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Short communication

Rapid quantitation of testosterone hydroxyl metabolites by ultra-performance liquid chromatography and mass spectrometry

Dandan Wang, Minli Zhang *

Drug Metabolism and Pharmacokinetics, AstraZeneca Pharmaceuticals LP, 1800 Concord Pike, Wilmington, DE 19850, USA

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Abstract

A rapid and sensitive ultra-performance liquid chromatography and mass spectrometry (UPLC/MS) method was developed to simultaneously quantify seven monohydroxyl testosterone metabolites (16α-, 2α-, 7α-, 6α-, 2β-, 6β-, and 16β-hydroxyl testosterones) in rat liver microsomes. The UPLC system used a short 1.7-μm particle size column coupled to a Sciex 4000 Q trap in multiple reaction monitor (MRM) mode. All hydroxyl testosterones were resolved within 2.5 min. A 4-day validation was performed to determine the linearity, repeatability, reproducibility and accuracy of the method in rat liver microsomes. This method is applicable to the measurement of the testosterone hydroxylase activity in biological matrices such as the liver microsome incubates.

Keywords: UPLC; LC/MS; Quantitation; Testosterone; Metabolites

1. Introduction

Cytochrome P450 (CYP) family metabolizes a large number of structurally diverse endogenous and exogenous compounds. When a chemical inhibits CYPs, such as CYP3A4, it would likely to cause drug–drug interaction. The induction of CYPs could also potentially impair normal biosynthesis and metabolism of endogenous molecules and thereby interfere with regular biological processes leading to toxicological consequences. CYP inhibition and induction by drug candidates are closely monitored in drug discovery and development. Formation of different hydroxyl testosterone (OHT) metabolites has been widely used as the important marker for specific cytochrome P450 enzyme activities such as the activity of CYP3A4 in human [1–3]; CYP3A12 in dog [4]; CYP2A1, CYP2B1/2, CYP2C11 and CYP3A1/2 in rat liver microsomes [5–7].

HPLC methods with UV detection were originally used to quantify OHTs [8,9]. Several LC/MS methods have been developed to improve detection limits [10,11]. HPLC methods with on-line column switching techniques were applied to further increase sensitivity [12,13]. Since OHTs share the same parent and product ion patterns, it is impossible to use mass spectrometry to distinguish them without good chromatographic separation. Therefore, to positively identify and quantify one of the OHTs, it is necessary to have a LC method that can separate these OHTs.

Most HPLC methods reported so far require a complex gradient and long chromatographic run time (usually 30 min or longer) to obtain OHT separation. Recently, ultra-performance liquid chromatography (UPLC) with <2 μm particle size column was introduced for rapid and efficient compound separation [14]. UPLC coupled with mass spectrometry is increasingly being used for rapid multiple component quantitation [15] for *in vitro* [16] and *in vivo* [17] metabolite characterization. However, no reported chromatographic method is available for full separation of OHTs, especially the efficient separation of 6β-hydroxyltestosterone, a testosterone metabolite by CYP3A, from 7α-hydroxyltestosterone. No unique mass spectrometry method is available for unambiguous identification of these OHTs simultaneously. The objective of this study was to develop a sensitive, rapid and simple UPLC/MS method to separate and simultaneously quantify seven hydroxyl testosterone metabolites.

* Corresponding author. Tel.: +1 302 886 1044; fax: +1 302 886 5345.
E-mail addresses: dandan.wang@astrazeneca.com (D. Wang), minli.zhang@astrazeneca.com (M. Zhang).

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2. Experimental

2.1. Chemicals and microsomal samples

Seven most commonly analyzed hydroxyl testosterones metabolites, 16α-hydroxytestosterone (16α-OHT), 2α-hydroxyltestosterone (2α-OHT), 7α-hydroxyltestosterone (7α-OHT), 6α-hydroxyltestosterone (6α-OHT), 2β-hydroxytestosterone (2β-OHT), 6β-hydroxytestosterone (6β-OHT) and 16β-hydroxytestosterone (16β-OHT) of analytical grade were obtained from Steraloids (Wilton, NH, USA). The analytical grade corticosterone was obtained from Sigma–Aldrich (Saint Louis, MO, USA). All organic solvents were of HPLC grade from Sigma–Aldrich (Saint Louis, MO, USA). Milli-Q water was generated in house. All other reagents were of analytical grade from Fisher Scientific (St. Louis, MO). Rat liver microsomes were purchased from In Vitro Technologies, Inc. (Baltimore MD, USA).

2.2. Standard solution and sample preparation

A standard working solution containing the mixture of OHTs, each at 50 μg/mL, was prepared in methanol water (1:1, v/v). Further series dilutions with methanol–water (1:1, v/v) resulted in 10, 5, 0.5, 0.1 and 0.05 μg/mL standard working solutions. The 50, 0.5 and 0.1 μg/mL QC mixture working solutions were prepared in the same way. A 2-μg/mL corticosterone solution was prepared in methanol as internal standard (IS) solution.

Standards for calibration curve were prepared in 200-μL rat liver microsome incubates at 0.005, 0.01, 0.05, 0.5, 1 and 5 μg/mL by adding 20 μL of the standard working solution into 180 μL of rat liver microsome incubates without NADPH. Likewise, QC solutions were also prepared at 0.01, 0.05 and 5 μg/mL in the microsome incubates. Following protein precipitation by adding 200 μL of IS solution and centrifugation at 14000 × *g* for 5 min, 2.5 μL of the supernatant was injected to UPLC.

To generate OHT metabolites *in vitro*, testosterone was incubated for 20 min at 37 °C in a 200-μL rat microsome incubate that contained potassium phosphate buffer (100 mM, PH 7.4), $MgCl_2$ (5 mM), reduced form of nicotinamide adenine dinucleotide phosphate (NADPH, 1 mM), testosterone (100 μM) and rat liver microsomes (0.5 mg protein/mL). The reaction was initiated by the addition of NADPH. Incubation was terminated by adding 200-μL aliquot of IS solution. Following protein precipitation by centrifugation, 2.5 μL of the supernatant was injected to UPLC.

2.3. Chromatographic conditions

The UPLC system, including a sample manager and a binary solvent manager from Waters (Boston, MA USA) was used for sample injection and separation. Sample separation was performed in a Waters AQUITY BEH C18 column (2.1 mm × 50 mm, 1.7 μm) with flow rate of 0.5 mL/min at 40 °C. The mobile phase consisted of 5% acetonitrile in water with 0.1% formic acid (A) and 0.1% formic acid in methanol (B). The gradient started from 5% B to 45% B in 0.25 min, then to 50%B in 3.5 min, back to 5% B in 0.1 min followed by 0.9 min equilibration.

2.4. Mass spectrometry conditions

The LC/MS/MS analysis was carried out in a PE Sciex 4000 Q trap (Applied Biosystems, Foster City, CA) with a Turbospray interface in positive ionization under multiple reaction monitoring (MRM) mode. The Turbospray temperature was set at 500 °C, curtain gas at 25 psi, ion spray energy at 5000 V, Nebulizer gas (gas 1) and Turbo gas (gas 2) at 60 psi. The protonated molecules were fragmented by collision-activated dissociation (CAD) under 10 psi nitrogen collision gas. The collision energy (CE) was set to 23 eV. All OHTs were monitored at *m/z* 305 → 269 transition. The internal standard corticosterone was monitored at *m/z* 347 → 121 transition. Data correction, quantitation and limit of detection (LOD) were performed in Sciex Analyst Software (Version 1.4.1).

2.5. Method accuracy, precision and specificity

The OHT to IS peak area ratio was applied for quantitation of OHT concentration in microsomal incubates. Two sets of calibration curves were constructed in linear regression plots every day from six calibration points, ranging from 0.005 to 5 μg/mL. Three QC concentrations at 0.01, 0.5 and 5.0 μg/mL were included in each assay over 4 days to determine the intra-day and inter-day precision and accuracy. Accuracy was presented as the percent bias of the calculated concentration in comparison to the actual concentration. Precision was calculated as percent relative standard deviation (RSD%) of the analyte concentration. Specificity was determined by monitoring matrix interference of three separate batches of pooled microsomes, each from eight individual rat liver microsomes, on ionization and *m/z* 305 → 269 transition.

3. Results and discussions

3.1. UPLC separation and detection of hydroxyl testosterone metabolites

Since OHTs share the same mass transition fragments in mass spectrometer analysis, LC separation before MS analysis is the only way for their confirmation and quantitation in biological matrices, such as the liver microsomes. The UPLC method developed from this study was able to achieve baseline separation for the seven OHTs within 2.5 min (Fig. 1). The separation of 6β-OHT from 7α-OHT is critical for testosterone based liver microsomes assays monitoring CYP 3A activity. The 2.1 mm × 50 mm BEH C18 column, with 1.7 μm particle size, provided efficient separation in short analysis time. Chromatograms in Fig. 2 show OHTs formed in non-induced rat microsome incubates (A) and dexamethasone-induced rat microsome incubates (B) in which the 6β-OHT (2) peak increased, suggesting an increase of CYP3A activity in induce-rat liver microsomes. In rat microsomal incubates, monitoring at *m/z* 305 → 269 transition, a peak was detected following 16β-

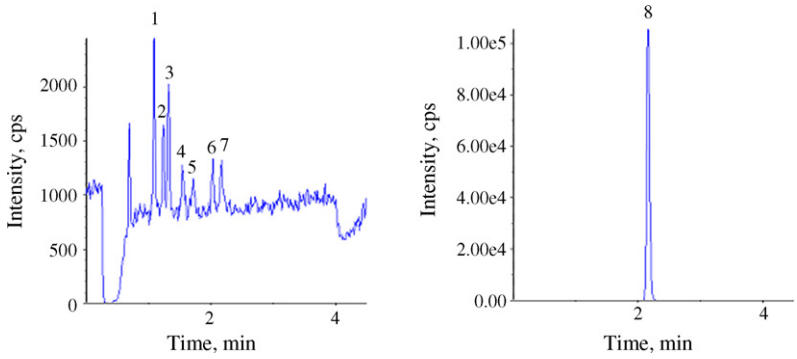

Fig. 1. Extracted ion chromatogram of 6α-OHT (1), 6β-OHT (2), 7α-OHT (3), 16α-OHT (4), 16β-OHT (5) 2α-OHT (6), 2β-OHT (7) spiked in rat liver microsomes at 0.005 μg/mL (at LLOQ level); transition of *m/z* 305 → 269 was set to monitor OHTs, transition of *m/z* 347 → 121 for internal standard corticosterone (8) at 2 μg/mL.

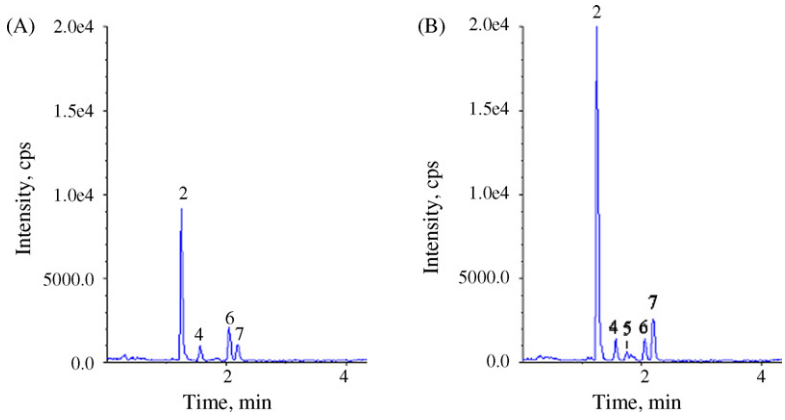

Fig. 2. Extracted ion chromatogram of OHTs in rat liver microsomes (0.5 mg protein/mL) incubated with testosterone at 100 μM for 20 min. Incubates were diluted 100-fold before analysis. (A) Normal rat liver microsome incubate. The 6β-OHT (2), 16α-OHT (4), 2α-OHT (6) and 2β-OHT (7) concentrations were 27.2, 4.78, 4.80 and 1.75 μg/mL, respectively. (B) Dexamethasone induced rat liver microsome incubate. The 6β-OHT (2), 16α-OHT (4), 16β-OHT (5) 2α-OHT (6) and 2β-OHT (7) measured concentrations were 58.9, 5.68, 2.04, 2.96 and 4.06 μg/mL, respectively.

OHT (5) (Fig. 2, A and B), suggesting an additional hydroxyl testosterone metabolite different from the hydroxyl testosterone standard references used.

3.2. Linearity

Linearity was achieved in a wide concentration range from 0.005 to 5 μg/mL for six standard points in rat liver microsomes. Under $1/x^2$ weighing, the mean values of the correlation coefficient (R^2) were >0.99 for all seven OHTs. Under current conditions the calculated LOD according to the 3σ signal-to-noise ratio were 0.32, 1.01, 0.55, 1.42, 2.0, 1.59, and 1.27 ng/mL for 6α, 6β, 7α, 16α, 16β, 2α, and 2β-OHT, respectively. The lowest calibration standard (LLOQ) was set at 0.005 μg/mL in rat liver microsome and achieved at least 5× noise level for all OHTs. Fig. 1 represents extract ion chromatogram of these OHTs at LOQ levels.

3.3. Specificity

Blank extracts of three pooled rat liver microsome incubates were tested in the UPLC/MS system to assess microsomal matrix interferences. Following injection of blank microsome extracts, the UPLC eluent was mixed through a tee with OHT solutions delivered continually by a syringe pump into mass spectrometer monitoring *m/z* 305 → 269 transition. Though strong ion suppression was observed in retention time before 0.8 min, the suppression was not in OHT and IS retention time regions between 1 and 2.5 min (Fig. 3). When blank microsome extracts were analyzed by UPLC/MS monitoring

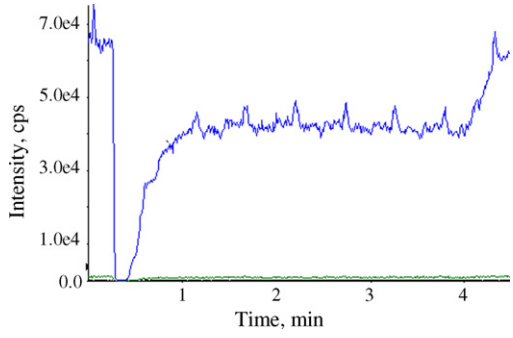

Fig. 3. Representative chromatogram monitoring ion suppression/enhancement effects: extracts of blank pooled rat liver microsome incubates were injected into UPLC/MS. Eluent was mixed with OHT infusion through a tee before MS analysis at *m/z* 305 → *m/z* 269 transition.

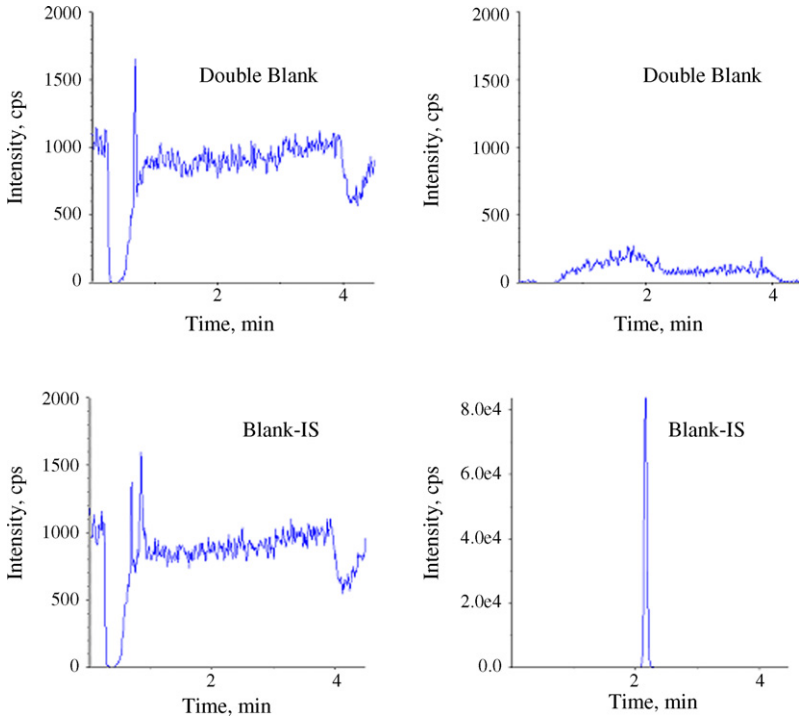

Fig. 4. Extracted ion chromatograms of the blank pooled rat liver microsomes. On the left were ion chromatograms monitoring *m/z* 305 → 269 transition for OHTs. On the right were ion chromatograms monitoring *m/z* 347 → 121 transition for IS. Double blank, microsomes without OHTs and IS; Blank-IS, microsomes without OHTs but spiked with IS.

m/z 305 → 269 and *m/z* 347 → 121 transitions, no endogenous interferences were detectable (Fig. 4). Results from these observations indicate that OHT and IS retention time regions around 1–4 min were free from endogenous interference and strong ion suppression.

3.4. Precision and accuracy

This method was developed for CYP induction, enzyme profiling and inhibition studies. In order to accommodate these studies with a single UPLC/MS method, a wide OHT dynamic concentration range is necessary. Precision and accuracy data were therefore determined at concentration range from 0.005 to 5 μg/mL in rat microsomes over 4 different days. For all standards the inter-day precisions (RSD%) ranged from 0.8 to 7.7%, the inter-day accuracy (%) ranged from 87 to 107% (Table 1), suggesting that this UPLC/MS method is able to provide a wide linear dynamic range (0.005–5 μg/mL) for all seven OHTs. The QC intra-day RSD% ranged from 3.8 to 11% with accuracy ranging from 87 to 107% (Table 2). The QC inter-day RSD% ranged from 1.0 to 10% with accuracy ranging from 85

Table 2
Intra-day precision (RSD%) and accuracy ($n = 4$) for QCs in pooled rat liver microsomes

Steroid	5 μg/mL		0.5 μg/mL		0.01 μg/mL	
	RSD%	Accuracy	RSD%	Accuracy	RSD%	Accuracy
6α-OHT	4.1	87	0.8	107	5.0	105
7α-OHT	3.8	89	1.2	106	5.8	107
16α-OHT	3.9	92	1.4	107	11	98
16β-OHT	4.5	94	0.7	103	9.2	105
2α-OHT	4.7	100	1.0	103	8.5	99
2β-OHT	4.0	100	1.0	105	8.4	96
6β-OHT	4.3	94	2.1	104	7.0	105

Table 1
Inter-day precision (RSD%) and accuracy ($n = 8$) for standards in pooled rat liver microsomes over 4 days

Steroid	0.005 μg/mL		0.01 μg/mL		0.05 μg/mL		0.5 μg/mL		1 μg/mL		5 μg/mL	
	RSD%	Accuracy	RSD%	Accuracy	RSD%	Accuracy	RSD%	Accuracy	RSD%	Accuracy	RSD%	Accuracy
6α-OHT	2.1	98	4.0	103	4.8	102	1.1	105	5.6	106	2.1	87
7α-OHT	0.8	98	1.0	103	5.1	100	0.8	104	6.0	105	2.0	89
16α-OHT	3.1	99	5.2	102	6.0	100	2.2	104	6.3	105	1.9	90
16β-OHT	3.6	99	6.0	101	6.0	99	1.6	102	6.9	104	1.4	94
2α-OHT	3.9	100	7.7	101	3.4	96	1.9	101	5.3	104	2.5	99
2β-OHT	0.4	102	1.3	97	4.7	96	0.9	102	5.3	105	2.3	99
6β-OHT	1.3	96	2.3	107	3.7	100	1.0	101	5.4	105	3.0	92

Table 3
Inter-day precision (RSD%) and accuracy ($n = 16$) for QCs in pooled rat liver microsomes over 4 days

Steroid	5 μg/mL		0.5 μg/mL		0.01 μg/mL	
	RSD%	Accuracy	RSD%	Accuracy	RSD%	Accuracy
6α-OHT	3.0	85	1.2	106	3.6	107
7α-OHT	2.8	88	1.3	106	3.0	107
16α-OHT	2.7	89	1.9	105	10	97
16β-OHT	1.6	93	1.0	103	9.9	107
2α-OHT	3.2	98	2.1	102	7.1	104
2β-OHT	2.6	98	1.4	103	4.6	97
6β-OHT	2.6	92	1.8	104	1.0	106

to 107% (Table 3). There was an apparent trend of a slightly low bias in accuracy (85–98% recovery) at 5 μg/mL level and a slightly high bias in accuracy (97–107% recovery) for 0.5 and 0.01 μg/mL levels. Factors causing the biases remained to be further investigated. However, the 85–107% recovery was within 15% of the actual value, and within 20% at LLOQ according to Guidance for Industry Bioanalytical Method Validation by FDA [18].

4. Conclusion

A sensitive, rapid and simple UPLC/MS/MS method was developed and partially validated to simultaneously quantify 16α-, 2α-, 7α-, 6α-, 2β-, 6β-, and 16β-OHTs in rat liver microsomal incubates. The UPLC/MS/MS method covers a dynamic range from 0.005 to 5 μg/mL with good accuracy and reproducibility. This method reduces analysis time to less than 5 min. It is suitable for quantitation of hydroxyl testosterone metabolites in liver microsomes generated from P450 inhibition and induction assays.

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