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Urinary 6 β -hydroxycortisol: a validated test for evaluating drug induction or drug inhibition mediated through CYP3A in humans and in animals

Received: 20 June 2003 / Accepted: 17 September 2003 / Published online: 6 November 2003
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Abstract 6 β -Hydroxycortisol (6 β -OHF) urinary excretion has, for a long time, been considered a marker of drug induction and, more recently, of drug inhibition in humans and in laboratory animals, but its specificity is still under debate. In this work, we review 277 papers devoted to 6 β -OHF urinary excretion. We have evaluated factors that could modify 6 β -OHF excretion and, thus, could explain contradictory results. We have examined the effect of the analytical techniques on physiological values. Intra- and inter-individual variability and the effect of circadian rhythms on urinary excretion of 6 β -OHF as well as cortisol and 17-hydroxycorticosteroids have been evaluated. We also give an overview of drugs that induce, inhibit or have no effect on 6 β -OHF. For inducing and inhibiting drugs, we calculated the ranges of variation of 6 β -OHF excretion from the results indicated in the different papers. This work was done for well-known inducers, such as anti-convulsants, but also for other inducing or inhibiting drugs found in the literature. The time-course of variation in 6 β -OHF excretion when different drugs are co-administered was also investigated. The potential relationship between cytochrome P_{450} 3A4 (CYP3A4) polymorphism and 6 β -OHF excretion was studied. Finally, the interest of 6 β -OHF urinary excretion was compared with that of other tests proposed to measure CYP3A4 activity. This review demonstrates that 6 β -OHF urinary excretion is a good test to evaluate

drug-metabolising enzyme inducing or inhibiting properties of drugs when the subjects are their own controls, but this test is not reliable enough to measure actual CYP3A4 activity.

Keywords 6 β -Hydroxycortisol · Drug induction · Drug inhibition

Introduction

The activity of many enzymes that are involved in drug or pollutant metabolism can be increased after exposure to these substances in humans as well as in animals. This induction can have beneficial (protective) effects, but can also be at the origin of drug–drug interactions. Clinical consequences of the induction or the inhibition of drug-metabolising enzymes have been largely reviewed in the past [1, 2, 3, 4, 5].

In vivo assessment of enzyme induction in humans has been performed by the measurement of the plasma half-life of probe drugs. Antipyrine has been widely used for this purpose. Although a decreased plasma half-life of antipyrine may often reflect induction of drug-metabolising enzymes, many other factors may alter the absorption, distribution, metabolism and elimination of antipyrine. Several non-invasive procedures for measuring enzyme induction in humans have been proposed. Among these procedures, CO₂ breath tests have become the more commonly used. After administration of drugs, usually labelled with the stable isotope ¹³C, the rate of exhalation of ¹³CO₂ is measured by mass spectrometry. Erythromycin *N*-demethylation has been demonstrated to be mediated by cytochrome P_{450} 3A4 (CYP3A4) [6] and a breath test using erythromycin as substrate has been suggested to be a marker of cytochrome P_{450} 3A (CYP3A) activity [7]. However, these techniques are time consuming and expensive, and, therefore, enzyme induction has also been studied indirectly by measuring certain potential markers of drug-metabolising enzymes

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in urine. Among these markers, 6 β -hydroxycortisol (6 β -OHF: 6 β , 11 β , 17 α , 21-tetrahydroxypregn-4-ene-3,20-dione) has been widely used, since its discovery in human urine after cortisol loading by Burstein et al. in 1954 [8]. However, its usefulness as a non-invasive marker of microsomal CYP3A activity is still under debate [9].

The aim of this review was to report what is known about the variations of urinary 6 β -OHF and its ratio to free cortisol. This ratio is considered a safe and simple test to evaluate induction [5, 10, 11, 12] or inhibition [13, 14] due to drug treatment or pollutant exposure in humans and in animals, as no probe is administered.

Metabolism

Cortisol is metabolised by reduction of the A ring, reduction of the 20-ketone function and by 6 β -hydroxylation. The latter reaction leads to the formation of 6 β -OHF, essentially in the liver [15, 16, 17], but also in the adrenal glands [16, 17, 18, 19], kidney [16] and placenta [16]. These two last organs represent only very low metabolic capacities in humans [16]. 6 β -OHF is excreted in urine by the kidney in a polar unconjugated form [20, 21]. It represents about 1% of the total cortisol metabolites in urine [22]. Ged et al. [23], using human liver microsomes, were the first to demonstrate that the transformation of cortisol to 6 β -OHF is catalysed by CYP3A. The CYP3A subfamily is the most abundant cytochrome P_{450} in the liver. It consists of at least three isoforms, CYP3A4, CYP3A5, and CYP3A7. CYP3A4 accounts for approximately 30–40% of total cytochrome P_{450} content in human adult liver and small intestine. CYP3A5 presents 83% homology with CYP3A4. It is also expressed in human adult liver, but usually at a much lower level than CYP3A4 [24]. However, relatively high levels of metabolically active CYP3A5 are expressed in the liver of 30–40% of Caucasians [25]. CYP3A7 is expressed in fetal liver and is undetectable in most individuals older than 1 year [26]. Cortisol is a substrate for CYP3A4, which is also responsible for the metabolism of a large variety of drugs—approximately half of all in use today [27]. Using human livers, Abel et al. [28] demonstrated that the human metabolism of cortisol is very complex (Fig. 1) and highly variable among individuals. There was considerable inter-individual variation in metabolic profiles obtained after incubation with liver microsomes. 6 β -OHF varied from 2.8% to 31.7% of the total metabolites produced, and this could explain the large inter-individual variability in 6 β -OHF excretion. The same authors, using various inhibitors, confirmed that 6 β -OHF was produced by CYP3A [29]. The specificity of the measurement of CYP3A4 activity is mainly provided by the use of the ratio 6 β -OHF/cortisol. Another confirmation came from Lemoine et al. [30], who demonstrated that a patient grafted with a liver with no CYP3A4 activity excreted very low amounts of 6 β -OHF after transplantation. CYP3A4 (as well as CYP3A5) is also present in human intestine [31] and in the kidney, but only in 14% of human adults [32],

and their contribution to cortisol metabolism is not completely elucidated.

Analytical methods

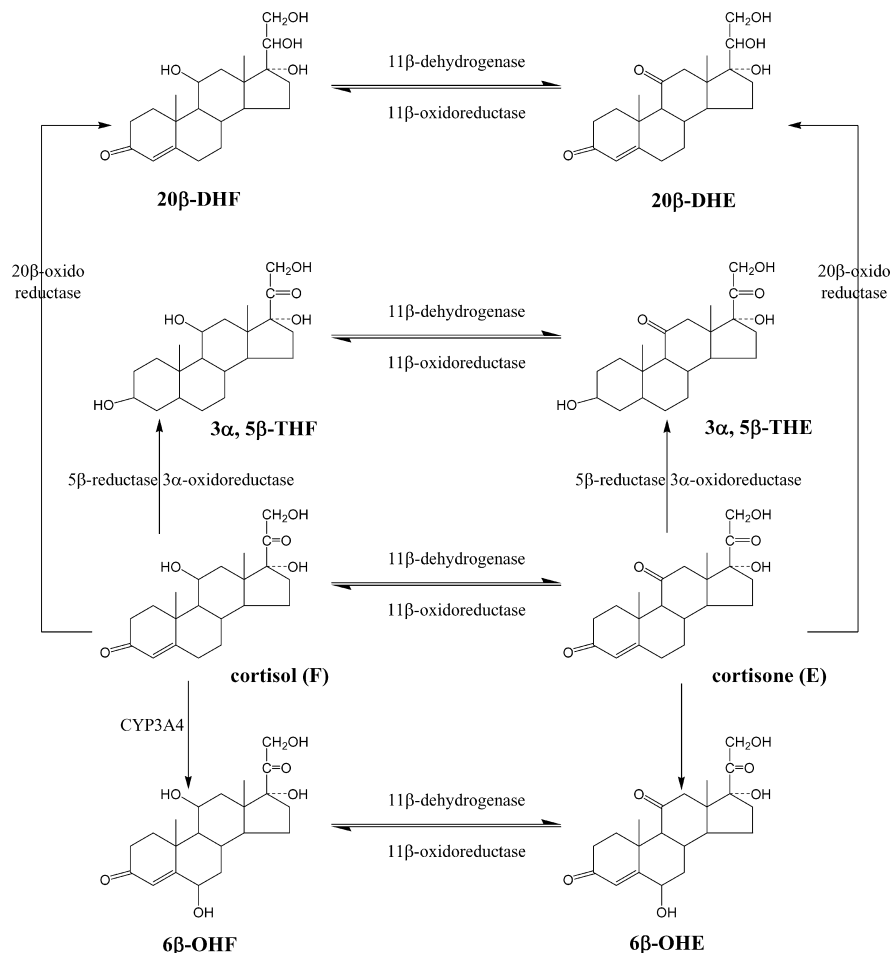
Many techniques have been described for the determination of 6 β -OHF in urine. The earliest ones used paper chromatography [18, 20, 33], thin layer chromatography [34, 35, 36] or column chromatography [37, 38]. The detection of 6 β -OHF was done using the Porter and Silber colorimetric reaction [18, 20, 35, 36, 39] or the blue tetrazolium reaction [40, 41]. However, these techniques lack specificity, and values were generally largely higher than those obtained with other techniques (Table 1). They are now completely abandoned and replaced by more specific methods.

Since 1977, three radio immunoassays (RIA) have been published, all using the same technique [42, 43, 44]. In each laboratory, rabbit polyclonal antibodies were raised according to the method of Park et al. [45]. 6 β -OHF–21-hemisuccinate conjugated to bovine serum albumin was used, except by Nahoul et al. [44], who did not remove the acetate group at C-21. They used the 3 (O-carboxymethyl) oxime derivative coupled to bovine serum albumin as immunogen. ^3H -6 β -OHF was used as the competitive ligand. Separation of free and bound 6 β -OHF was performed by the addition of dextran-coated charcoal. The analytical characteristics of the three techniques were very similar. A protein-binding assay was developed in 1981 by Bowler-Wong et al. [46].

The first technique using high performance liquid chromatography (HPLC) for the measurement of 6 β -OHF in urine was reported in 1979 by Roots et al. [47]. Since that date, several other HPLC techniques have been published. The oldest ones allowed only to measure 6 β -OHF by HPLC, the other steroids being determined by various other techniques such as colorimetry [Porter and Silber reaction for total 17-hydroxycorticosteroids (17-OHCS¹)] or RIA (for free urinary cortisol). Most HPLC methods necessitated a preliminary extraction step, and separation of 6 β -OHF from other steroids was achieved either by liquid–liquid partitioning [47, 48] or by reverse phase chromatography [49, 50]. Dumont et al. [51] used the true first internal standard (Δ^4 -pregnenediol-3one), gradient elution and ultraviolet (UV) detection. Recently, Homma et al. [52] have proposed a new HPLC method for measuring 6 β -OHF in the urine of pregnant women. Goto et al. [53] proposed a HPLC technique in which 6 β -OHF was quantitatively transformed into the 21-(9-anthroyl) derivative and then chromatographed in a normal phase column. Detection was achieved by fluorescence. Thereafter, some

¹ Urinary 17-OHCS group: all the urinary steroids possessing a function 17,21 dihydroxy-20 ketone, i.e. di and tetrahydroxy derivatives of cortisol, cortisone and 11 deoxycortisol. This old nomenclature is no more in use as each of these compounds is now specifically measured.

Fig. 1 Proposed metabolic pathways of cortisol in human liver microsomes [28]



techniques allowing for the simultaneous determination of 6β-OHF and cortisol, either with UV detection [54, 55, 56] or fluorescence [57] have appeared. In this last method, 6β-OHF, cortisol and fludrocortisone (internal standard) were extracted and dissolved in methanol and then oxidised by cupric acetate. The resulting compounds were converted into fluorescent derivatives with 1,2-diamino-4,5-methylenedioxybenzene. Then, the derivatives were separated on a reversed-phase column and detected by fluorimetry. Simultaneous determination of six urinary glucocorticoids is now possible using HPLC [58]. The intra-assay and inter-assay coefficients of variation were less than 5% and 10%, respectively. Different protocols have been applied to the determination of 6β-OHF in different studies.

The simultaneous detection of 6β-OHF and cortisol permitted the reduction of the fluctuations observed during the course of the day for these two compounds [59]. The daily excretions of 6β-OHF as a function of the different methods are presented in Table 1. In spot urine, Lykkesfeldt et al. [56] found a 6β-OHF/cortisol ratio of 10.09 ± 6.89 (mean \pm SD). This value is very similar to that obtained by Bienvenu et al. (9.34 ± 4.50) [54].

In 1981, Nambara's team [60] developed a direct competitive enzyme immunoassay in order to measure 6β-OHF in urine. The hapten was 6β-OHF hemisucci-

nate conjugated to bovine serum albumin, and polyclonal antiserum was raised in rabbits. Competition was performed using 6β-OHF conjugated to β galactosidase. This technique was highly specific for 6β-OHF. 6β-hydroxycortisol was the only interfering steroid among those tested (1.4% for 50% inhibition). Subsequently, Zhiri et al. [61] and Patel et al. [62] developed competitive enzyme-linked immunosorbent assays (ELISAs). They used the antiserum raised in Nambara's laboratory, but the competitive ligands were 6β-OHF conjugated to horseradish peroxidase and to penicillinase, respectively. These three techniques showed the same analytical performances in terms of sensitivity, specificity and precision. More recently, new ELISAs have been developed by Yeung et al., using a polyclonal [63] or a monoclonal antibody [64]. This last technique had a lower sensitivity than the three techniques using polyclonal antibodies (200 ng/ml versus 1 ng/ml), which can be a problem when investigating decreased levels of 6β-OHF, in case of CYP3A4 inhibition. A ready-to-use kit based on the technique developed by Zhiri et al. [61] is now commercially available (Stabiligen, 54600 Villers-Les-Nancy, France). It has been used in several studies [65, 66, 67, 68, 69, 70, 71, 72, 73, 74].

As early as in 1988, a first technique using gas chromatography/mass spectrometry (GC/MS) (selected ion

Table 1 Usual values of 6β -hydroxycortisol (6β -OHF) urinary excretion in healthy males, according to different analytical methods. UV ultraviolet

	Number of subjects	6β -OHF daily excretion ($\mu\text{g/day}$)	Range
Paper chromatography			
Frantz et al. [20]	6	436	309–510
Katz et al. [18]	8	298 ± 82.5	156–378
Werk et al. [33]		400 ± 220	150–840
Thin-layer chromatography			
Thrasher et al. [36]	27	350 ± 200	100–800
Yamaji et al. [35]	8	346 ± 115	
Column chromatography			
Berman and Green [37]	13	490 ± 240	180–1160
Pal [38] fluorimetric detection	100	377 ± 60	289–457
Radio immunoassay			
Kishida et al. [42]	6	487 ± 144.8	316–726
Park [43]	10	286 ± 54	216–333
Nahoul et al. [44]	14	238 ± 66	137–348
Enzyme immunoassay			
Hosoda et al. [60]	27	237 ± 84	
Zhiri et al. [61]	10	228 ± 64	
Yeung et al. [63]	4	370 ± 105	
Yeung et al. [64]	/	485 ± 100	
High-performance liquid chromatography			
Roots et al. [47] (UV)	12	273 ± 74.8	143–421
Dumont et al. [51] (UV)	23	199 ± 79.8	
Ono et al. [49] (UV)	10	214 ± 41.4	174–295
Bienvenu et al. [54] (UV)	19	163.7 ± 70.1	93.2–345.8
Bidart and Lesgards [55] (UV)	76	165 ± 30	
Goto et al. [53] (fluorimetry)	7	303 ± 110	212–503
Inoue et al. [57] (fluorimetry)	47	145 ± 52.9	
Ohno et al. [84] (mass spectrometry)	10	167 ± 45	113.8–270.3
Gas chromatography/mass spectrometry			
Palermo et al. [76]	17 ^a	164 ± 59	70–261

^aMale subjects

monitoring) was developed by Ishibashi et al. [75] for the simultaneous determination of 6β -OHF and free cortisol in urine. [$^2\text{H}_3$] cortisol was used as an internal standard. Palermo et al. [76] proposed another GC/MS technique for the measurement of cortisol and related 3-oxo-4-ene steroids (particularly 6β -OHF) in urine. Furuta et al. [77] have published a new GC/MS method for the determination of 6β -OHF in urine, and Luceri et al. [78] have proposed a new quantitative GC/MS method with isotope dilution. In all these methods, steroids are derivatised in methoxime-trimethylsilyl analogues. The specificity and sensitivity of these techniques are very good. Liquid chromatography/atmospheric pressure chemical ionisation–mass spectrometry has been applied to the simultaneous determination of cortisol and 6β -OHF in human [79] and monkey urine [80]. Normal values determined by HPLC or GC/MS are generally lower than those found by other techniques (Table 1). Among all these techniques, only specific ones should be recommended, i.e. ELISA, HPLC and GC/SM.

Biological variations

Intra-individual and inter-individual variations

6β -OHF excretion has been found to be variable from one day to another in the same subject. For instance,

Ohnhaus et al. [81] found that 6β -OHF excretion varied from $297 \pm 43 \mu\text{g/day}$ to $341 \pm 60 \mu\text{g/day}$ in seven adults tested on three different days. Zhiri et al. [82] measured 6β -OHF daily excretion in six and eight subjects during two or three different days. In these studies, the observed variations were not statistically significant. In contrast, Gerber-Taras et al. [83] found a highly significant variation when they measured 6β -OHF excretion at two separate occasions in six subjects. More recently, Ohno et al. [84] measured 6β -OHF and cortisol excretion with a very specific LC/MS technique in ten subjects during a 2-week period (daily urinary collection for 9 days). They showed that intra-individual variations in 6β -OHF (5.8–23.3%) and cortisol (7.1–22.2%) excretion were relatively low, which indicated that 6β -OHF/cortisol ratios are constant for a single individual. Similarly, Lee [85] did not find any significant variation when the ratio 6β -OHF/cortisol was measured during six consecutive days.

As 6β -OHF formation has been related to CYP3A4 activity, this inter-individual variability in 6β -OHF excretion is the consequence of the variability in the level of expression of CYP3A4, which has been shown to vary up to 60-fold in human adult livers [86]. Yamamoto et al. [87] hypothesised that if a considerable amount of exogenous cortisol is administered, the inter-individual variability of measuring 6β -OHF would be estimated more precisely. This precision is necessary when drugs with a narrow therapeutic index are metabolised

through CYP3A4. Indeed, an excellent correlation ($r=0.867$; $P<0.001$) between 24h-excretion of 6β -OHF after the administration of exogenous cortisol and doxetaxel clearance was obtained. This indicated that inter-patient variability of CYP3A4 activity could be estimated by this method [88].

Variations due to gender

Fifteen studies have reported comparison of 6β -OHF excretion in adult males and females (Table 2). For the studies published before 1990, the results are controversial; some authors found higher excretion in males than in females [43, 44, 61], and others found the opposite [18], but the variations were generally not statistically significant. The only statistically significant results were those obtained by Pal et al. [38], who found an 11% higher excretion ($P<0.001$) in 100 women compared with 100 men. More recent studies, performed after 1990 and which used HPLC [85, 89], ELISA [70] or GC/MS [76], found 6β -OHF excretion slightly lower in females than in males. However, these differences were statistically significant in only three studies [52, 70, 90]. The study of Inagaki et al. [90] presents a very significant difference between the 6β -OHF/cortisol ratio of males and females in Japanese subjects. It is the first study demonstrating such a high difference in 6β -OHF/cortisol

urinary excretion according to gender. Additional studies should be performed in order to verify these data in other groups of populations.

In order to take into account the diurnal variation in both 6β -OHF and cortisol excretion, it has been recommended to measure these two compounds in each urine sample. For example, Palermo et al. [76] found the excretion of cortisol clearly higher in males (35.2 ± 12.6 $\mu\text{g/day}$) than in females (23.2 ± 13.2 $\mu\text{g/day}$) and Ng et al. [70] obtained similar results. The urinary ratio 6β -OHF/creatinine was slightly higher in women (median = 47 $\mu\text{g}/\text{mmol}$) than in men (median = 29 $\mu\text{g}/\text{mmol}$) [91]. Before phenytoin dosing, no difference in the ratio 6β -OHF/17-OHCS was observed between males and females. After the administration of phenytoin, the increase of this ratio was more pronounced in females than in males [92].

In children, several authors [36, 93, 94, 95] have found no effect of sex on the urinary excretion of 6β -OHF, but Nakamura and Yakata [96] found 6β -OHF excretion higher in boys (5–10 years) than in girls. This difference disappeared when 6β -OHF/cortisol ratios were considered. Palermo et al. [76] found that young females (before the age of 12 years) excreted more urinary 6β -OHF (+23%) than males of the same age. Inversely, after the age of 13 years, females excreted lower quantities (–22%) than males. These variations according to gender are generally low and not

Table 2 Comparison of 6β -hydroxycortisol (6β -OHF) urinary excretion ($\mu\text{g/day}$) between male and female adults. *ELISA* enzyme linked immunoassay, *GC/MS* gas chromatography/mass spectrometry, *HPLC* high performance liquid chromatography, *RIA* radioimmunoassay

Reference	Age	Males		Females		Variation %	Technique Used
		Number	Mean \pm SD	Number	Mean \pm SD		
6β-OHF excretion ($\mu\text{g/day}$)							
Katz et al. [18]		8	298 \pm 74	8	399 \pm 95	+34*	Colorimetry
Thrasher et al. [36]	15–69	27	350 \pm 200	10	260 \pm 110	–26°	Colorimetry
Yamaji et al. [35]	20–29	8	346 \pm 115	7	517 \pm 143	+49*	Colorimetry
Park [43]	-	10	286 \pm 54	8	233 \pm 80	–19°	RIA
Pal [38]	18–62	100	377 \pm 60	100	417 \pm 81	+11*	Fluorimetry
Bowler-Wong et al. [46]	30 \pm 6	9	300 \pm 60	10	313 \pm 55	–15°	Protein-binding
Nahoul et al. [44]	20–40	14	238 \pm 66	15	210 \pm 93	–12°	RIA
Nakamura and Yakata [96]	26–30	28	200.6 ^a	14	239.5 ^a	–19°	HPLC
Zhiri et al. [61]		10	228 \pm 64	10	193 \pm 42	–15°	ELISA
Krause et al. [244]	29 \pm 7	10	350 \pm 70	10	240 \pm 50	+45	RIA
Barton et al. [89]	< 35	8	238.4 ^b	6	223.3 ^b	–7°	HPLC
Lee [85]	20–60	18	168.8 \pm 92.7	22	177.5 \pm 117.7	+5°	HPLC
Ng et al. [70]	15–84	75	311 \pm 231	68	233 \pm 147	–25*	ELISA
Palermo et al. [76]	-	17	164 \pm 59.2	18	108 \pm 55.1	–34**	GC/MS
Ratio 6β-OHF/free cortisol excretion (%)							
Nakamura and Yakata [96]	26–30	28	6.1 \pm 2.6	14	7.4 \pm 2.4	+21°	HPLC
Iganaki et al. [90]	18–24	238	7.96 \pm 4.59	249	15.91 \pm 7.76	+100**	HPLC
Ng et al. [70]	15–84	/	5.83 \pm 3.16	68	6.01 \pm 2.76	+3°	ELISA
Palermo et al. [76]	-	17	5.10 \pm 2.32	18	4.88 \pm 1.37	–4°	GC/MS

^aStandard deviation not indicated

^bMedian value

^cNon-significant

* $P<0.05$

** $P<0.01$

*** $P<0.001$

statistically significant. Thus, it does not appear necessary to separate populations as a function of gender in further studies.

Variations due to age

In adults, all studies comparing young and old subjects found a decrease in urinary excretion of 6 β -OHF in elderly (Table 3). These decreases were comprised between 17% and 46%. Nakamura and Yakata [96] found the same trend of decreasing excretion with age. In males and in females, the differences (calculated on mean values) between groups aged 14–20 years and 50–67 years were –34% and –40%, respectively. In contrast, Yamagi et al. [35] did not find any decrease between the age of 20 and 29 years and 50 and 59 years in males or in females. Similarly, Dolara et al. [97] did not find any difference in the 6 β -OHF/17-OHCS ratio in control subjects before and after the age of 40 years.

In children aged 7–12 years, 6 β -OHF excretion was largely lower than in adults, in males as well as in females [76]. Similar results were found by Thrasher et al. [36], but the difference was not significant when the ratio 6 β -OHF/17-OHCS was taken into account. On the contrary, Nakamura and Yakata [96] found the 6 β -OHF/cortisol excretion ratio higher in children than in adults.

In newborns, it is quite impossible to collect 24-h urine, so the results have been expressed in ng/ml. Kishida and Fukushima [42] found a very high urinary excretion of 6 β -OHF in neonates (530 \pm 156 ng/ml). Patel et al. [98] found the lowest values in children aged between 15 days and 90 days (31.67 \pm 6.55 ng/ml). These values increased with age by 52% when comparing babies aged 90 days and 195 days [99]. In breast-fed infants whose mothers had been taking anti-tuberculosis drugs (ethambutol and isoniazid), 6 β -OHF excretion was lower than in control infants. These drugs, thus, significantly inhibited 6 β -OHF excretion in infants [99]. Vauzelle-Kervroedan et al. [100] found higher 6 β -OHF/cortisol ratios in premature and in full-term neonates (1–15 days after birth) than in 30- to 359-day-old infants (7.2 \pm 3.8; 7.9 \pm 5.6 and 5.0 \pm 4.7,

respectively). However, Nakamura et al. [101] obtained the opposite results. In premature neonates (less than 37 weeks gestational age), the mean 6 β -OHF/cortisol ratio was 5.3, and, in mature infants, it was 16.5. They found no difference due to gender in neonates and no effect of the mode of delivery—caesarean section or vaginal delivery—on the 6 β -OHF/cortisol ratio, but they did observe a correlation between 6 β -OHF/cortisol and gestational age and also birth weight. In addition, in premature neonates, the ratio was relatively stable between the day of birth and 14 days after. In contrast, in mature neonates, this ratio decreased until day 5 and then stabilised. No relationship between 6 β -OHF/cortisol ratio in mature neonates on the day of birth and the ratio measured in their mothers was found [102]. These variations could, at least in part, explain contradictory results found in literature.

In conclusion, in adults 6 β -OHF/cortisol excretion decreases after the age of 50–60 years. Therefore, this variation factor must be taken into account in studies of 6 β -OHF/cortisol excretion. In children and neonates, the results are controversial, which obviates the need for additional studies.

Effect of hormonal status

In a limited number of healthy children, Palermo et al. [76] demonstrated that 6 β -OHF and cortisol excretions were increased with puberty both in males and in females compared with younger children, but the excretion of both 6 β -OHF and cortisol remained lower than in adults. The ratio 6 β -OHF/cortisol did not vary with age nor with puberty [76].

Katz et al. [18], and Burstein et al. [103] did not find any significant variation in the 6 β -OHF/cortisol ratio in 13 healthy females during the four different weeks of their menstrual cycle. Similarly, Lin et al. [104] did not find any significant difference in the ratio 6 β -OHF/cortisol in Caucasian nor in Asian women during the different periods of their menstrual cycle.

In healthy menopausal women, the 6 β -OHF/cortisol ratio (measured more than 1 year after the last menstruation) did not significantly vary from the values

Table 3 6 β -Hydroxycortisol urinary excretion (μ g/day) as a function of age. HPLC high performance liquid chromatography, RIA radioimmunoassay

Reference	Young adults			Elderly				
	Age (years)	Number	Mean \pm SD	Age (years)	Number	Mean \pm SD	Variation (%)	Technique used
Vestal et al. [209]	19–31	10	478 \pm 142.3	71–84	10	371 \pm 88.5	–22°	RIA
Crowley et al. [156]	20–33	10	333 \pm 139.1	68–88	10	275 \pm 136.0	–17°	RIA
Barton et al. [89]	25–35	8	238.4 ^a (136–1333)	66–81	8	155.2 ^a (91–564)	–35°	HPLC
Lee [85]	20–60	40	175.5 \pm 106.7 (70.8–471.2)	> 61	39	95.8 \pm 82.5 (40.8–346.3)	–45*	HPLC

^aMean values and (ranges)

°Non-significant

* $P < 0.10$

obtained in non-menopausal women, and there was no variation during the different periods of the month [103].

Frantz et al. [105] were the first to demonstrate that 6β -OHF excretion increased during pregnancy—results confirmed by Nahoul et al. [44] and Bowler-Wong et al. [46]. Compared with non-pregnant women, 6β -OHF excretion was increased by about a factor of two ($P < 0.001$) during the third trimester of pregnancy [41, 44, 46, 52]. The amount of daily urinary excreted 6β -OHF and the ratio 6β -OHF/17-OHCS was studied previously, 1 week and 3 months after parturition in a group of nine healthy pregnant women. The values observed before delivery were largely higher (6β -OHF: +212%; 6β -OHF/17-OHCS: +299%) than those obtained in non-pregnant women [106]. The values for 6β -OHF as well as for the ratio 6β -OHF/cortisol returned to control values 3 months after delivery [106]. Pregnant women in true premature labour who were spontaneously delivered less than 24 h after admission had significantly higher 6β -OHF levels (1144 ± 210 ng/ml) than subjects in false premature labour (544 ± 88 ng/ml) [107]. However, Hunter et al. [108] found a too-high diurnal variation to distinguish false from true premature labour with a single urine sample.

Circadian rhythm

Cortisol is well known to be excreted according to a circadian rhythm. Saenger [109] was, in 1983, the first to demonstrate that 6β -OHF excretion paralleled cortisol excretion and was the highest between 0800 hours and 1200 hours and was the lowest around midnight. He demonstrated in non-treated controls, as well as in phenobarbital-treated patients, that the excretions of 6β -OHF and cortisol varied throughout the day, reflecting the changes in adrenal secretion. However, there was no significant diurnal variation when the 6β -OHF/cortisol ratio was taken into account. Since this first report, several authors have obtained similar results with 6β -OHF/cortisol [59, 61, 85, 108] or with 6β -OHF/17-OHCS [109]. 6β -OHF/cortisol ratios are relatively stable around a 24-h period. Similar results have been obtained in 32-week pregnant women [110]. But Ohno et al. [84] and McCune et al. [111] have demonstrated that diurnal variations of 6β -OHF and of cortisol were not really parallel, and, therefore, the ratio varied from 4.3 between 0900 hours and 1300 hours to 12.6 between 1800 hours and 2200 hours. These results are in disagreement with those obtained by other authors who found a good correlation between morning and 24-h 6β -OHF/cortisol ratios [49, 54, 112, 113].

In conclusion, 24-h urine would be the optimal sampling, but it is difficult to collect qualitatively and quantitatively. It could, therefore, be recommended to use a single-spot morning urine sample to measure both 6β -OHF and cortisol and to calculate the ratio 6β -OHF/cortisol. This, indeed, is the usual way of practice in most studies.

Other variations

Obesity

Mean urinary excretion of 6β -OHF, cortisol and 17-OHCS are not different in obese and in non-obese subjects [114].

Genetic factors

Geographic origin In healthy non-smoking and non-alcohol consuming Chinese subjects, 6β -OHF and cortisol output was similar to those reported for healthy Caucasians [71].

Polymorphism of CYP3A4 The ratio 6β -OHF/17-OHCS was measured in the urine of 102 healthy volunteers. There was a wide distribution ranging from 17 to 233.3. No clear bimodality was observed [66]. Rifampicin treatment of these healthy subjects increased 6β -OHF excretion, and, again, there was a wide distribution for the ratio (91.3–917.9). However, here again, no clear bimodality was observed. Moreover, the mean 6β -OHF level was higher in women than in men. These observations do not indicate the existence of a CYP3A4 genetic polymorphism reflected by 6β -OHF excretion, but evoke a sexual dimorphism [66], which is not really confirmed when variations of 6β -OHF excretion according to gender are considered. These results do not mean that CYP3A4 is not polymorphic, but rather that, as some polymorphisms do exist on this gene, they have no influence on 6β -OHF excretion. A recent study in 487 healthy Japanese individuals obtained the same results [90]. Collecting the results obtained between 1966 and 1999 by the use of repeated drug administration, Özdemir et al. [115] suggested that approximately 90% of inter-individual variability in hepatic CYP3A4 might be of genetic origin.

It has now been demonstrated that CYP3A4 presents several polymorphisms. Rebeck et al. [116] found a point mutation ($A_{290} \rightarrow G$) on the 5'-flanking region of the human *CYP3A4* gene with an allele frequency in the U.S. Caucasian population estimated to be 9.6%. It was significantly higher in African Americans (53%) and absent in Taiwanese [117]. The allele frequency for this polymorphism was found to be 5.3% in 199 healthy Dutch Caucasians [118]. This mutation seemed to be associated with higher clinical stage and grade in men with prostatic cancer [116]. Wandel et al. [119] observed a lower CYP3A4 activity associated with this variant allele designated *CYP3A*1B* (systemic clearance in G/G lower by about 30% than in A/A, tested in vivo using midazolam), but Rivory et al. [120] did not find any association between this polymorphism and cyclosporin clearance in liver transplant patients. On the other hand, Sata et al. [121] found that 2.7% of a white population presented another variant allele (*CYP3A*2*: Ser222Pro). In these subjects, the intrinsic clearance for nifedipine was lower than in the wild-type enzyme carriers, but there was no difference for testosterone β -hydroxylation.

In addition, Weslind et al. [122] did not find any influence of this polymorphism on microsomal testosterone hydroxylation in vitro. Another rare allele, designated *CYP3A4*3* (Met445Thr), was discovered in a single Chinese individual by the same team [121]. The allelic frequency of *CYP3A4*3* was 1.1% in Caucasians [123]. Lamba et al. [124] studied the genetic variability of *CYP3A4* in 82 individuals. They found 28 single nucleotide polymorphisms in different regions of the *CYP3A4* gene. None of these polymorphisms was associated with a low in vivo *CYP3A4* activity evaluated through midazolam hydroxylation rate. However, Hsieh et al. [74] demonstrated the presence of three novel mutations on the *CYP3A4* gene. *CYP3A4*4* (Ile118Val) and *CYP3A4*5* (Pro218Arg) mutations were present in respectively 3 and 2 of 102 Chinese subjects. Another allele was found in a single heterozygous Chinese individual (*CYP3A4*6*: A17776, which causes frameshift and an early codon stop in exon 9). These three gene modifications seem to be associated with decreased *CYP3A4* activity, measured using the urinary 6β -OHF/cortisol ratio. These results need confirmation in further studies.

Effects of other polymorphisms of drug metabolising enzymes: Park et al. [125] and Eichelbaum et al. [126] did not find any significant difference between extensive and poor metabolisers of sparteine (*CYP2D6*). In 1989, Leclercq et al. [127] found the same results using debrisoquine as probe drug. 6β -OHF excretion was also very similar in extensive and in poor metabolisers of S-mephenytoin (*CYP2C19*) [68].

After isoniazid treatment alone or in combination with rifampicin, there was no difference in 6β -OHF/17-OHCS excretion between slow and rapid acetylors [128].

Effects of drugs

Since early studies, the excretion of 6β -OHF has been used as an endogenous marker of drug-inducing effects on *CYP450* activity in vivo [129, 130, 131], but not of cytochrome P_{448} ² [132].

Effects of classical inducing drugs

6β -OHF excretion has been demonstrated to be increased by a large number of drugs. Antipyrine was able to induce 6β -OHF excretion as well as the ratio 6β -OHF/17-OHCS, but the induction was quite moderate (maximum twofold) [10, 11, 81, 126, 130, 131, 133, 134, 135, 136, 137, 138].

Carbamazepine increased 6β -OHF excretion in adults by 1.5 to 3.0 times ($P < 0.001$) as a function of the dosage and of the duration of the treatment [71, 139, 140, 141, 142, 143]. In six children (6–14 years old), a treatment with this drug for 4 weeks provoked a statistically significant mean increase of 2.5 times [144].

Phenobarbital also induced 6β -OHF excretion but the increases were quite low (7–88%). They were a function of the dosage and of the duration of treatment [10, 11, 81, 126, 127, 129, 130, 131, 134, 138, 145, 146, 147, 148, 149, 150, 151, 152]. No significant change in the cortisol production rate and its excretion was observed after phenobarbital treatment [22]. Phetharbital (*N*-phenylbarbital) presented larger inducing properties (161–291%) on 6β -OHF excretion [153, 154]. Pentobarbital is also a good inducer of 6β -OHF [37, 155]. Phenytoin has been demonstrated to largely increase 6β -OHF excretion (2 to 7 times) [40, 42, 71, 93, 139, 145, 156, 157, 158, 159]. Valproic acid, another anticonvulsant, was a slight inhibitor of urinary 6β -OHF excretion [140]. Rifampicin has been shown as the best inducer of 6β -OHF excretion. McColl et al. [160] observed an increase in 6β -OHF excretion by 6 times in patients treated with a dose of 600 mg/day for 30 days ($P < 0.001$). Several other studies found an increase in 6β -OHF excretion [43, 47, 64, 67, 126, 131, 137, 138, 161, 162, 163, 164, 165, 166] as well as in the 6β -OHF/cortisol excretion ratio [23, 167, 168, 169, 170, 171] or in the 6β -OHF/17-OHCS ratio [10, 11, 47, 81, 127, 134, 172, 173, 174, 175]. Reichel et al. [176] observed that the induction of cytochrome P_{450} by rifampicin was associated with a less than 10% increase in liver volume and with no variation of portal perfusion. It kept its inducing properties in patients with primary biliary cirrhosis [177]. Rifampicin treatment increased the hepatic clearance of cortisol to 6β -OHF from 13 ± 15 ml min⁻¹ to 72 ± 5 ml min⁻¹, which corresponded to the observed rise in 6β -OHF excretion [178]. Some other anti-tubercular agents, derivatives of rifampicin, such as rifabutine [164] or rifapentine [175, 179], are also inducers, but to a lesser extent than rifampicin. The induction due to rifapentine was dose dependent [175].

It has been observed that different drugs have various lag times to switch on their inducing effect on the hepatic microsomal enzyme activities. It is this latent period that allows speculation on the induction property of a drug, rather than a simple direct enzyme activation. It is very important to know exactly the time-course of drug induction or inhibition for the co-administration of drugs.

Latham et al. [148] performed a study in 17 healthy male subjects that were randomly divided into three groups: 6 subjects received placebo or 120 mg phenobarbital and the 5 subjects in the remaining group received 240 mg phenobarbital for 14 days. At the end of day 14, treatment was abruptly terminated. Placebo had no significant effect, but 240 mg phenobarbital per day produced a highly significant increase in the log-normalised values of the urinary excretion of 6β -OHF

² Cytochrome P_{448} is the old name of a family of cytochromes whose absorbance peak of the complex with carbon oxide shifted from 450 nm to 448 nm. In modern nomenclature, it refers mainly, but not exclusively, to *CYP1A*.

($P < 0.001$ during treatment and $P < 0.01$ after treatment) when compared with the mean of the corresponding pre-treatment control values. Two important observations from these studies were the absence of steady-state conditions for 6β -OHF after 7 days of treatment with phenobarbital and the duration to return to steady-state baseline concentrations after the withdrawal of treatment. 6β -OHF started to decrease after termination of treatment and almost reached baseline values in the fifth week after treatment was stopped. Therefore, the time necessary for a return to baseline concentrations, if dependent on the peak 6β -OHF excretion, may be considerably longer than 1 month. A study was conducted by Crowley et al. [156] to correlate 6β -OHF daily excretion levels with age, smoking habits and duration of treatment with phenytoin. 6β -OHF daily excretion was not significantly influenced by age or by smoking, as indicated before. In contrast, depending on the duration of treatment with phenytoin, 6β -OHF excretion was significantly increased ($P < 0.001$). Induction of urinary 6β -OHF excretion due to antipyrine, phenobarbital or rifampicin as a function of time was studied in 71 non-smoker healthy volunteers by Ohnhaus et al. [131]. Drugs were given chronically for either 7 days or 14 days. Significant increases in 6β -OHF excretion occurred only 2 days after start of administration of rifampicin (600 mg/day or 1200 mg/day), 4 days after administration of antipyrine (1200 mg/day),

but 13 days after administration of phenobarbital (100 mg/day). When rifampicin was administered for 14 days (1200 mg/day), 6β -OHF excretion reached a maximum between day 11 and day 14, and the excretion was significantly greater than on day 7. After stopping rifampicin treatment in a 7-day study, 6β -OHF excretion decreased over the next 6 days, but still remained significantly elevated compared with the original control values [180]. These studies show that measurement of urinary 6β -OHF provides a simple, non-invasive method for monitoring the time-course of enzyme induction by drugs in man.

Effects of other inducers

It is now well known that enzymatic induction can lead to unexpected effects after drug co-administration, and, therefore, the inducing properties of new candidate drugs are usually tested during their development. Table 4 presents the inducing effects of several drugs on 6β -OHF excretion. Most of them have low-inducing properties, except for enflurane and miconazole, which increased the ratio 6β -OHF/17-OHCS by a factor of two. Pentobarbital, which is structurally very similar to phenobarbital, produced a 2.5-fold induction of this ratio. Induction is dose dependent and it is also dependent on the duration of administration. For example, a

Table 4 Inducing properties of drugs (not referred as classical inducers) on 6β -hydroxycortisol (6β -OHF) excretion in adults (expressed as percentage of variation between control and treated subjects)

Drug	Dosage	6β -OHF Variation (%)	6β -OHF/cortisol Variation (%)	6β -OHF/17-OHCS Variation (%)	Reference
52028RP	200 mg/day/15 days	227**			[65]
Bosentan	500 mg/day/8 days	60°			[73]
Dichloralphenazone	1300 mg/day	60**			[133]
Fenofibrate	300 mg/day/8 days	40*			[245]
Glutethimide	500 mg/day/30 days	18***			[246]
Pravastatin	10 mg/day/60 days	69***			[223]
St John's Wort ^a	1200 mg/day/14 days		114**		[184]
Ticlopidine	250 mg/day/14 days	32**			[201]
Zidovudine	1000 mg/day/7 days	56***			[14]
Miconazole	2–10 mg/kg/day/14 days		240°		[247]
Pravastatin	20 mg/day/15 days		233°		[198]
Sulfinpyrazone	800 mg/day/30 days		285***		[248]
Troglitazone	400 mg/day/10 days		118***		[249]
Valproate	15 mg/kg		127*		[224]
Amobarbital	200 mg/day/21 days	56*		84*	[250]
Enflurane	9.6 + 0.4SEMMac/h			187**	[251]
Feprazone	300 mg/day/5 days			60**	[252]
Mandrax	275–500 mg/day/21 days	37*		34*	[250]
Miconazole	1000 mg/day			185*	[253]
Nortryptilline	50–75 mg/day/7 days	164*		143*	[254]
Pentobarbital	600–1200 mg/day/years	213**		250***	[155]
Pentobarbital	4 mg/kg/day/1 day	39–70°		59–69**	[37]
Simvastatin	20 mg/day/15 days			24**	[255]
Sulfinpyrazone	800 mg/day/25 days	52**		70**	[206]

^a*Hypericum perforatum* extract

°Non-significant

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

derivative of isoquinoline (52028RP) was highly inducing 6β -OHF excretion after 5–15 days of treatment, but after 20 days, there was no induction [65]. Probenecid [181] as well as phenylbutazone [182] significantly increased 6β -OHF urinary excretion. Saint John's Wort is an herbal extract (*Hypericum perforatum*) used in mild to moderate depression. This extract was shown to interact with different variety of drugs. Bauer et al. [183] demonstrated a significant mean increase from 9.9 to 14.3 of 6β -OHF/cortisol ratio in subjects treated with Saint John's Wort for 14 days. These results confirm those previously obtained by Roby et al. [184].

A first paper published in 1998 by Seidegard et al. [72] demonstrated the inhibitory effect of grapefruit juice. A review [185] confirmed this inhibitory effect on intestinal CYP3A4. The effect of grapefruit juice largely varies from patient to patient, probably because of wide variability in intestinal concentrations of CYP3A4. In a very recent paper, Fuhr et al. [186] considered grapefruit juice as an inducer of hepatic CYP3A4, evaluated by the increase of renal excretion of 6β -OHF in humans. Similar results were obtained in the rat [187]. This example demonstrates the difficulty of such studies, which are dependent upon several factors and among which the treatment conditions are of primary importance. Further studies must be performed in order to evaluate the respective contributions of hepatic and of intestinal CYP3A4 on the excretion of urinary 6β -OHF.

Effects of inhibitors

If there is no contest about the usefulness of 6β -OHF/cortisol ratio in detecting induction, some controversy exists about detection of inhibition. Some drugs inhibit 6β -OHF excretion, but usually their inhibiting properties are fairly low (Table 5).

Inhibitory effects are highly dependent upon the dose administered. For example, ozagrel, a thromboxane inhibitor, did not reduce 6β -OHF/cortisol when 400 mg/day was given, but this ratio was decreased by 15–20%

when the dose was 800 mg/day [188]. Stiripentol, a new anticonvulsant, has been demonstrated to inhibit CYP3A4 in vitro and in vivo when dextromethorphan was used as probe drug, but showed no inhibiting properties on 6β -OHF/cortisol [189]. Macrolide antibiotics, such as troleandomycin (2 g/day for 10 days) or josamycin at the same dose, caused a decrease in both urinary 6β -OHF and cortisol excretion, but when the 6β -OHF/cortisol was considered, these variations were not statistically significant [190].

Ketoconazole was the most potent inhibitor of cortisol metabolism by human liver microsomes ($IC_{50}=0.6\ \mu M$) and itraconazole also showed marked inhibition of cortisol metabolism ($IC_{50}=2.4\ \mu M$) [191]. However, in a study with tirilazad-ketoconazole co-administration, 6β -OHF/cortisol did not reflect this inhibitory effect [192]. Fluconazole was also a good inhibitor of the 6β -OHF/cortisol ratio [13]. Danazol, a synthetic androgen, has been shown to probably inhibit CYP3A4, as demonstrated by the decrease of the ratios 6β -OHF/cortisol and 6β -OHF/17-OHCS [159]. Certain HIV-protease inhibitors, i.e. indinavir, ritonavir and amprenavir, are known CYP3A4 inhibitors, but none of these drugs caused any significant variation in 6β -OHF/cortisol excretion [193]. Nelfinavir reduced the 24-h urinary excretion of 6β -OHF by an average of 27% [194]. A single dose of methadone administered to healthy volunteers decreased 6β -OHF/cortisol urinary excretion over the first 24 h following drug administration by $33\pm 33\%$ [195]. Amiodarone (800 mg per day for 3 days) significantly decreased 6β -OHF/cortisol [196]. Ushiyama et al. [197] demonstrated that clarithromycin (400 mg/day or 800 mg/day for 7 days) led to significant decreases of 6β -OHF/cortisol ratios by 47% and 65%, respectively. They obtained parallel results when using the mean partial cortisol clearance to 6β -OHF, which was supposed to better reflect CYP3A4 activity than 6β -OHF/cortisol urinary excretion.

These different studies demonstrate that only weak correlations exist between in vitro and in vivo inhibition

Table 5 Drugs inhibiting 6β -hydroxycortisol (6β -OHF) excretion in human adults (expressed as percentage of variation between control and treated subjects)

Drug	Dosage	6β -OHF Variation (%)	6β -OHF/cortisol Variation (%)	6β -OHF/17-OHCS Variation (%)	Reference
Clarithromycin	400–800 mg/day/7 days		47*–65*		[197]
Cimetidine	1000–1200 mg/day/7–8 days	20*		25–35**	[11, 130, 209, 256, 257]
Danazol	400 mg/day/28 days		52*	35*	[159]
Fluconazole	400 mg/day/7 days	28***			[14]
Fluconazole	200 mg/day/3 days		50*		[13]
Levonorgestrel	0.15 mg/day/28 days		19***		[199]
Nitrazepam	5–10 mg/day/21 days			11*	[250]
Oral contraceptive medication		27***			[162]
Ozagrel	800 mg/day/60 days		15–20*		[188]
Ranitidine	300 mg/day/15 days			9	[131]

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

studies. This is probably due to the lack of sensitivity and high inter-individual of in vivo measurements of CYP3A activity. For that reason, some drugs could be presented as inhibitors or as drugs having no effect on 6β -OHF excretion, but for different dosages.

Drugs having no effect

Finally, some drugs have been demonstrated to have no effect on 6β -OHF production. Table 6 gives the list of such drugs having no statistically significant effect on 6β -OHF, 6β -OHF/cortisol or 6β -OHF/17-OHCS excretion at the doses indicated.

Among these drugs, oral contraceptive formulations containing ethinylestradiol and levonorgestrel or gestodene or cyproterone acetate administered to healthy young women did not cause any difference in 6β -OHF/cortisol excretion [82, 198, 199, 200].

Urinary 6β -OHF fluctuation after drug co-administration

In drug monotherapy, the categorisation will be relatively simple to assign, but in drug combination therapy the situation is different, especially when an enzyme inducer and an enzyme inhibitor are administered together. When different inducers were associated, the rise observed was usually higher. For example, various anti-convulsants administered in association gave mean increases of 6β -OHF excretion of 4.9 to 7.6 times, and Zhiri et al. [61] found an increase of 6β -OHF/17-OHCS by more than 10 times.

An enzyme inducer will influence the pharmacokinetics of some co-administered drugs and lead to lowered plasma concentration and half-life. The antipyrene

half-life value in eight barbiturate-treated patients was significantly shortened, and the urinary output of 6β -OHF was approximately three times that in a control population [155].

In the case of enzyme inhibitors, the reverse will be observed and the plasma concentration and half-life of the co-administered drug will be increased. However, the results may be different according to the route of administration. For example, the ratio 6β -OHF/cortisol significantly decreased after ketoconazole + oral tirilazad administration, but not after ketoconazole + i.v. tirilazad treatment [192].

The administration of fluconazole for 7 days led to a significant increase in zidovudine half-life and a significant decrease of 6β -OHF excretion (-28%). Inversely, a treatment with zidovudine had no effect on fluconazole half-life and significantly increased 6β -OHF excretion ($+56\%$) [14].

The interaction of ciclosporin A (immunosuppressive agent) and ticlopidine (anti-thrombotic agent) on 6β -OHF excretion was studied in heart-transplant recipients [201]. Urinary 6β -OHF excretion was increased in the ticlopidine-treated patients compared with the placebo group ($+32\%$, $P < 0.05$), suggesting an induction of CYP3A4 possibly leading to a reduced ciclosporin exposure. The 6β -OHF/cortisol ratio did not significantly vary in patients receiving delavirdine mesylate and fluconazole for 1 day to 15 days [202]. After antipyrene administration, a fall in steady-state plasma warfarin concentration in five subjects, a shortening of the plasma warfarin half-life in two subjects and increased urinary excretion of 6β -OHF were observed [133].

Amitriptyline co-administered with atenolol or with metoprolol produced a non-significant reduction of 6β -OHF excretion when compared with atenolol or metoprolol monotherapy [203]. A relatively high dose of lansoprazole did not affect the bioavailability of

Table 6 Drugs which have been shown to have no effect on 6β -hydroxycortisol (6β -OHF) excretion in humans

6β -OHF excretion	6β -OHF/17-hydroxycortisosteroids
Cimetidine (800 mg/day/15 days) [258]	Atenolol (100 mg/day/14 days) [203]
Diazepam (15 mg/day/30 days) [259]	Clemastine (6 mg/day/14 days) [146]
Encainide (75 mg/day/5 days) [221]	Metoprolol (200 mg/day/14 days) [203]
Ethinylestradiol (30 μ g/day/6 days) [260]	Nifedipine (40 mg/day/15 days) [268]
Indomethacin (75 mg/day/21 days) [43]	Nitrazepam (5–10 mg/day/21 days) [250]
Lansoprazole (60 mg/day/21 days) [261, 204] ^a	Pravastatin (20 mg/day/17 days) [255]
Omeprazole (120 mg/day/7 days) [68]	Primaquine (45 mg/1 day) [269]
Oxcarbazepine (300 mg/day/14 days) [262]	Ranitidine (300 mg/day/14 days) [270, 205]
Pantoprazole (40 mg/day/1 day) [240]	Roxatidine (150 mg/day/3 days) [187]
Pranlukast (112.5–675 mg/day/1 day) [263]	SB216469 (120 mg/day/7 days) [174] ^b
Rabeprazole (20 mg/day/14 days) [264]	6β -OHF/cortisol
Reboxetine (2–4mg/day/9 days) [69]	Alprazolam (0.8 mg/day/1 day) [271]
Spirolactone (15 mg/day/14 days) [265]	Artemisinin (500 mg/day/7 days) [272]
Stanozolol (10 mg/day/14 days) [266]	Co-artemether (40 mg artemether + 480 mg lumefantane every 10 h for 60 h [273])
Terguride (0.5–1.5 mg/day/21 days) [244]	Doxylamine (50 mg/day/17 days) [152]
Valproate (1.5 g/day/3 days) [267]	Eletriptan (160 mg/day/7 days) [274]
	Interferon alpha (3.10 ⁶ U 3 times/week) [275]
	Ozagrel (400 mg/day/3 days) [188]
	Pioglitazone (45 mg/day/14 days) [276]
	Stiripentol (1.0–3.0 g/day/12 days) [189]
	Vigabatrin (3.0 g/day/30 days) [277]

^aHealthy women under oral contraceptives (ethinylestradiol 0.03 mg + levonorgestrel 0.15 mg)

^bSelective α 1-adrenergic receptor antagonist

ethinylestradiol and levonorgestrel, and 6 β -OHF excretion was not significantly influenced either [204]. Ranitidine did not impair the hepatic elimination of diazepam, although excretion of 6 β -OHF ($P=0.029$) and 17-OHCS ($P=0.041$) were significantly increased by ranitidine. However, the ratio did not change [205]. In a study to investigate the effect of sulphinpyrazone on antipyrine metabolism and on the urinary excretion of 6 β -OHF, there was no change in the renal clearance of antipyrine after 23 days of sulphinpyrazone treatment, but the renal clearance of each antipyrine metabolite was significantly increased, as was the quantity of urinary 6 β -OHF in 24 h urine before and on days 3, 9, and 24 of sulphinpyrazone treatment. The calculated ratio to 17-OHCS excretion also increased significantly in a time-related manner [206]. In another study directed to evaluate the interaction of cimetidine and rifampicin with the β 1-selective adrenoceptor antagonist bisoprolol, no significant interaction of bisoprolol with cimetidine, a well-known inhibitor of monooxygenase system in the liver, occurred, but rifampicin, the most potent enzyme inducing drug known, significantly decreased the maximal plasma level and increased the elimination of bisoprolol [207]. In this study, the daily excretion of 6 β -OHF was significantly increased, but no effect of bisoprolol on urinary 6 β -OHF was reported.

As a conclusion on this section on the effects of various drugs, it can be recommended to pay attention to the conditions of treatment when interpreting the variations of urinary 6 β -OHF. It must also be strongly recommended to use the ratio 6 β -OHF/cortisol and when possible to test at least two doses for a drug. The results will also depend on the number of subjects included in the study as well as the statistical methodology used.

Effects of xenobiotics

Alcohol intake

Urinary 6 β -OHF/cortisol was significantly higher in chronic alcoholics than in control subjects (10.21 ± 2.4 versus 3.97 ± 0.47 ; $P < 0.01$). Ethanol might have a slight inducing effect on CYP3A4 in humans, as it is the case in cultured hepatocytes. On the contrary, in patients with acute alcohol intoxication, the mean urinary 6 β -OHF/cortisol excretion was lower than in controls [78].

Coffee intake

Following 21 days to 52 days of caffeine restriction, no significant variation in the excretion of 6 β -OHF/17-OHCS was observed in 11 non-smoker volunteers [208]. The authors observed increases in the ratio in 6 subjects (from 30% to 176%) and decreases in 5 subjects (from -4% to -73%).

Smoking

Vestal et al. [209] demonstrated that smoking had no effect on 6 β -OHF excretion in young as well as in old healthy males. In smokers, 6 β -OHF was slightly lower than in non-smokers (-24% to -7%, according to age) but these variations were not statistically significant. Similar results were obtained by other authors [91, 156, 213]. In addition, smoking did not influence 6 β -OHF/creatinine excretion [91].

Environmental chemicals

Several studies have been undertaken to study the effect of environmental chemicals on the mixed function oxidase enzyme activity on the basis of urinary 6 β -OHF excretion. The pesticides dieldrin-aldrin and endrin affected 6 β -OHF excretion differently [210]. Dieldrin-aldrin had no effect on the ratio 6 β -OHF/17-OHCS (30.7 ± 18.2 versus 30.5 ± 16.2 for exposed and non-exposed workers, respectively), whereas endrin largely influenced 6 β -OHF/17-OHCS excretion (91.0 ± 27.6 versus 30.5 ± 16.2 for exposed and non-exposed subjects, respectively). Dichloro-diphenyl-trichloroethane provoked a 57% mean increase in 6 β -OHF excretion [39].

Styrene was also found to induce 6 β -OHF production as judged by the ratio 6 β -OHF/17-OHCS [211], which was 18.8 ± 4.2 in non-exposed and 36.9 ± 15.3 in exposed workers. But Bragt et al. [212] did not confirm this observation on styrene when they analysed 6 β -OHF in the 24 h urine from 40 styrene workers and 37 laboratory workers used as controls. They did not find significant alterations in 6 β -OHF excretion levels. 6 β -OHF levels in exposed workers were 236 ± 83 μ g/day versus 256 ± 126 μ g/day in controls. Moretti et al. [213] did not observe any induction, but rather an inhibition of 6 β -OHF/17-OHCS in workers in the rubber industry. Occupational exposure to cis-1,3-dichloropropene did not affect 6 β -OHF/cortisol ratio [214].

A decreased urinary excretion level was observed in lead-intoxicated children [215]. The decrease was all the more important when the lead storage was elevated [216].

Variations due to pathological states

Few studies have been conducted to study the variations in 6 β -OHF excretion in pathological cases. Nevertheless, 6 β -OHF excretion has been reported to be much more increased than cortisol itself in hypercortisolemic patients [20, 41, 42, 114, 156, 217], although Nahoul et al. [44] observed the opposite. Nakamura and Yamata [112, 114] did not find any difference in 6 β -OHF excretion between Cushing's syndrome (overproduction of cortisol by adrenal glands) and Cushing's disease (overproduction of ACTH by pituitary glands), although both 6 β -OHF and cortisol excretion were higher than in control subjects.

Werk et al. [33] found increased excretion of 6 β -OHF in terminal illnesses, especially in advanced cancer, but these increases, although very high, were not statistically significant due to the wide scattering of values. In six patients with bronchogenic carcinoma, Kishida and Fukushima [42] did not find any significant rise in 6 β -OHF excretion.

Several studies have been performed to evaluate the variation of 6 β -OHF excretion in different hepatic diseases. In nine patients with hepatic disease, Borkowski et al. [218] did not find any difference in 6 β -OHF excretion compared with control subjects. A slight increase in the ratio 6 β -OHF/cortisol was found in healthy Hb_sAg carriers [219]. In subjects presenting an abnormal liver function profile, Eade et al. [220] found a mean 6 β -OHF/17-OHCS increase of 75% ($P < 0.001$). The largest rises were observed in patients suffering from acute hepatitis (+273%; $P < 0.05$) or hepatic carcinomas (+327%; $P < 0.05$). However, Wensing et al. [221] did not find any variation in the daily excretion of 6 β -OHF in patients with liver cirrhosis. After liver transplantation, 6 β -OHF excretion increased by about 4 times, even in a patient where liver biopsy was unable to detect CYP3A4 [29]. Ng et al. [70] did not find any significant variation in the 6 β -OHF/cortisol ratio in chronic liver disease, in cirrhosis and in resectable carcinoma. In contrast, in non-resectable carcinoma they found a significant rise (10.55 ± 7.3) compared with control subjects (5.92 ± 2.97). More recently, Wild et al. [222] showed that 6 β -OHF/cortisol did not vary in patients with chronic hepatitis B.

A significant increase ($P < 0.01$) in 6 β -OHF excretion was observed in five glucose-6 phosphate dehydrogenase deficient patients [218]. In patients suffering from thyrotoxicosis, 6 β -OHF excretion was lower than in controls and the opposite was observed in myxedematous patients [35]. In heterozygous familial hypercholesterolemia 6 β -OHF as well as cortisol excretion were similar to those observed in control subjects [198, 223].

As mentioned in a previous section, 6 β -OHF urinary excretion was increased by about 2 times during the third trimester of normal pregnancy. In toxemia pregnancy, this increase was demonstrated to be much higher (by about 5 times) [105]. 6 β -OHF excretion was elevated in traumatic brain injury [224] and did not vary in rheumatoid disease [18].

In a recent case-control study, the risk of breast cancer was increased with increasing levels of 6 β -OHF/cortisol urinary excretion [225]. The association 6 β -OHF/cortisol and breast cancer was stronger among older women who had a high body mass index, late age at menopause and early age at menarche.

Comparison with the other tests measuring CYP3A activity in humans

Watkins' team [7, 226] has demonstrated that the erythromycin breath test well reflects the hepatic activity

of CYP3A4. It was well correlated with 6 β -OHF in some patients, but not in others [168]. Hunt et al. [227] did not find any correlation between erythromycin breath test and 6 β -OHF urinary excretion in a small number of patients with hypertension. Kinirons et al. [228] confirmed these results in young healthy men. More recently, the same team failed to find any correlation between erythromycin breath test, 6 β -OHF/cortisol excretion and midazolam clearance, all three tests supposed to represent CYP3A liver activity [229]. Similarly, Gotzowsky et al. [230] found a poor correlation between the 24-h urinary 6 β -OHF/cortisol molar ratio and midazolam clearance. These results strongly suggest that cortisol 6- β hydroxylation, erythromycin *N*-demethylation, and midazolam hydroxylation are catalysed by distinct CYP3A4 isoforms, genetically different or other CYP3A forms like CYP3A5. Moreover, overall CYP3A5 protein content accounted for 31% of the variability in hepatic midazolam hydroxylation activity [231]. For Watkins et al. [232], the ideal probe for CYP3A is not discovered yet, and it seems that a single probe would not be appropriate to test CYP3A4 activity.

The use of 6 β -OHF as test of induction in different animal species

Hepatic cortisol metabolism is highly variable across species. Rat and mouse liver microsomes produce 6 β -OHF as a minor metabolite (2%) [233]. Dog liver is also able to produce small quantities of 6 β -OHF from cortisol [17]. In the guinea-pig, 6 β -OHF production *in vivo* is strain-dependent, and two distinct phenotypes exist with high or low urinary excretion of 6 β -OHF [234]. In the hamster, 6 β hydroxylation is a major pathway of cortisol biotransformation (30%) [233].

Pre-treatment of rats, guinea-pigs or mice with phenobarbital increases the mean rate of *in vitro* cortisol transformation to 6 β -OHF in liver microsomes [235, 236, 237]. Dexamethasone, but not β -naphthoflavone, also induced cortisol 6 β hydroxylation in mice [237]. Ethanol was not an inducer of 6 β -hydroxylase in the rat [235].

Concerning 6 β -OHF excretion, marmoset and rhesus monkeys have been demonstrated to be good animal models of CYP induction due to phenobarbital [57, 238] or rifampicin [80, 239]. These animals represent the best model for estimating drug induction. The 6 β -OHF/cortisol ratio was increased approximately 4-fold on the eighth day after the beginning of phenobarbital treatment (30 mg/kg/day) [240].

In guinea-pigs, 6 β -OHF excretion was increased by pre-treatment of the animals by phenobarbital or rifampicin, but no variation was observed after 3-methylcholanthrene, pregnenolone-16 α -carbonitrile or spironolactone treatment [241]. The induction due to phenobarbital has been demonstrated to be variable with the strain [242].

When mouse liver microsomes were incubated in vitro with gestodene, 6 β -OHF formation was inhibited [237]. In rats, Abu-Qare and Abou-Donia [243] found a significant increase in urinary 6 β -OHF after a single dermal dose of an insect repellent, *N,N*-diethyl-*m*-toluamide (DEET), but permethrin, another insecticide, had no effect on 6 β -OHF excretion.

Conclusion

The measurement of 6 β -OHF excretion has to be considered as a useful test for estimating CYP3A drug induction or inhibition in human and in laboratory animals, particularly monkeys and guinea pigs. Urinary cortisol must be evaluated in the same patients as the ratio 6 β -OHF/cortisol minimises the effect of circadian rhythms. Because of large inter-individual variations in cortisol metabolism, each patient must be his own control for the evaluation of drug induction or inhibition and the time course of induction or inhibition should be followed.

Some limitations appeared in the use of 6 β -OHF/cortisol ratio to measure CYP3A4 activity. First, cortisol is a substrate for CYP3A4 and also for CYP3A5, and the contribution of this last enzyme on the metabolism of cortisol to 6 β -OHF is poorly understood. Is it possible that CYP3A5 variations should mask changes in CYP3A4? That does not seem very probable, as CYP3A5 is not inducible and is present in much smaller concentrations than CYP3A4 [173], except for those people who are carriers of the CYP3A5*1 allele [25]. Second, 6 β -OHF/cortisol ratio reflects liver CYP3A4 activity and also extrahepatic activity. Nevertheless, most authors consider that 6 β -OHF/cortisol ratio is useful non-invasive test to evaluate inducing or inhibiting properties of drugs, totally validated for induction, still under debate for some inhibition. The great advantage of this test is that there is no drug to be administered, as cortisol and 6 β -OHF are natural metabolites.

Further studies are needed to establish possible correlations between CYP3A4 polymorphisms and 6 β -OHF/cortisol urinary excretion. Comparative studies between 6 β -OHF/cortisol excretion and other tests supposed to evaluate CYP3A activity must be performed. 6 β -OHF/cortisol urinary excretion does not seem to be the appropriate test to measure the actual CYP3A activity, but the ideal probe has not yet been discovered.

Finally, the measurement of 6 β -OHF excretion presents also new interest in certain pathologies such as breast cancer, as has been demonstrated very recently. Further studies must be conducted in different pathologies to examine the interest of 6 β -OHF as biomarker of disease states.

Acknowledgement We would like to warmly thank G. Sanderink, Aventis, for his help.

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