-over using calibration and QC samples. Calibration standards were prepared in eight different concentrations ranging from 5 to 500 ng/mL by dilution with methanol. The QC samples (lower limit of quantification [LLOQ], low, medium, high) were prepared to 5, 15, 150, and 375 ng/mL in urine sample and 5, 15, 80, and 350 ng/mL in plasma sample, respectively. The range of the calibration standards used in this method was determined based on previous reports to cover the levels of endogenous steroids. All samples were quantified based on the ratio of peak area to IS peak area. The validation results comprised accuracy, precision as coefficient of variation (% CV), calibration linearity (r^2), and carry-over for 3 replicates over 3 different days. In addition, auto-sampler stability (short-term stability) of the analytes was assessed by injecting the prepared QC samples stored for 10 hours in an auto-sampler.

Table 1. Retention times and analytical parameters for quantification of the endogenous steroids and internal standards

Compound name*	Precursor ion (m/z)	Product ion (m/z)	Qualifier ion (m/z)	Retention time (min)	Collision energy (eV)
Cortisol	632.3	234.2	245.2	17.57	30
Cortisone	615.2	243.2	227.2	16.91	35
6β-hydroxycortisol	720.4	317.2	217.1	18.30	40
6β-hydroxycortisone	703.2	407.2	331.1	17.50	30
Cortisol-d_4	636.3	235.2	191.1	17.59	40
Cortisone-d_8	621.2	247.2	284.2	16.89	20
6β-hydroxycortisol-d_4	724.4	319.2	218.2	18.29	45
4β-hydroxycholesterol	366.4	158	211.1	17.40	20
4β-hydroxycholesterol-d_7	373.5	158.1	143.1	17.36	20

*All compounds were derivatized with the N-methyl-N-(trimethylsilyl) trifluoroacetamide, ammonium iodide, and dithioerythritol (200:2:1, v/w/w).

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Subjects and sample collection

All urine and plasma samples were provided by biobank. These were collected from 30 healthy Korean males and females who agreed to consent forms approved by the Institutional Review Board (IRB) of Seoul National University Bundang Hospital (Seoul, Republic of Korea). The IRB registry number is B-1802/453-002. The samples were stored at −80°C freezer until use.

RESULTS

Optimization of GC-MS/MS analysis

For the GC-MS/MS analysis, the spectra of the scan mode (precursor ion), product ion of all analytes, qualifier ion, retention time, and collision energy are shown in **Table 1**. The GC-MS/MS chromatograms of the five endogenous steroids are shown in **Fig. 2**. The analytical time per sample was shortened to 20 minutes and sample volume required for analysis reduced to 1 mL.

Calibration and linearity

The calibration curve consisted of a blank (matrix sample prepared without an internal standard), zero (matrix sample prepared with an internal standard), and eight different concentrations of standards (STD1–STD8) in the expected range. Eight-point calibration curves ($1/x^2$ weighting) were generated for five steroids. Good linearity was achieved for all compounds analyzed with a correlation coefficient (r^2) ranging from 0.9969 to 0.9998 (**Table 2**).

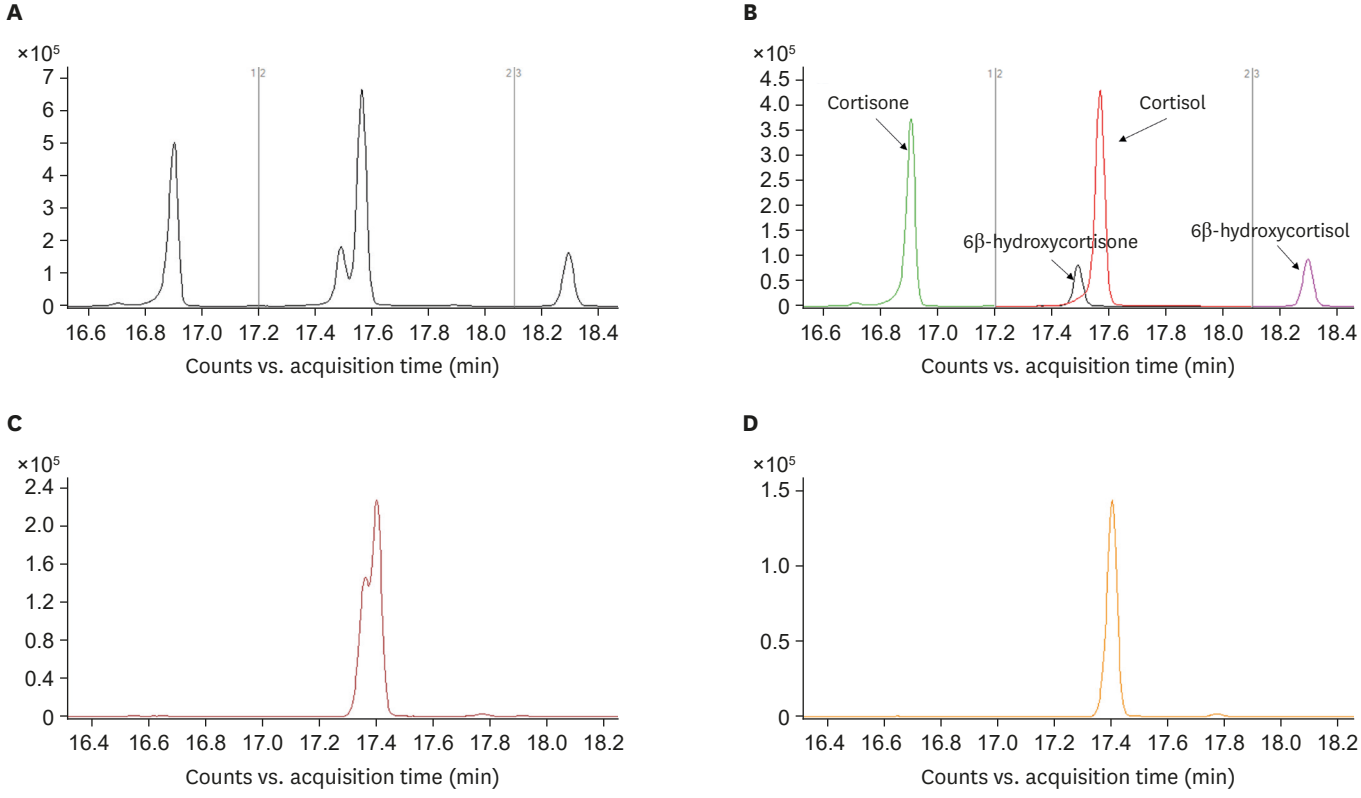

Figure 2. The gas chromatography interfaced with triple-quadrupole mass spectrometry chromatograms of the five endogenous steroids. (A) TIC and (B) EIC of urinary cortisol, cortisone, 6β-hydroxycortisol, and 6β-hydroxycortisone. (C) TIC and (D) EIC of plasma 4β-hydroxycholesterol.
TIC, total ion chromatogram; EIC, extracted-ion chromatogram.

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Table 2. Between-run accuracy and precision of validation batches for compounds

Compound	Between-run accuracy (%) (% CV)*			
	LLOQ	LoQC	MeQC	HiQC
Cortisol	99.36 (4.791)	100.0 (2.294)	107.0 (3.043)	101.7 (1.956)
Cortisone	100.3 (4.566)	93.18 (4.541)	109.1 (2.779)	103.9 (3.710)
6β-hydroxycortisol	103.6 (3.032)	101.4 (3.107)	106.2 (3.078)	103.4 (2.179)
6β-hydroxycortisone	110.0 (4.412)	104.1 (4.465)	104.3 (3.181)	96.64 (3.785)
4β-hydroxycholesterol	106.8 (5.339)	98.57 (4.422)	97.52 (2.218)	103.6 (4.780)

CV, coefficient of variation; LLOQ, lower limit of quantification; LoQC, low quality control; MeQC, medium quality control; HiQC, high quality control.
*The precision and the accuracy of the method were determined from the QC samples at four different concentrations. For between-run repeatability, 3 batches were analyzed and the results are the average of all batches.

Accuracy and precision

Between-run accuracy and precision were determined by analyzing the QC samples with the LLOQ, low QC (LoQC), medium QC, and high QC (HiQC), in the urine and plasma samples (**Table 2**). The between-run accuracy in urine and plasma samples ranged from 93.18% to 110.0% and 97.52% to 106.8%, respectively. The between-run precision (CV) in urine and plasma samples ranged from 1.96% to 4.79% and 2.22% to 5.34%, respectively. All QC concentrations were consistent with the calculated amounts; the acceptable accuracy and CV were within at least 15% in all analytes.

Short-term stability

The short-term stability of processed samples was evaluated at room temperature (20°C) using LoQC and HiQC samples (n = 5). The samples were stable for 10 hours in the auto-sampler. The short-term stability was determined by considering the average analytical time per batch and storage time of samples (10 hours) in the auto-sampler. Cortisol, cortisone and their metabolites at low and high concentrations in urine were stable and the difference in the CV ranged from 0.007% to 10.7%. 4β-hydroxycholesterol in plasma was stable and the difference in the CV ranged from 1.4% to 10.8% (data not shown).

Carry-over

Carry-over was evaluated by analyzing the highest concentration (500 ng/mL, STD8) sample followed by the blank sample. Carry-over was not detected in the blank sample right analyzing the highest concentration sample. The carry-over values ranged from 0.0002% to 0.06% and 0.04% to 0.08% in urine and plasma samples, respectively (data not shown).

Application of the quantitative method to urine and plasma samples

This developed method was used to determine CYP3A activity by quantifying five endogenous steroids in urine and plasma samples obtained from healthy males and females. The five endogenous steroids were successfully detected. All calibration curves of the steroids showed good linearity and the r^2 ranged from 0.9953 to 0.9999 (**Fig. 3**). The concentrations of endogenous steroids are summarized in **Fig. 4** and the concentration of each compound in all 30 subjects was within the calibration curve. Thus, this method can be used to predict CYP3A activity at the baseline.

DISCUSSION

In this study, GC-MS/MS was used to develop an accurate, sensitive, efficient, and fast method for the quantification of five endogenous steroids in human samples owing to its

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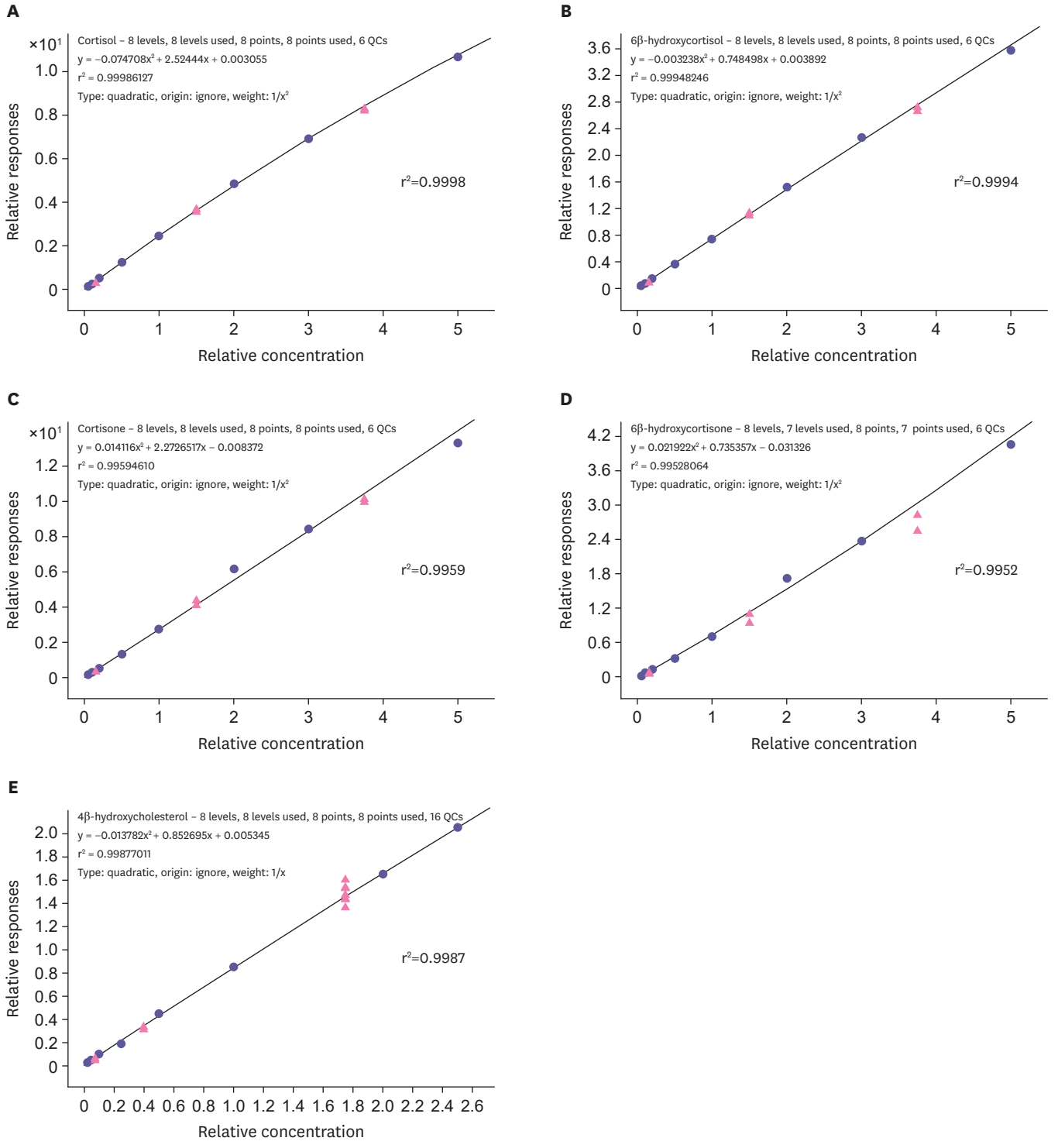

Figure 3. Calibration curves for endogenous steroids from male and female samples. Urinary endogenous steroids; (A) cortisol, (B) 6β-hydroxycortisol, (C) cortisone, and (D) 6β-hydroxycortisone and plasma endogenous steroid, (E) 4β-hydroxycholesterol. X-axis is relative concentration and y-axis is relative responses.
QC, quality control.

selectivity for corticosteroids with similar structures and retention times. In addition, an MRM mode was selected because of its sensitivity and accuracy, which reduced interferences and enhanced the quality of quantitative results. To achieve specific quantified results,

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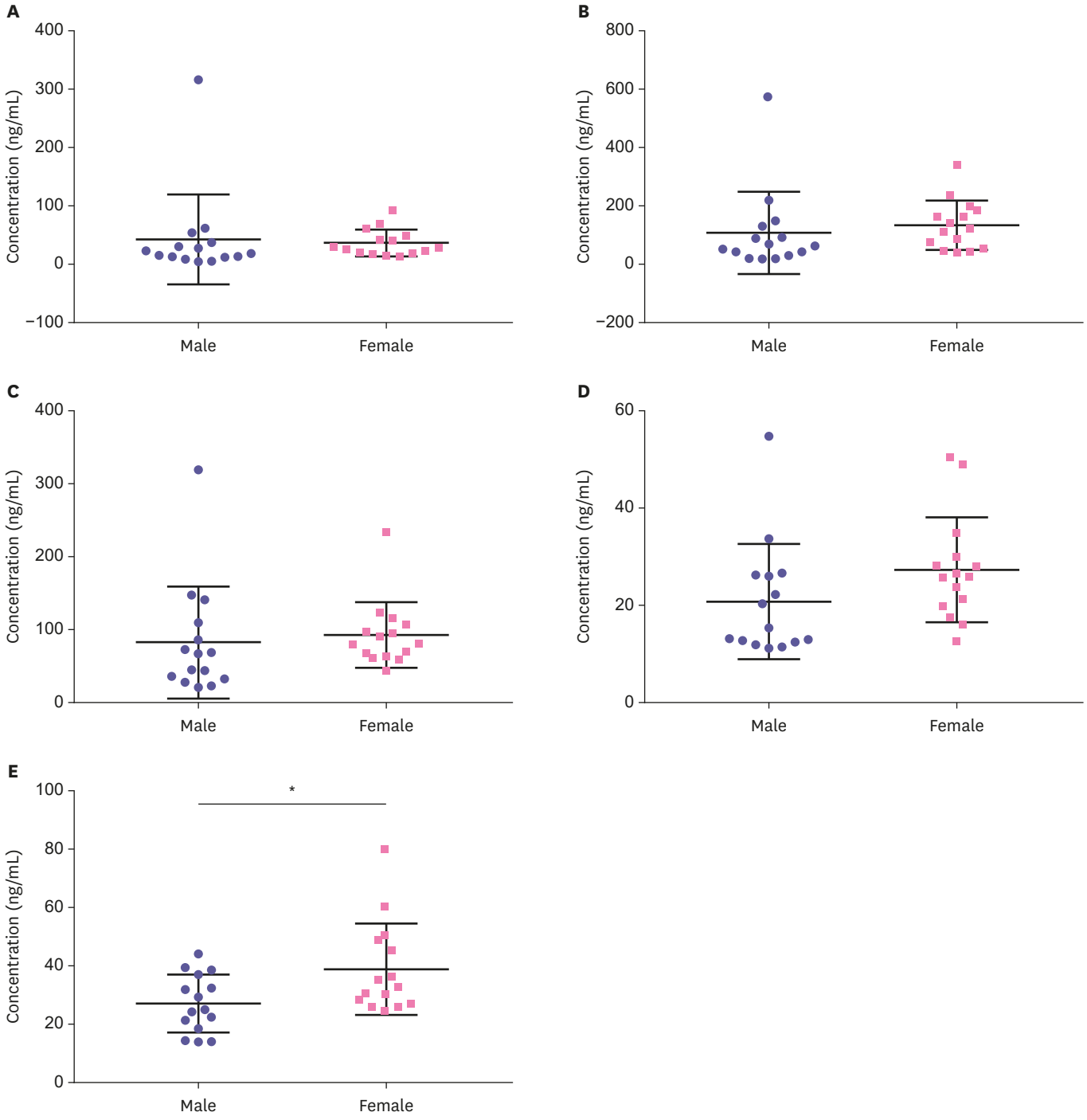

Figure 4. Grouped column scatter plots of quantified endogenous steroids in males and females. Concentration is presented with the mean ± standard deviation. Urinary endogenous steroids; (A) cortisol, (B) 6β-hydroxycortisol, (C) cortisone, and (D) 6β-hydroxycortisone and plasma endogenous steroid, (E) 4β-hydroxycholesterol. The analysis was conducted by diluting the concentration of 6β-hydroxycortisol in a male subject out of the calibration range.
*$p < 0.05$.

the accuracy was increased by reducing interferences, the sensitivity was increased at low concentrations of steroids, the analytical time was reduced, and the sample volume was smaller. Moreover, shortening the analytical time and reducing the sample volume required for analysis will help the clinical use of these biomarkers.

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This developed method was used to determine CYP3A activity by quantifying four urinary endogenous steroids and one plasma endogenous steroid. Urinary cortisol, cortisone, 6β-hydroxycortisol, and 6β-hydroxycortisone were quantified using 12h-interval urine samples to adjust the circadian rhythm effect of cortisol and cortisone [24-26]. Due to low abundance of 4β-hydroxycholesterol in urine, it was quantified in plasma. This method can accurately measure the changes in CYP3A activity by a new drug using pre- and post-dose samples in early-phase clinical trials. The changes of CYP3A activity can characterize inducible or inhibitory properties of the new drug without using probe drugs, which may inform researchers about potential DDIs when taking the new drug and CYP3A substrate together.

In conclusion, to more efficiently measure endogenous biomarkers to predict CYP3A activity, a GC-MS/MS method was developed. The devised method enables the efficient, selective, and sensitive quantification of cortisol, cortisone, 6β-hydroxycortisol, 6β-hydroxycortisone, and 4β-hydroxycholesterol using a small amount of sample and GC-MS/MS. Therefore, the validated method can be a useful technique to measure endogenous biomarkers to predict CYP3A activity and might be applicable in the prediction of CYP3A-mediated ADRs and DDIs.

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