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Short communication

Quantification of cortisol and 6 beta-hydroxycortisol in human urine by LC-MS/MS, and gender-specific evaluation of the metabolic ratio as biomarker of CYP3A activity

Ursula Lutz[a], Nataly Bittner[a], Mike Ufer[b], Werner K. Lutz[a,*]

[a] Department of Toxicology, University of Würzburg, 9 Versbacher St., D-97078 Würzburg, Germany
[b] Institute of Experimental and Clinical Pharmacology, University Hospital Schleswig-Holstein, D-24105 Kiel, Germany

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ABSTRACT

Drug–drug and food–drug interactions are often due to an inhibition or induction of drug-metabolizing cytochrome P450 (CYP) enzymes and may result in non-response or adverse reactions. Hence, phenotypic biomarkers of CYP activity appear as useful tools for individualized pharmacotherapy. The metabolic ratio (MR) of the concentration of 6β-hydroxycortisol (6β-OHC) to cortisol (MR 6β-OHC/cortisol) in human urine had been proposed as an endogenous marker for CYP3A activity. Here, we report on the improvement of published LC-MS/MS methods for the simultaneous quantification of cortisol and 6β-OHC, using on-line sample cleanup by column switching and isotope-labeled analogues as internal standards. [$^2H_2$]6β-OHC was prepared by incubation of human recombinant CYP3A4 with commercially available [$^2H_2$]cortisol. Analytical sensitivity could be increased about 10-fold. The first morning urine of 69 female and 27 male healthy volunteers was analyzed for cortisol and 6β-OHC. Concentrations ranged from 1.0 to 142 and 24 to 670 ng/mL, respectively. Individual MR 6β-OHC/cortisol varied more than 20-fold and we were able to show for the first time for a Caucasian population significantly higher MR values in females as compared to males. This non-invasive biomarker for CYP3A activity lends itself for the study of genetic differences as well as enzyme induction or inhibition in the clinical setting without the need of using a probe drug.

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1. Introduction

The cytochrome P450 (CYP) subfamily CYP3A is of major importance for drug metabolism since it is the most abundant human CYP isoform with a broad substrate specificity [1,2]. CYP3A enzymes contribute to the metabolism of about half of all marketed drugs [3]. There are large interindividual differences in the expression and activity of CYP3A due to genetic and non-genetic factors, including enzyme inhibition and induction [4–6].

For phenotyping for CYP3A activity [7] several probe drugs, such as midazolam and erythromycin, are used. These assays have the drawback that a test compound needs to be administered, which could induce adverse reactions. Alternatively, the urinary concentration ratio of endogenous 6β-hydroxycortisol to cortisol (6β-OHC/cortisol) was found to be a valid biomarker for evaluating xenobiotic-mediated induction or inhibition of CYP3A when each subject served as its own control. This approach of a metabolic ratio (MR) based on endogenous urinary excretion products has the advantage that no probe drug is required, and that it is truly non-invasive [3,8].

Several methods have been published for the quantification of cortisol and 6β-OHC in human urine and they have been reviewed in 2003[9]. More recently, an on-line solid phase extraction (SPE) LC-MS/MS method [10] was reported. It is based on a special on-line SPE equipment (Prospect-2^{TM}, Spark) that is not routinely available. 6α-Methylprednisolone was used as one single internal standard for the quantification of both cortisol and 6β-OHC.

Human urine samples vary considerably in composition and concentration due to differences in food and liquid intake. As a consequence, ionization efficiency in mass spectrometry fluctuates from sample to sample. This phenomenon is known as ion suppression and is commonly seen with electrospray ionization [11]. These problems are compensated by using internal standards, preferentially with stable isotope labels, as these elute together with the analytes and therefore experience the same conditions in the ion source. Labeled standards are often not available. Compounds of similar chemical structure are then used instead. This may give good results if they differ only in the carbon backbone, whereas compounds with differences in functional groups are less appropriate [12]. As a consequence, 6α-methylprednisolone

* Corresponding author. Tel.: +49 931 201 48 402; fax: +49 931 201 48 446.
E-mail address: lutz@toxi.uni-wuerzburg.de (W.K. Lutz).

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appears as an adequate internal standard for the quantification of cortisol by LC-MS methods, but may not be optimal for 6β-OHC.

In this communication we present a number of analytical improvements, using on-line sample cleanup by column switching instead of proprietary on-line SPE equipment and two stable isotope-labeled internal standards. [$^2H_2$]6β-OHC was synthesized by biotechnological oxidation of commercial [$^2H_2$]cortisol with human CYP3A4-expressing liver supersomes. We then applied the more sensitive method to determine the MR 6β-OHC/cortisol in human urine as an endogenous biomarker of CYP3A activity and show significant differences between males and females for Caucasians.

2. Experimental

2.1. Chemicals

Rotisolv™ HPLC gradient grade MeOH and water were from Roth, Karlsruhe, Germany. Cortisol (purity ≥98%), 6β-hydroxycortisol (purity ≥98%) and other chemicals used were from Sigma/Aldrich, Taufkirchen, Germany. Cortisol-1,2-d_2 ([$^2H_2$]cortisol) was from CDN isotopes, Dr. Ehrenstorfer GmbH, Augsburg, Germany. The isotopic purity was 98 at.% deuterium. Human CYP3A4 supersomes (enzyme baculovirus-insect cell-expressed) were purchased from Gentest, BD Biosciences, Woburn, MA, USA.

2.2. Stable isotope-labeled 6β-hydroxycortisol

Stable isotope-labeled 6β-hydroxycortisol [$^2H_2$]6β-OHC was obtained by incubating [$^2H_2$]cortisol with human CYP3A4-expressing liver supersomes. A reaction mixture of 325 μL potassium phosphate buffer (KPP, 100 mM, pH 7.4), 16 μL $MgCl_2$ (10 mg/mL), 50 μL $NADP^+$ (10 mg/mL), 47 μL glucose-6-phosphate (10 mg/mL), 2 μL glucose-6-phosphate dehydrogenase (0.1 U/μL), all in KPP buffer, and 10 μL [$^2H_2$]cortisol (2.5 μL of 1 mg/mL in MeOH + 7.5 μL KPP) was preincubated at 37 °C for 5 min. The reaction was started by adding 50 μL CYP3A4 supersomes (0.05 nmoles). At first the time course of the reaction was investigated by taking aliquots of 10 μL after 10, 20, 30, 40, 50, 60 and 90 min. The analysis showed that the concentration of [$^2H_2$]6β-OHC increased up to 60 min. Therefore after 60 min the reaction was stopped by adding 250 μL ice-cold acetonitrile and mixing. After centrifugation at 10 000 × *g* for 10 min 5 μL of the supernatant were added to 105 μL H_2O and 100 μL were injected into a C18-AQ column 33 mm × 3 mm to monitor the reaction products. The solvents were H_2O (A) and MeOH (B) with a flow rate of 600 μL/min. The gradient started with 30% B for 3 min, increased to 100% B within 0.5 min, was held at 100% B for 4.5 min, decreased to 30% B within 1 min. The eluant was splitted with a graduated microsplitter valve (Upchurch Scientific, Oak Harbor, WA, USA) so that one third entered the MS and two thirds went to waste. RTs were 1.75 min for [$^2H_2$]6β-OHC and 5.5 min for [$^2H_2$]cortisol. To isolate the formed [$^2H_2$]6β-OHC, aliquots of 100 μL of the supernatant were injected and the fraction between RT 1.5 and 2.5 min was collected. The fractions of all aliquots were combined. The purity was checked by the monitoring method and showed no residual [$^2H_2$]cortisol. The concentration of [$^2H_2$]6β-OHC was determined by LC-MS/MS as described in Section 2.4 with a standard curve of 6β-OHC. Aliquots of 80 μL (containing 19 ng [$^2H_2$]6β-OHC) were prepared and stored at −20 °C until use. This procedure ensured that the same amount of the same solution of [$^2H_2$]6β-OHC could be added as internal standard to each calibration standard and to each sample.

2.3. Calibration curve and sample preparation

Stock solutions of cortisol and 6β-OHC were dissolved in MeOH at a concentration of 1 mg/mL. Calibration standards were prepared by dilution with water to give concentrations corresponding to: 0, 0.5, 1, 5, 10, 25, 50, 100, 200 ng/mL for cortisol and 0, 2, 4, 20, 40, 100, 200, 400, 800 ng/mL for 6β-OHC in urine.

Samples were prepared by acidifying 500 μL urine with 2 μL formic acid (FA) and centrifugation at 10 000 × *g* for 10 min. One hundred microliters of the supernatant were added to 130 μL H_2O, 10 μL [$^2H_2$]cortisol (2.5 ng) and 80 μL [$^2H_2$]6β-OHC (19 ng).

2.4. Liquid chromatography/mass spectrometry

The on-line extraction LC-MS/MS system as used in our laboratory [13] was adapted to the new analytes. The autosampler (Agilent Series 1100, Waldbronn, Germany) introduced the sample (300 μL) into the system and pump 1 (Agilent Series 1100) carried the mobile phase (70% H_2O containing 0.1% FA/30% MeOH, v/v) at 600 μL/min to load the sample on the trap column (ReproSil-Pur C18-AQ, 33 mm × 3 mm, 5 μm, Maisch, Ammerbuch, Germany). After 1.4 min the valve switched to the elution position. Pump 2 supplied a gradient to back flush the trapped analytes from the trap column and to transfer them onto the analytical column (ReproSil-Pur C18-AQ, 100 mm × 2 mm, 5 μm, Maisch). The mobile phase was 0.1% FA (A) and MeOH containing 0.1% FA (B) with a flow rate of 240 μL/min. The gradient started with 50% B for 4 min, increased to 70% B within 0.5 min, was held for 6.5 min at 70% B, increased to 100% B for 5 min and equilibrated back to 50% B for 7 min. An API 3000 mass spectrometer (MDS Sciex, Applied Biosystems, Darmstadt, Germany) with a TURBO-Ionspray source in negative ion mode was operated with Analyst software 1.4.1. using the following parameters: IS voltage −4000 V, TEM 400 °C with N_2 (CUR = 12, NEB = 11, CAD = 4); DP −36 V, FP −140 V, EP −7 V. Cortisol: quantifier *m/z* 407 → 331, CE −26 V, CXP −19 V, qualifier *m/z* 407 → 297, CE −44 V, CXP −17 V; [$^2H_2$]cortisol: *m/z* 409 → 333, CE −26 V, CXP −19 V; 6β-OHC: quantifier *m/z* 423 → 347, CE −26 V, CXP −19 V, qualifier *m/z* 423 → 313, CE −46 V, CXP −17 V; [$^2H_2$]6β-OHC: *m/z* 425 → 349, CE −26 V, CXP −19 V; dwell time 125 ms for each transition.

2.5. Method validation and application

Urine of five donors was used as validation samples. Each sample was analyzed five times on the same day to obtain the intra-day variation and on five different days to determine the inter-day variation. The relative analytical recovery was assayed by spiking the urine with 10 ng/mL cortisol and 100 ng/mL 6β-OHC or 40 ng/mL cortisol and 200 ng/mL 6β-OHC in duplicate.

Ninety-six healthy German volunteers of Caucasian origin (69 females and 27 males), aged from 22 to 63 years collected their first morning urine. None of them received any concomitant drug treatment known to inhibit or induce CYP3A. The samples were frozen and stored at −20 °C until analysis. The study was approved by the Ethics Committees of the Christian Albrechts University Kiel and of the University of Wuerzburg and the participants gave informed written consent.

For statistical analysis the free "R" statistics software www.r-project.org was used.

3. Results and discussion

3.1. Internal standards

The study aim was to develop a sensitive and accurate method to quantify cortisol and 6β-OHC (chemical structures in Fig. 1) in

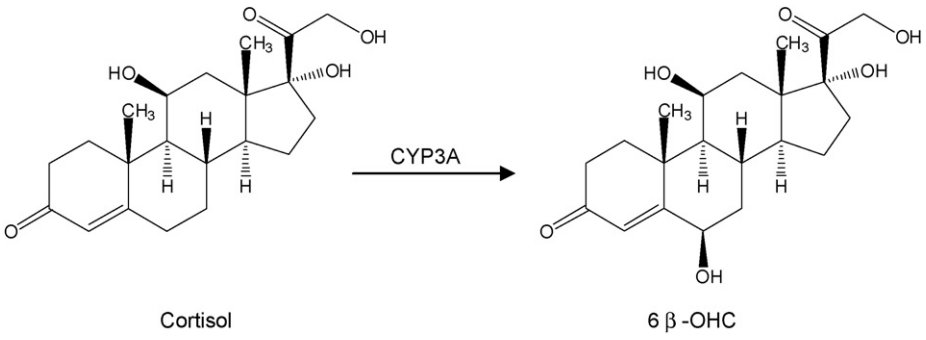

Fig. 1. Chemical structure s of cortisol and 6β-hydroxycortisol (6β-OHC). CYP3A, cytochrome P450 3A subfamily.

human urine with on-line sample cleanup using two HPLC pumps and a switching valve, in order to use the MR 6β-OHC/cortisol to phenotype humans for CYP3A activity. To compensate for ion suppression stable isotope-labeled internal standards were sought for both analytes. Deuterium-labeled cortisol was commercially available. A chemical synthesis of stable isotope-labeled 6β-OHC has been described [14], but is technically demanding. We therefore used a novel biotechnological approach by incubating deuterated cortisol with human liver supersomes expressing CYP3A4 activity. After optimizing the incubation conditions a yield of 700 ng [$^2H_2$]6β-OHC from 2.5 μg [$^2H_2$]cortisol was achieved. The product was isolated from the reaction mixture by LC and the purity confirmed by LC-MS/MS. It was used as a stock solution for internal standardization. The use of supersomes represents a convenient method to obtain stereochemically correct metabolites and may be applied to other compounds, enzymes, and reactions.

3.2. LC-MS/MS method validation

The most sensitive measurement was obtained in the negative ion mode by monitoring the transition of the adduct of cortisol or 6β-OHC with the formic acid anion (*m/z* 407 or *m/z* 423) in the first quadrupole and the fragments *m/z* 331 for cortisol or *m/z* 347 for 6β-OHC [10], corresponding to the loss of CH_2O from the molecular ion after breakage between carbon atoms 20 and 21 of the steroid moiety [15]. The amount of urine used, injection volume and washing time over the trap column were optimized to give the best signal-to-noise ratio (S/N). Chromatography was developed for the separation of the analytes from isobaric compounds. The most sensitive transition was used for quantification and a second transition for analyte confirmation. Chromatograms of the standards and of a human urine sample containing deuterated internal standards are shown in Fig. 2.

The deuterated internal standards were checked for the question whether they contained unlabeled compounds that give a peak in the analyte channels. The isotopic purity of [$^2H_2$]cortisol was 98 at.% deuterium as declared by the provider. With the amount used in our method, a marginal peak (S/N = 3) was detected in the cortisol channel. [$^2H_2$]6β-OHC gave a small peak (S/N = 21) in the 6β-OHC channel, in agreement with the larger amount of [$^2H_2$]6β-OHC (19 ng) compared to that of [$^2H_2$]cortisol (2.5 ng). In order to adjust for this minor contribution, a "calibrator zero sample" containing only the internal standard and no analyte was measured for each standard curve and the resulting value considered for the calculation of the standard curve. Calibration curves from 0.5 to 200 ng/mL for cortisol and 2 to 800 ng/mL for 6β-OHC comprising eight calibration points were established by fitting a linear regression model with $1/x$ weighting using the peak area. The resulting equations were $y = 1.14 \times 10^{-8} + 0.033 \times x$, $r = 0.999$ for cortisol and $y = 0.00311 + 0.00368 \times x$, $r = 0.998$ for 6β-OHC. The accuracy was in the range of 92–111% for cortisol and 89–110% for 6β-OHC, as measured in five analytical runs on five different days.

The internal standards contained two deuterium isotopes, which results in a mass difference of two between analyte and internal standard. Natural isotope abundance in the analyte was calculated to result in a theoretical contribution of 4% to the channel of the internal standard. This may incorrectly increase the amount of internal standard detected, when high concentrations of analyte are present with low concentrations of internal standard. Following the procedure published by Duxbury et al. for cortisol and [$^2H_2$]cortisol [16], we therefore chose the concentration of the internal standard high enough to avoid deviation from linearity of the calibration curve.

A lower limit of quantitation of 0.5 ng/mL for cortisol and of 2 ng/mL for 6β-OHC as used for our lowest calibrator was adequate for the analysis of all our samples. The limit of detection was 1 pg on column for both cortisol (S/N = 8) and 6β-OHC (S/N = 6), which corresponds to a concentration of 0.01 ng/mL. This is more sensitive by more than a factor of 10 than the LC-MS/MS method with the Spark on-line SPE sample cleanup [10] that showed S/N = 10 for 0.2 ng/mL cortisol and S/N = 42 for 1 ng/mL 6β-OHC.

The intra- and inter-day assay precision for the analysis of cortisol and 6β-OHC is presented in Table 1 for five urine samples. Coefficients of variation (%CV) comply with the FDA guidelines that allow for up to 15% CV [17]. Table 2 shows the relative recovery of the analytes spiked at two levels into urine samples of different background concentration. The values spanned 95–107% for cortisol and 88–101% for 6β-OHC, indicating that the response ratio of analyte/internal standard was not affected by sample differences. Analyte concentrations were stable at −20 °C, as indicated by repeated measurement of 5 samples over 6 months of storage (data not shown).

3.3. Metabolic ratio MR 6β-OHC/cortisol and gender differences

Cortisol and 6β-OHC were measured in the first morning urine of 96 healthy German volunteers of Caucasian origin. Concentrations ranged from 1.0 to 142 ng/mL for cortisol and 24 to 670 ng/mL for 6β-OHC. The MR 6β-OHC/cortisol spanned from 2.1 to 44.9, with a right-skewed distribution and a median MR of 7.9. Literature data based on APCI LC-MS showed a range from 1.8 to 20.3 for 30 Japanese [18], the Spark on-line SPE LC-MS/MS method resulted in MR values of 3.1–13.5 for percentiles 2.5–97.5 for Americans [10]. The interindividual variability found in these and our studies, based on an endogenous CYP3A substrate, is in line with the 5–20-fold range in metabolic clearance determined with CYP3A-dependent drugs [4]. Using HPLC/UV analysis MR ranges of 2.8–26.9 in 11 Danish individuals [19] and of 0.09–130.8 for 487 Japanese were reported [20]. GC/MS analysis resulted in a range of 1.9–8.3 in 35 individuals [21].

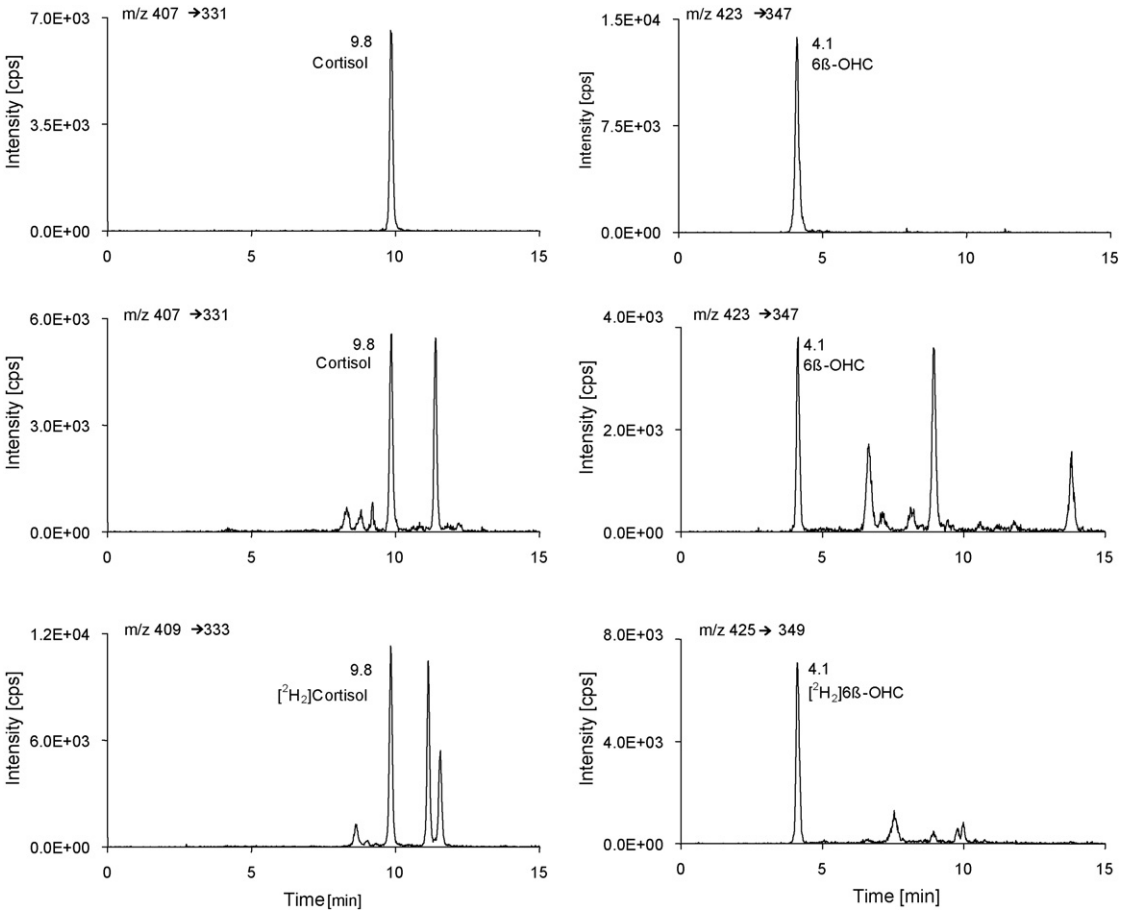

Fig. 2. Chromatograms of LC-MS/MS analysis of cortisol and 6β-hydroxycortisol (6β-OHC) using on-line sample preparation by HPLC column switching. Top row: cortisol and 6β-OHC standards; center and bottom row: human urine sample containing [$^2H_2$]cortisol, and [$^2H_2$]6β-OHC internal standards.

Table 1
Intra- and inter-day assay variation in the mass spectrometric analysis of cortisol and 6β-hydroxycortisol in 5 human urine samples.

	Intra-day variation					Inter-day variation				
	Donor					Donor				
	1	2	3	4	5	1	2	3	4	5
Cortisol [ng/mL]										
Mean (n = 5)	8.4	11.8	51.9	34.3	30.3	8.3	11.2	52.3	34.9	29.9
SD	0.2	0.7	1.3	0.7	0.8	0.3	1.0	1.2	2.4	0.8
%CV	2.9	5.7	2.4	2.1	2.8	3.7	9.3	2.4	6.9	2.5
6β-Hydroxycortisol [ng/mL]										
Mean (n = 5)	127	361	229	107	130	136	358	238	105	130
SD	3.8	10	9.8	7.1	4.4	14	12	18	5.0	3.3
%CV	3.0	2.8	4.3	6.7	3.4	10	3.4	7.5	4.8	2.6

Table 2
Concentrations [ng/mL] of cortisol and 6β-hydroxycortisol in 5 human urine samples.

	Donor 1		Donor 2		Donor 3		Donor 4		Donor 5	
	[ng/mL]	[%]	[ng/mL]	[%]	[ng/mL]	[%]	[ng/mL]	[%]	[ng/mL]	[%]
Cortisol										
Endogenous (n = 5)	8.4		11.8		51.9		34.3		30.3	
Endo + 10 (n = 2)	18.9	103	22.9	105	59.0	95	45.8	103	43.3	107
Endo + 40 (n = 2)	50.3	104	53.8	104	94.5	103	79.0	106	72.4	103
6β-Hydroxycortisol										
Endogenous (n = 5)	127		361		229		107		130	
Endo + 100 (n = 2)	217	95	453	98	332	101	195	94	221	96
Endo + 200 (n = 2)	294	90	494	88	414	97	278	91	310	94

Results of standard addition experiment and calculation of relative analytical recovery [%].

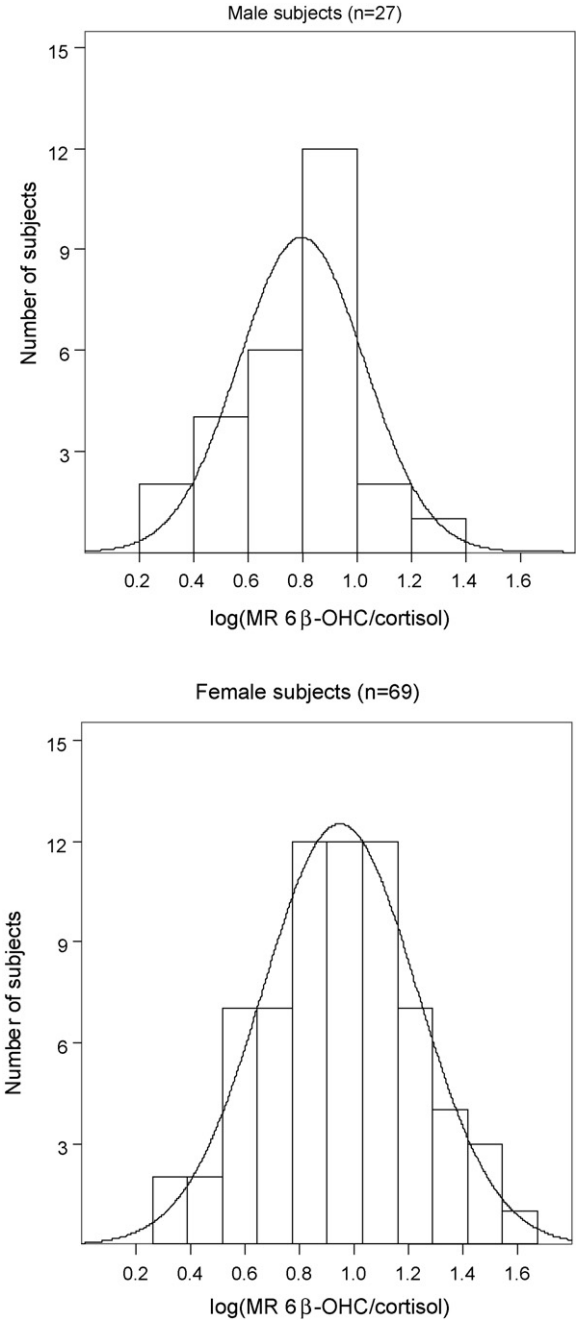

Fig. 3. Frequency distribution histogram of $\log_{10}(MR) = \log_{10}(6\beta\text{-OHC/cortisol})$ determined in the first morning urine of 27 males (top) and 69 females (bottom), with fitted normal distributions. Mean ± SD was 0.80 ± 0.23 for males and 0.95 ± 0.28 for females. The Welch two-sample *t*-test showed that the difference was significant ($p = 0.01$).

Separate analysis of males and females in our study showed a significant gender difference, with median MR values of 6.8 and 9.2, respectively, and a *p*-value for the difference in rank sum of 0.02 (Wilcoxon test). The frequency distributions of the $\log_{10}$-transformed MR values are shown in Fig. 3 for both males and females. The Shapiro–Wilk normality test did not reject normal distribution, with mean $\log_{10}(MR) \pm SD$ of 0.80 ± 0.23 for males and 0.95 ± 0.28 for females. The Welch two-sample *t*-test corroborated the significance of the difference ($p = 0.01$).

Few studies only had reported a significant gender difference for the MR 6β-OHC/cortisol before. Higher mean MR for females had been determined in a small cohort of American patients of undefined ethnicity using radioimmunoassay [22]. In a Japanese population investigating 487 individuals, females also showed significantly higher $\log_{10}(MR)$ values (mean = 0.87) than males (0.63), determined in a day-time urine sample [20]. For Caucasians, our data represent the first respective information. The higher MR 6β-OHC/cortisol in females may indicate higher CYP3A activity relative to males, possibly resulting in a gender-specific drug disposition and response [23]. This hypothesis is supported by a recent meta-analysis indicating a small but significant difference in clearance of CYP3A substrates [24].

3.4. Outlook

Our sensitive and specific LC-MS/MS method to determine the metabolic ratio of 6β-OHC/cortisol is based on as little as 100 μl of urine. The simplicity of sample collection and work-up renders this approach well suited as an indicator of CYP3A activity in humans. As half of all drugs on the market are metabolized by CYP3A isoenzymes, and since CYP3A activity varies considerably between individuals, it appears of clinical relevance to develop a non-invasive phenotyping strategy based on endogenous biomarkers to ultimately enable individualized drug dosing. In addition, the method may also be used to study circadian rhythms of CYP3A activity, time course or dose response of enzyme induction and inhibition by drugs of both synthetic and herbal origin [9] or by food items, such as grapefruit juice [25]. Furthermore, the MR 6β-OHC/cortisol may serve as supplementary biomarker of individual metabolic capacity in clinical phase-I or II trials.

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