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Liquid chromatographic determination of urinary 6β-hydroxycortisol to assess cytochrome p-450 3A activity in HIV positive pregnant women

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Abstract

Assessing the activity of CYP3A4 is important for predicting the pharmacokinetic behavior of protease inhibitors in HIV positive patients, especially in pregnant women. The endogenous hormonal ratio of 6β-hydroxycortisol (6β-OHF) to cortisol (F) in the urine is an index for metabolic enzyme activity of cytochrome p-450 (CYP) 3A4. Because the ratio is a unique way to assess the enzyme activity without using any exogenous probes for this isozyme, it is practical for use in pregnant women. In this paper, we describe a method using high performance liquid chromatography (HPLC) for 6β-OHF in urine from pregnant women to estimate the ratio of 6β-OHF/F. Urinary 6β-OHF was measured by using C18-cartridge solid phase extraction and isocratic HPLC. Aliquots (1 ml) of urine samples spiked with internal standard, 6β-hydroxyprednisolone (6β-OHPSL), were alkalinized with NaOH, then applied to C18-cartridges, which were washed with water and hexane and eluted with ethyl acetate. After the effluents were dried and reconstituted in 10% acetonitrile, the samples were analyzed by HPLC using an isocratic mobile phase (acetic acid/acetonitrile/50 mM potassium dihydrogenphosphate; 0.2/9/90.8; v/v) and ultraviolet detection at 245 nm. The recoveries of 6β-OHF from C18 cartridges were 93.2 and 93.9% when the authentic 6β-OHF was added to the urine sample at the concentration of 50 and 300 ng/ml, respectively. Intra- and inter-day variations estimated at concentrations of 113–674 ng/ml were 2.9–5.6 and 4.9–8.1%, respectively. The method was applied to morning urine samples collected from HIV-positive pregnant women managed with protease inhibitor containing anti-retroviral regimens.

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1. Introduction

Maternal anti-retroviral therapy effectively reduces the risk of vertical transmission of the human immunodeficiency virus (HIV) from mother to fetus [1]. Zidovudine monotherapy, a Food and Drug Administration (FDA) approved regimen, given during pregnancy and labor and delivery reduces HIV transmission by two-thirds [1]. However, transmission of resistant strains remains unresolved [2]. Because of these concerns, many women continue to take highly active antiretroviral therapy including protease inhibitors throughout pregnancy. Unfortunately, dosing regimens in pregnant women have not been clearly established owing to changes in the pharmacokinetics of anti-retrovirals during pregnancy. Because the metabolism of protease inhibitors is mediated by the hepatic microsomal CYP enzyme system, specifically by the CYP3A4 isozyme [3], assessing this isozyme activity during pregnancy is of importance in predicting the pharmacokinetic disposition of these drugs.

Biotransformation of endogenous cortisol (F) to 6β-hydroxycortisol (6β-OHF) is known to be mediated by CYP3A isozymes, CYP3A4 and 3A5 [4–6], and is affected by CYP3A4 inducers (rifampicine and anti-epileptic drugs) and CYP3A4 inhibitors (e.g. ketoconazole) of drug metabolism. The ratio of 6ß-OHF/F measured in the urine is an endogenous index of CYP3A activity, likely CYP3A4 [7]. Changes in drug metabolism during pregnancy have not been well established. In general, women when they become pregnant limit their use of unnecessary medications to avoid potential exposure of the fetuses to drugs with unknown teratogenecity. When assessing enzyme activity, the use of conventional probes for CYP3A4 such as dapsone and erythromycin [7] is undesirable. Therefore, estimating the endogenous hormonal ratio of 6β-OHF/F may provide a suitable alternative to measure enzyme activity in pregnant women.

Several assay methods for 6ß-OHF have been developed by using high-performance liquid chromatography (HPLC) and immunoassay techniques [4–6,8–10]. Using the immunoassay to analyze urine samples from pregnant women is problematic due to the cross-reactivity of anti-6β-OHF anti-body to other nonspecific steroids which circulate at high levels during pregnancy. An alternative approach is to adopt chromatographic techniques. The HPLC assay methods for 6β-OHF described thus far have involved tedious sample pre-treatment, liquid–liquid extraction followed by alkaline wash [4,10], or gradient elution [4,5]. The purpose of the present study is to develop a simple method for the routine measurement of 6β-OHF in urine by using isocratic HPLC combined with a C18 cartridge column extraction. The method could be applied to urine samples from pregnant women receiving antiretroviral agents to determine the hormonal ratios, which, in turn, can be used to differentiate CYP3A activity.

2. Experimental

2.1. Chemicals and instruments

All chemicals were of HPLC or reagent-grade and were obtained from Fisher Scientific (Fair Lawn, NJ) or Sigma (St. Louis, MO). 6β-Hydroxycortisol (6β-OHF) and 6β-hydroxyprednisolone (6β-OHPSL) as an internal standard purchased from Steraloids Inc. (Wilton, NH). Octadecylsilyl (C18) cartridges (Extract-Clean Hi-Load C18, 100 mg) used for solid phase extraction were obtained from Alltech Associate Inc. (Deerfield, IL)

2.2. HPLC apparatus and conditions

The HPLC system used consisted of a Waters M-510 HPLC pump, a model 717 Plus (WISP) auto injector, a C18 reversed-phase column (Novapak, 3.9 mm × 300 mm), a column oven mod-

ule with the temperature control module set at 45°C, a model 441 UV detector (Waters Assoc., Milford, MA) and a HP integrator model 3396A (Hewlett–Packard, Avondale, PA). The detection wavelength was fixed at 245 nm and the sensitivity was 0.002–0.01 aufs. The mobile phase consisting of acetic acid/acetonitrile/50 mM KH_2PO_4, (0.2/9/90.8, v/v) was pumped at a flow rate of 1.1 ml/min for analysis of 6β-OHF.

2.3. C18-column extraction

One milliliter aliquot of each urine sample, to which was added 6β-OHPSL (500 ng) as the internal standard, was alkalinized by adding 100 μl of 1 M NaOH. After vortex-mixing, the mixture was immediately loaded onto the C18-cartridge, which had been pretreated with 1 ml of methanol followed by 1 ml of distilled water. After the cartridges were positioned on a 10-port vacuum elution manifold (Varian), samples were drained away and the cartridges were washed under the reduced pressure with 1 ml of water followed by 1 ml of hexane. 6ß-OHF and internal standard were subsequently eluted with 2 ml of ethyl acetate into glass tubes. The effluents were dried under nitrogen and reconstituted in 200 μl of 10% acetonitrile. Twenty microliters of each reconstituted sample was injected into the HPLC.

2.4. Determination of urinary F concentration

Urinary F was determined by HPLC with minor modification of a previously published method [11]. In brief, 1 ml of each urine sample spiked with internal standard (methylprednisolone 250 ng) was alkalinized by adding 100 μl of 1 M NaOH. After vortexing, each sample was loaded onto a C18-cartridge, which was pre-activated with methanol and water (1 ml each). The cartridge was washed subsequently with 20% acetone, water, and hexane (1 ml each), and then eluted with 1 ml of ethyl ether. The ether extracts were evaporated to dryness and were reconstituted with 200 μl of 25% acetonitrile. Twenty microliters of each sample was injected onto the HPLC column. The HPLC conditions were identical to those used for 6β-OHF, except for a column temperature setting of 25°C and a mobile phase solvent consisting of acetonitrile/50 mM KH_2PO_4 (22/78, v/v).

2.5. Clinical sample collection

Morning urine samples (07:45–09:30 h) were collected from 6 HIV-positive pregnant women at 2nd or 3rd trimester. Further sample collection during the post partum period were done in three of the six subjects. Pregnant subjects were recruited, who were treated with highly active antiretroviral regimens including protease inhibitors. Informed consent was obtained from all subjects and the study was approved by the Committee for Human Research of the University of California San Francisco, Institutional Review Board, which was constituted according to the US FDA guidelines.

3. Results

A typical chromatogram from a pregnant urine sample is shown in Fig. 1. The retention times of internal standard and 6β-OHF were 9.9 and 12.6 min, respectively. The detection limit of urinary 6β-OHF was 20 ng/ml (2 ng injected onto the column) with a signal to noise ratio better than 3.

Linearity of the calibration curve was determined by plotting the peak height ratio of 6β-OHF to internal standard against 6β-OHF concentration in water. A linear response was obtained for 6β-OHF concentrations from 30 to 1000 ng/ml. The equation of the line calculated by regression analysis was $Y = 0.0011X - 0.0036$ ($r = 0.9999$), where Y is the peak height ratio and X, the concentration (ng/ml).

The assay precision and recovery of 6β-OHF from the C18-cartridge were examined by adding known amounts of authentic 6β-OHF (50 and 300 ng/ml) to urine samples containing 135 ng/ml of 6β-OHF (Table 1). The difference (relative error) between the theoretical concentration and the observed concentration was $\sim 1\%$ (0.9 and 1.1%). The recovery of 6β-OHF from urine was determined by comparing the peak height ratio of 6β-OHF to internal standard from the sample to

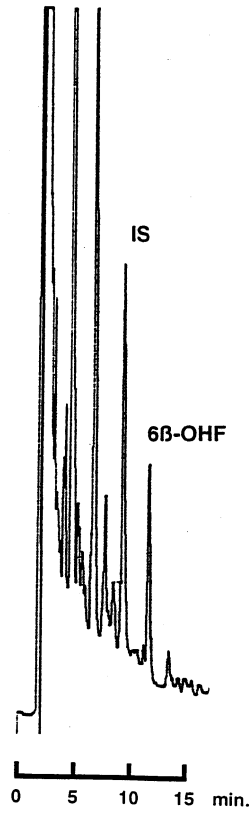

Fig. 1. Typical chromatogram for determination of 6β-OHF in a pregnant urine. The concentration of 6β-OHF was 442.2 ng/ml.

which 6β-OHF was added before and after C18-cartridge extraction. The recoveries were more than 93% for both concentrations.

Analytical precision for urinary 6β-OHF was further evaluated by intra- and inter-day assay validation at the three different concentrations, 112.9, 193.5 and 673.5 ng/ml (Table 2). For intra-day assay precision, five sets of each of the control samples were assayed on the same day. For the inter-day assay, five samples of each concentration were assayed on 5 different days over 4 weeks. The CV values of the intra-day assay and inter-day assay assessments were less than 5.6 and 8.1%, respectively.

We applied our method to the urine samples collected from six HIV positive pregnant women receiving highly active anti-retroviral regimens including protease inhibitors (Table 3). The ratio of 6β-OHF/F were different between the trimesters. Case 2 reveals an increase in the ratio from 5.4 during the 2nd trimester to as high as 20.5 during the 3rd trimester. This was followed by a decrease to 5.8 post partum. The fall of the ratio post partum was also observed for cases 1 and 3. These observations suggest that CYP3A4 activity increase during pregnancy and decreases following delivery.

4. Discussion

The use of a C18-cartridge provides a simple and rapid sample clean-up procedure compared with liquid–liquid extraction which was used previously for extraction of 6β-OHF [4,10]. Lykkesfeldt et al. described a C18-cartridge column extraction for 6β-OHF and successively achieved its quantitative determination with gradient HPLC [5]. We further simplified the C18-cartridge extraction for routine isocratic HPLC. In the previous reports, organic extracts from the C18-cartridge were washed with alkaline solution to remove acidic contaminants from the extracts [5]. With this current method, urine samples were alkalinized prior to the C18-cartridge extraction to eliminate the washing procedure from the sample clean-up process. This modification does not alter the recovery or the chromatographic read-

Table 1
Assay precision and recovery of urinary 6-OHF from C18-cartridge

Added concentration (ng/ml)	Observed Concentration (ng/ml)	Relative error (%)	Recovery (%)	CV (%)
0 ($n=5$)	135.0 ± 4.2			
50 ($n=5$)	182.9 ± 7.7	1.1	93.2 ± 3.8	4.1
300 ($n=5$)	437.7 ± 17.5	0.9	93.9 ± 3.5	3.7

Table 2
Intra-day and inter-day assay precision for urinary 6-OHF

Sample	Intra-day ($n = 5$)		Inter-day ($n = 5$)	
	Concentration (ng/ml)	CV (%)	Concentration (ng/ml)	CV (%)
Low	112.9 ± 6.3	5.6	108.2 ± 8.8	8.1
Medium	193.5 ± 8.5	4.4	189.5 ± 9.2	4.9
High	673.8 ± 20.2	2.9	653.1 ± 32.1	4.9

Table 3
CYP3A4 activity determined by the ratio of 6-OHF/F in six pregnant women receiving antiretroviral agents[a]

Case	Trimester (weeks)	Concentration (ng/ml)		Ratio	Medication	
		6-OHF	F	6-OHF/F	PI	RTI
1	3rd (34)	416.4	37.2	11.2	IDV	AZT, 3TC
	Postpartum	90.0	25.1	3.6	IDV	AZT, 3TC
2	2nd (21)	461.7	85.3	5.4	IDV	d4T, 3TC
	3rd (33)	630.3	30.8	20.5	IDV	d4T, 3TC
	3rd (34)	674.1	38.9	17.3	IDV	d4T, 3TC
	Postpartum	140.9	24.4	5.8	IDV	d4T, 3TC
3	3rd (36)	442.2	105.1	4.2	NFV	AZT, 3TC
	Postpartum	154.9	40.9	3.8	NFV	AZT, 3TC
4	3rd (33)	540.4	99.6	5.4	NFV	AZT, 3TC
5	2nd (29)	204.7	34.0	6.0	NFV	ABC, NVP
6	2nd (21)	483.3	126.2	3.8	NFV	AZT, 3TC

[a] PI, protease inhibitors; RTI, riverse transcriptase inhibitors; IDV, indinavir; NFV, nelfinavir; AZT, zidovudine; 3TC, lamivudine; d4T, stavudine; ABC, abacavir; NVP, nevirapine.

ing. Although 6β-OHF and internal standard are labile under alkaline conditions, no peak height reduction was observed if the urine sample was loaded onto the C18-cartridge within 5 min of adding NaOH solution to the sample. The reproducibility confirmed in the recovery (Table 1) and intra- and inter-day variations (Table 2) was comparable to those of previous reports [4,5].

Simultaneous determination of urinary F and 6β-OHF has been reported by Lykkesfeldt [5]. They used gradient HPLC followed by C18-cartridge extraction. The method, however, could not be applied to the urine samples collected from pregnant women receiving anti-retroviral medication owing to an interference with 6ß-OHF. Since many interfering peaks, which may be derived from other steroidal contaminants circulating high level during pregnancy or polar metabolites of anti-retroviral drugs, appeared around capacity ratio of 6β-OHF, quantitative determination was hard to be achieved with gradient elution. We, therefore, need to optimize HPLC conditions for determination of 6β-OHF in pregnant urine with isocratic elution.

Isocratic reversed-phase HPLC for 6β-OHF has been reported by Fleishaker et al. [6]. However, their chromatographic conditions when attempted in our group could not be applied to pregnant urine samples without any modifications. We optimized elution conditions including analytical column, mobile phase, and increased column temperature to achieve sufficient peak separation without using gradient elution. The most important factor for peak separation was the increase in column temperature to 45°C.

Optimized HPLC conditions could be applied to six HIV positive pregnant women receiving highly active anti-retroviral therapy. At least, zi-

dovudine monothrapy and triple therapy including indinavir, nelfinavir, lamivudine, stavudine, abacavir or nevirapine did not cause any problems on the chromatogram for determination of 6β-OHF. Clinical application in the context of other anti-HIV medications needs to be determined.

The ratios of 6β-OHF/F were 3.8–6.0 at 2nd trimester, 4.2–20.5 at 3rd trimester and 3.6–5.8 post partum period. These values were comparable with reported ones (2.8–26.9) determined in spot urine samples collected from healthy subjects [5]. Significant suppression on the ratios was not observed in the patients even though they were all treated with protease inhibitor, indinavir or nelfinavir, both possessing inhibitory activity on CYP3A4 [3]. The 6β-OHF/F ratios seemed to be varied between trimester periods preferably rather than between anti-retroviral medications.

Frantz et al observed high levels of 6β-OHF in women in the 3rd trimester and toxemic patients [12]. They suggested increased conversion from F to 6β-OHF in these subjects. Our preliminary observations support these findings. Compared to post partum, higher ratios of 6β-OHF/F were observed during the 3rd trimester in three subjects (Table 3). The elevation of 6β-OHF/F ratio may correspond to the increased activity of CYP3A4 during pregnancy, which may be supported by the following evidence: messenger RNAs of CYP3A4 isozymes have been found in the placenta [13] and glucocorticoids (cortisol and corticosterone) circulating at high concentrations during pregnancy are known to act as a CYP3A4 inducer [3,14]. It is, therefore, considered that placental CYP3A4 and induction of the enzyme activity by high levels of glucocorticoids in the late gestational period contribute to the high 6β-OHF/F ratio observed in the 3rd trimester. Further investigation is required to confirm this hypothesis.

Changes in CYP3A4 activity during pregnancy can alter the pharmacokinetics of protease inhibitors and thereby dose adjustment may be required. We observed that plasma indinavir concentration decreased with the elevation of 6β-OHF/F ratio during the 3rd trimester in cases 1 and 2. The indinavir AUCs at third trimester (9040 and 7116 nM h in cases 1 and 2, respectively) were extremely low compare to post partum AUCs (24 187 and 50 086 nM h in cases 1 and 2, respectively) even though the anti-retroviral regimen remained unchanged. Similar changes were also observed in C_{max} and C_{min}. Thus, because the change in pharmacokinetic of indinavir is profound, it might be important for assessing change in metabolic enzyme activity to determine urinary 6β-OHF during the pregnancy. The method can be applied to characterize such pharmacokinetic alterations.

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