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CORTISOL METABOLISM BY HUMAN LIVER *IN VITRO*—I. METABOLITE IDENTIFICATION AND INTER-INDIVIDUAL VARIABILITY

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Summary—The measurement of urinary 6β-hydroxycortisol (6β-OHF) has been widely used as a non-invasive clinical test to detect cytochrome *P*450 induction. Although only a minor biotransformation, 6β-OHF formation represents a sensitive target for many *P*450-inducing drugs and environmental chemicals in man. There is good evidence that an isozyme of the *P*450IIIA subfamily is predominantly responsible for 6β-hydroxylase activity and therefore it has been suggested that urinary 6β-OHF is a marker of the induction of *P*450IIIA. The basis of the present study was that in order to realistically assign to 6β-OHF the status of a *P*450IIIA marker we should characterize all the metabolites of cortisol produced by human liver and assess inter-liver variability. Incubations at 37°C for 2 h contained [^{3}H]cortisol (0.1 μCi, 1 or 50 μM), $MgCl_2$ (10 mM), microsomal or cytosolic protein (3 mg), an NADPH-regenerating system and 1/15 M phosphate buffer (pH 7.4) to give a final volume of 0.5 ml. Extraction with ethyl acetate (2 × 2 ml) was followed by radiometric HPLC analysis. Metabolites were identified by co-chromatography with authentic standards and mass spectrometry (electron impact and chemical ionization). All the microsomal incubations ($n = 6$ livers) produced 6α-hydroxycortisol (6α-OHF), 6β-OHF, 20β-dihydroxycortisol, 20β-dihydroxycortisone, cortisone, and 3α,5β-tetrahydrocortisone (3α,5β-THE), while five produced 6β-hydroxycortisone and four produced 3α,5β-tetrahydrocortisol (3α,5β-THF). The cytosolic incubations gave a much simpler metabolic profile, with 3α,5β-THF the major metabolite and 3α,5β-THE a minor metabolite. There was considerable inter-individual variability in metabolite profiles from microsomal incubations. 6β-OHF varied from 2.8 to 31.7%. Major metabolites were cortisone and 3α,5β-THE. Inter-liver variability was less for cytosolic incubations, the major metabolite always being 3α,5β-THF. In conclusion we have rigorously identified the hepatic metabolites of cortisol formed *in vitro*. The highly complex and variable hepatic metabolism of cortisol clearly limits the use of urinary 6β-OHF excretion as a marker of baseline *P*450IIIA activity in man.

INTRODUCTION

The major routes of cortisol metabolism in humans determined from both urinary analysis [1, 2] and *in vitro* studies [3, 4] involve A-ring and side chain reduction followed *in vivo* by conjugation with glucuronic acid and sulphate. A minor metabolite is 6β-hydroxycortisol (6β-OHF; [1, 5]), which is formed mainly by hepatic oxidation and is excreted largely unchanged in urine. The urinary excretion of 6β-OHF has been widely used as a simple non-invasive marker of induction of the mixed function oxidase enzymes present in the endoplasmic reticulum which are responsible for the metabolism of many drugs. Recent advances in molecular biology have revealed that the enzymes involved in drug oxidation exist as a gene superfamily composed of more than 20 gene products which each exhibit variable degrees of selectivity. Ged *et al.* [6] suggested that *P*450IIIA is predominantly responsible for cortisol 6β-hydroxylase activity and that urinary 6β-OHF is a marker of the induction of this cytochrome *P*450. One aspect of the latter study was the identification from microsomal

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Abbreviations: Cortisol = 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; 6β-OHF = 6β,11β,17α,21-tetrahydroxy-4-pregnene-3,20-dione; 6α-OHF = 6α,11β,17α,21-tetrahydroxy-4-pregnene-3,20-dione; 6β-OHE = 6β,17α,21-trihydroxy-4-pregnene-3,11,20-trione; 20β-DHF = 11β,17α,20β,21-tetrahydroxy-4-pregnene-3-one; 20β-DHE = 17α,20β,21-trihydroxy-4-pregnene-3,11-dione; 3α,5β-THF = 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one; 3α,5β-THE = 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione; cortisone = 17α,21-dihydroxy-4-pregnene-3,11,20-trione.

incubations of only 6β-OHF, i.e. no other metabolites were identified. Since in none of the previous *in vitro* studies on cortisol metabolism has there been rigorous metabolite identification (i.e. by mass spectrometry), we have further examined the *in vitro* metabolism of cortisol in human liver, with particular emphasis on the characterization of all the metabolites and assessment of inter-liver variability. We believe that such an approach is important if we are to realistically assign to 6β-OHF the status of a marker of *P*450IIIA activity in either *in vitro* or *in vivo* studies.

MATERIALS AND METHODS

Chemicals

[1,2,6,7-^{3}H]Cortisol (80 Ci/mmol) was obtained from Amersham Int. (Bucks., England) Cortisol, 6β-hydroxycortisone (6β-OHE)-20β-dihydroxycortisol (20β-DHF), 20β-dihydroxycortisone (20β-DHE), cortisone, 3α,5β-tetrahydrocortisol (3α,5β-THF), 3α,5β-tetrahydrocortisone (3α,5β-THE), glucose-6-phosphate, NADP, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (Poole, England). 6α-Hydroxycortisol (6α-OHE) and 6β-OHF were synthesized by Dr J. Yeung (Chinese University of Hong Kong). HPLC solvents were of Analar grade and supplied by Fisons (Loughborough, England). Scintillation fluid (Flo-Scint A) was obtained from Canberra-Packard, (Pangbourne, Bucks., England). All other chemicals were from BDH (Poole, England).

Human liver samples

Histologically normal livers were obtained from kidney transplant donors. Ethical approval for the study was granted and consent to removal of the livers was obtained from the donors' relatives. Livers were transferred, on ice, to the laboratory within 30 min where they were divided into 10–20 g portions, placed in plastic vials and frozen in liquid nitrogen at −196°C. Liver samples were stored at −80°C until required.

Microsomes and cytosol incubations

Washed microsomes were prepared by the classical differential centrifugation technique [7]. Cytosol from the first 105,000 *g* spin was retained. Protein was assayed by the method of Lowry *et al.* [8].

Liver microsomes and cytosol (3 mg protein in each case) were incubated at 37°C in final volumes of 0.5 ml containing [^{3}H]cortisol (0.1 μCi), cortisol (1 or 50 μM), $MgCl_2$ (3 mM), glucose-6-phosphate (10 mM), NADP (5 mM), glucose-6-phosphate dehydrogenase (2 U) and 0.067 M phosphate buffer (pH 7.4). Incubations were for 2 h and were initiated by addition of the NADPH-regenerating system. The reaction was terminated by cooling in crushed ice and cortisol and metabolites extracted with ethyl acetate (2 × 2 ml). The solvent was evaporated under nitrogen and samples reconstituted in methanol (200 μl) before analyses by radiometric HPLC. Recovery of incubated radioactivity was >95%.

HPLC analysis

Cortisol and its metabolites were resolved on a Nucleosil $5C_8$ column (5 μm, 25 cm × 4.6 mm i.d.) protected by an in-line C_{18} guard column. Elution (50 min) was isocratic with a mobile phase of ammonium orthophosphate buffer (0.5% w/v) and acetonitrile (75:25). The flow rate was 0.7 ml/min. Absorbance was monitored between 220–280 nm. Analysis was carried out on an SP 8800 ternary pump, with an SP 100 variable wavelength detector (Spectra-Physics) linked to an on-line radiomatic A250 FLO-ONE detector (Canberra-Packard). Metabolites were initially identified by co-chromatography with authentic standards.

Mass spectrometry

Samples for metabolites for mass spectrometry were isolated by HPLC from incubations containing 50 μM cortisol to ensure sufficient mass and following chromatographic separation they were recovered from the eluate using SEP-PAK C_{18} cartridges. Steroids were eluted from the cartridges with methanol (3 ml).

Isolated metabolites and authentic standards were analysed via the solids probe of a VG tritech TS250 mass spectrometer. Electron impact (EI) and chemical ionization (CI) mass spectra were acquired over *m/z* 50–800 and 75–800, respectively, at resolution 800; scan time was 1 s. Operating conditions were as follows: accelerating voltage 4×10^3 V; source temperature, 180°C; electron energy, 70 eV (EI) or 50 eV (CI); emission current, 700 μA (EI) or 500 μA (CI). The reagent gas was either ammonia or isobutane at a source pressure of 2×10^{-4} mb.

RESULTS

A total of 6 livers were used in this study from male patients aged 18–55 years. The following metabolites were initially identified by co-chromatography with authentic standards and yielded mass spectra which confirmed these identifications: 6α-OHF, (retention time, R_t, 5.4 min), 6β-OHF (R_t, 8.0 min), 6β-OHE (R_t, 10 min), 20β-DHF (R_t, 17.5 min), 20β-DHE (R_t, 20 min), 3α,5β-THF (R_t, 28 min) cortisone (R_t, 31 min) and 3α,5β-THE (R_t, 40 min). Cortisol had a retention time of 26 min. The mass spectrometry data are summarized in Tables 1 (CI) and 2 (EI).

All the microsomal incubations produced 6α-OHF, 6β-OHF, 20β-DHF, 20β-DHE, cortisone and 3α,5β-THE, while five produced 6β-OHE and four produced 3α,5β-THF. The EI mass spectrum of 6α-OHF contained a molecular ion at m/z 378 and a dehydration ion fragment at m/z 360. The CI spectrum contained a pseudomolecular ion (M + 1) at m/z 379. The EI mass spectrum of 6β-OHF was similar to that of 6α-OHF and the CI spectrum was in agreement with the EI spectrum. 20β-DHF showed a molecular ion at m/z 364 and a dehydration peak at m/z 346; the CI spectrum confirmed the EI data with a pseudomolecular ion at m/z 365 and the corresponding dehydration peak (m/z 347). 20β-DHE yielded a molecular ion at m/z 362 and a dehydration fragment at m/z 344; its CI spectrum gave a pseudomolecular ion at m/z 363 and the dehydration fragment at m/z 345. Cortisone, gave a molecular ion at m/z 360, a product of side chain cleavage at m/z 301 and a pseudomolecular ion at m/z 361. The least polar metabolite (i.e. 3α,5β-THE) yielded an EI spectrum containing the molecular ion (m/z 364) and double dehydration peaks (m/z 346 and 328); CI gave a pseudomolecular ion at m/z 365 and the associated double dehydration peaks (m/z 347 and 329).

The cytosolic incubations produced a much simpler metabolic profile, with 3α,5β-THF the major metabolite and 3α,5β-THE a minor metabolite. The EI spectrum of 3α,5β-THF contained a molecular ion at m/z 366 and dehydration fragments (m/z 348 and 330). The CI spectrum showed a pseudomolecular ion (M + 1) at m/z 367 and the corresponding dehydration peaks (m/z 349 and 331). 3α,5β-THE was identified as above.

There was considerable inter-individual variation in metabolite profiles from microsomal incubations (Table 3). 6β-OHF varied from 2.8% (L22) to 31.7% (B1). Major metabolites were cortisone and 3α,5β-THE, although the latter varied between 5.8% (B1) to 28.1% (L14). HPLC-radiochromatograms for livers B1 and WT1 (Fig. 1) illustrate these differences in metabolite producton. Inter-liver variability was less for cytosolic incubations (Table 4); the major metabolite always being 3α,5β-THF (Fig. 2).

Table 1. Molecular ions and major fragments of cortisol metabolites isolated from hepatic *in vitro* studies (CI)

Metabolite	Chemical ionization Ion (relative intensity)
6α-OHF	379 (M + 1⁺; 7), 361 (7), 349 (12), 331 (3), 319 (100), 301 (19), 283 (16)
6β-OHF	361 (7), 349 (11), 343 (4), 333 (5), 319 (100), 301 (20), 283 (16)
6β-OHE	361 (M + 1⁺; 4), 347 (15), 317 (100), 299 (30)
20-DHF	365 (M + 1⁺; 100), 347 (93), 332 (75), 315 (36), 304 (81), 287 (34)
20β-DHE	363 (M + 1⁺; 100), 345 (26), 327 (5), 301 (17), 285 (5)
Cortisol	363 (M + 1⁺; 18), 345 (100), 327 (46), 315 (6), 303 (10), 285 (9)
3α5β-THF	367 (M + 1⁺; 12.5), 349 (21), 337 (11), 331 (100), 319 (14), 313 (72), 301 (27), 289 (26), 271 (34)
Cortisone	361 (M + 1⁺; 94), 343 (88), 331 (91), 301 (100), 283 (3)
3α,5β-THE	365 (M + 1⁺; 62), 347 (100), 329 (77), 317 (31), 305 (14), 287 (61)

Table 2. Molecular ions and major fragments of cortisol metabolites isolated from hepatic *in vitro* studies (EI)

Metabolite	Electron ionization Ion (relative intensity)
6α-OHF	378 (4), 360 (6), 348 (18), 331 (20), 318 (100), 303 (20), 285 (26), 267 (24)
6β-OHF	378 (4), 360 (6), 348 (15), 331 (19), 318 (100), 312 (10), 303 (18), 285 (27), 267 (24)
6β-OHE	346 (10), 329 (5), 316 (100), 301 (13), 287 (23), 273 (12), 255 (8), 241 (6)
20β-DHF	364 (54), 346 (36), 331 (15), 315 (43), 303 (50), 285 (100), 267 (41), 242 (40), 227 (45)
20β-DHE	362 (40), 344 (21), 326 (11), 313 (30), 301 (90), 283 (14), 257 (24), 243 (20), 227 (13), 122 (100)
Cortisol	362 (10), 344 (42), 329 (9), 311 (18), 303 (7), 297 (10), 285 (29), 267 (20), 257 (8), 242 (13), 227 (24)
3α5β-THF	366 (6), 348 (13), 330 (16), 317 (32), 301 (27), 289 (29), 273 (23), 271 (76), 255 (29), 253 (55), 246 (100), 213 (87)
Cortisone	360 (60), 342 (30), 330 (12), 313 (40), 301 (76), 283 (13) 272 (80), 258 (57), 243 (16), 225 (16), 122 (100)
3α,5β-THE	364 (22), 346 (31), 328 (17), 317 (23), 299 (47), 287 (51), 271 (29), 261 (21), 243 (100), 229 (29)

In each case the first ion is the molecular ion.

DISCUSSION

The measurement of urinary 6β-OHF has been widely used as a non-invasive, urinary clinical test to detect cytochrome *P*450 induction [5, 6, 9, 10]. Although only a minor

Table 3. Inter-individual variation in microsomal metabolism of [^{3}H]cortisol

Metabolite	R_t (min)	% Of each metabolite in liver						
		B1	L9	L14	L22	L23	WT1	Mean ± SD
6α-OHF	5.4	5.1	3.2	1.4	1.4	1.6	2.0	2.5 ± 1.5
6β-OHF	8	31.7	4.0	7.5	2.8	11.2	4.0	10.2 ± 11.0
6β-OHE	10	8.6	1.7	9.9	0	6.8	2.1	4.9 ± 4.1
20β-DHF	17.5	8.8	5.3	4.6	3.2	10.4	7.1	6.6 ± 2.7
20β-DHE	20	6.3	6.0	10.3	5.8	7.0	12.8	8.9 ± 2.9
Cortisol	26	25.7	41.7	18.7	54.1	29.6	24.5	32.4 ± 13.1
3α,5β-THF	28	3.6	13.9	0	0	16.9	10.1	7.4 ± 7.3
Cortisone	31	4.6	10.8	19.5	23.6	9.1	10.2	13.0 ± 7.1
3α,5β-THE	40	5.8	13.5	28.1	9.2	7.4	27.3	15.2 ± 10.0

R_t = retention time; B1, L9 etc. refer to individual human livers.

biotransforamtion, 6β-OHF formation represents a sensitive target for many *P*450-inducing drugs and environmental chemicals in man. Park and Kitteringham [5] have previously stressed the necessity of using changes in the amount of excreted 6β-OHF rather than absolute values i.e. each individual must be used as their own control. There is now good evidence that an isozyme(s) of the *P*450IIIA subfamily is predominantly responsible for 6β-hydroxylase activity. Thus, Ged *et al.* [6] showed rifampicin, a selective inducer of *P*450IIIA and of erythromycin demethylase in human liver microsomes [11], to strongly induced cortisol 6β-hdyroxylase. They also showed that in hepatic microsomes from both organ donors and rifampicin-treated patients, cortisol 6β-hydroxylase activity correlated with the *P*450IIIA

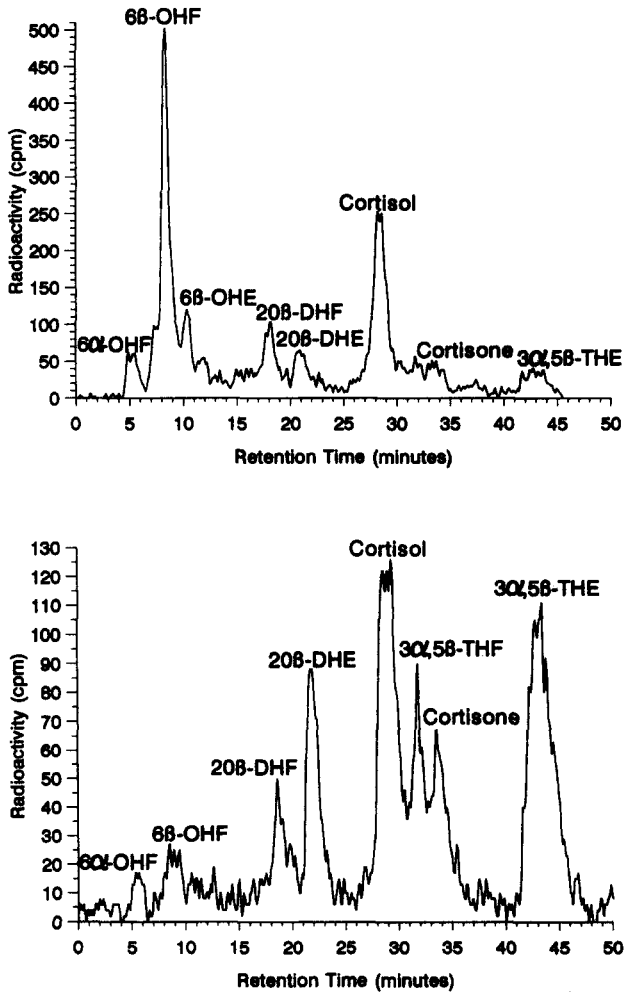

Fig. 1. HPLC separation of cortisol and metabolites produced by human liver microsomes. (a) Liver B1 (male, aged 27 years); (b) liver WT1 (male, aged 18 years).

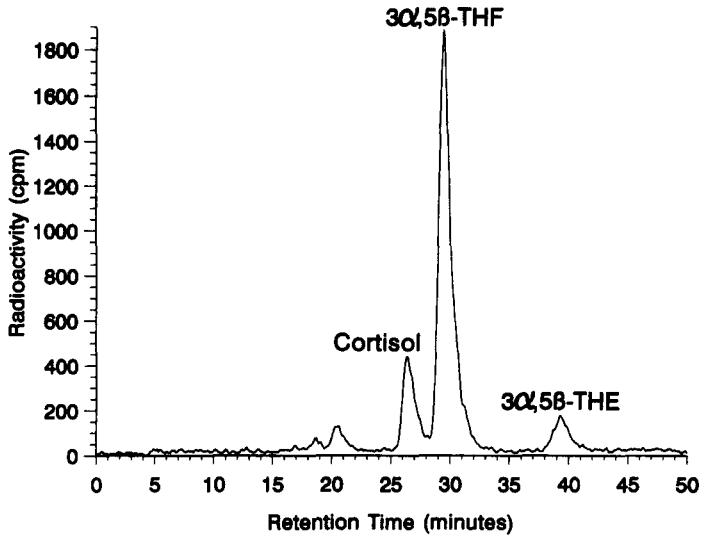

Fig. 2. HPLC separation of cortisol and metabolites produced by human liver cytosol.

level determined by Western blotting as well as with erythromycin demethylase activity. However, cortisol 6β-hydroxylase does not correlate with either ethoxyresorufin deethylase, a *P*450IA dependent activity [12], or the specific content of *P*450IA and *P*450IIC. Furthermore Vestal *et al.* [14], found no difference in 6β-OHF excretion between smokers and non-smokers, despite the fact that the former group showed enhanced clearance of theophylline, a *P*450IA substrate; smoking selectively induces *P*450IA enzymes. More recently, Hunt *et al.* [13], on the basis of a lack of correlation between erythromycin *N*-demethylation and 6β-OHF/cortisol ratios, suggested that cortisol 6β-hydroxylation and erythromycin *N*-demethylation are performed by distinct CYPIIIA isoforms.

From the foregoing, it is clear that urinary 6β-OHF provides an important marker for the induction of cytochrome *P*450IIIA. However, the present finding that the *in vitro* metabolism of cortisol is both extremely complex and variable between individuals indicates clearly that 6β-OHF is not a good marker of absolute levels of *P*450IIIA in different individual human livers and therefore that urinary 6β-OHF cannot be used to predict rates of drug metabolism *in vivo*.

Table 4. Inter-individual variation in cytosolic metabolism of [^{3}H]cortisol

Metabolite	R_t (min)	% Of each metabolite in liver L19	L20	L22	WT1	Mean ± SD
Cortisol	26	19.8	13.5	41.3	9.7	21.1 ± 12.2
3α,5β-THF	28	80.3	79.6	43.1	81.0	71.0 ± 16.1
3α,5β-THE	40	0	6.9	15.6	9.4	8.0 ± 5.6

R_t = retention time; L19, L20 etc. refer to individual human livers.

A number of early studies both *in vivo* [1, 2] and *in vitro* [3] provided evidence of numerous metabolites but without rigorous identification by mass spectrometry. In the context of understanding the overall hepatic metabolism of cortisol, the work of Iyer *et al.* [4] and the present work point to the crucial role of reductase enzymes in the cytosol. Cortisol is extensively reduced to 3α,5β-THF, the enzymes involved being a Δ^4-5β-reductase and a 3α-oxidoreductase. Thus, microsomal pathways constitute only part of the scheme of hepatic metabolism (Fig. 3). The profiles obtained with microsomal incubations point not only to cytochrome *P*450 activity but also to 11β-hydroxysteroid dehydrogenase, 5α/5β-reductase, 3α/3β-oxidoreductase and 20α/20β-oxidoreductase activities. The tremendous variability in cortisol metabolism shown in the 6 livers points clearly to the need to obtain complete metabolite analysis if 6β-OHF is to be used as a marker of *P*450IIIA activity. For example a relatively low turnover of cortisol to 6β-OHF may reflect either low *P*450IIIA activity or a higher activity of the reductase enzymes. Such inter-individual variation in the activity of the various reductases may partly explain the lack of correlation between urinary 6β-OHF and the rate of oxidation of drugs such as erythromycin [13]. However, changes in the urinary excretion of 6β-OHF can be used to monitor induction by drugs such as rifampicin and phenobarbitone which selectively induce CYP *P*450 isozymes but do not induce cytosolic and microsomal reductase enzymes.

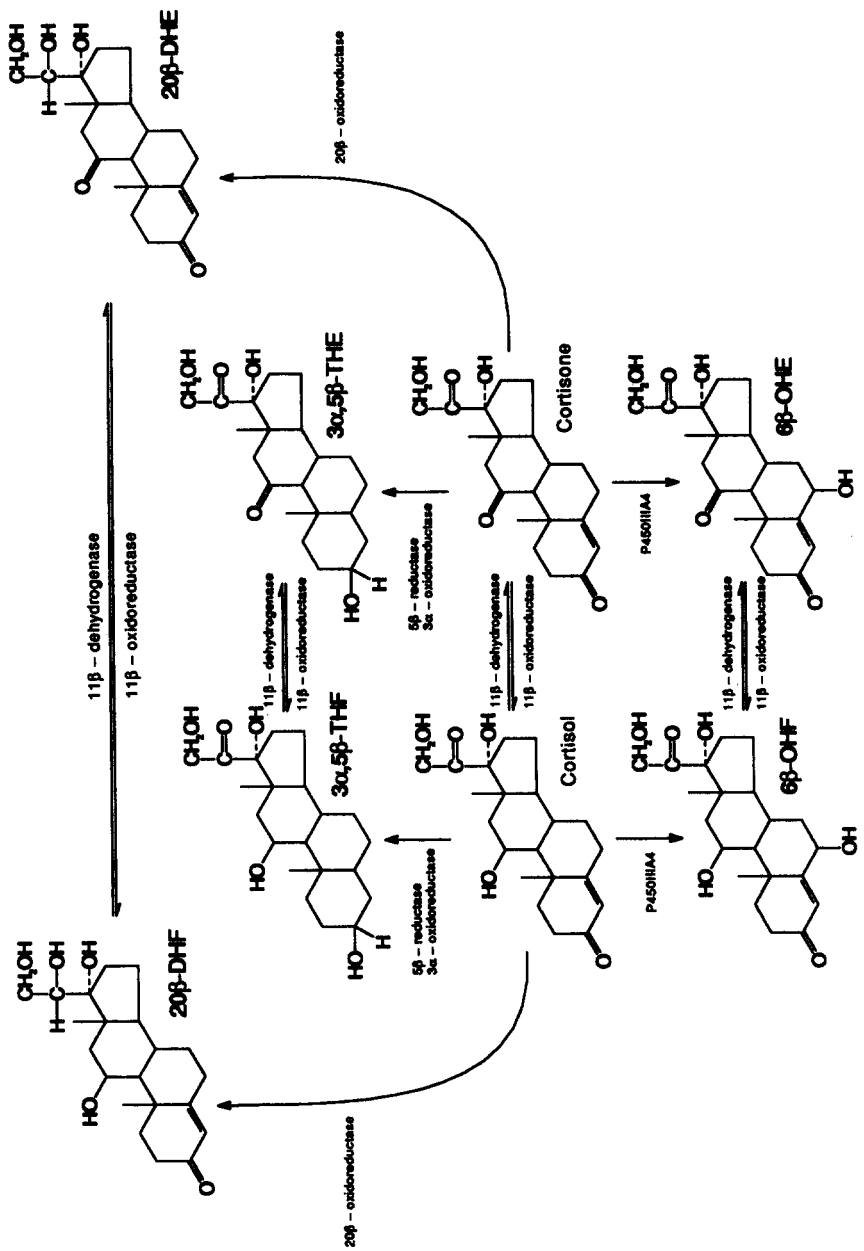

Fig. 3. The suggested metabolic pathways of cortisol in human liver microsomes.

In conclusion, we have rigorously identified the metabolites of cortisol produced by microsomal and cytosolic fractions of human liver and have described highly complex and variable metabolite profiles that impose limitations on urinary 6β-OHF excretion as a marker of baseline *P*450IIIA activity in man.

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