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# MEASUREMENT OF THE CORTISOL PRODUCTION RATE IN TWO SISTERS WITH 17α-HYDROXYLASE DEFICIENCY USING [1,2,3,4-$^{13}$C]CORTISOL AND ISOTOPE DILUTION MASS SPECTROMETRY

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**Summary**—[1,2,3,4-$^{13}$C]cortisol was i.v. administered to two sisters aged 11 yr (patient I) and 3 yr (patient II) who suffer from 17α-hydroxylase deficiency. This is the first time that the cortisol production rate (CPR) in patients with 17α-hydroxylase deficiency has been measured with a stable labelled tracer using the urinary method. The urine was collected for 3 days. High-performance liquid chromatography (HPLC) of ~100 ml urine extracts was carried out to isolate the small amount of cortisol metabolites excreted. The cortisol metabolites were oxidized to 11-oxo-aetiocholanolone. The isotope dilution in the methyl oxime tert-butyldimethylsilyl ether derivatives was measured by selected ion monitoring gas chromatography/mass spectrometry (GC/MS).

The CPR calculated from tetrahydrocortisone (THE) and the cortolones was 765 and 536 nmol/day, respectively in patient I. The CPR in patient II was only calculated from THE and was 62 nmol/day. If radioactive labelled cortisol had been used, much larger quantities of urine would have been needed for isolation of sufficient mass of metabolites, even then purification may have been difficult.

Steroid profiling of 1 ml urine samples by GC and identification by GC/MS revealed high concentrations of pregnenolone, progesterone, 11β-hydroxy progesterone and corticosterone metabolites. Tetrahydrocorticosterone and 5α-tetrahydrocorticosterone were found in urine at elevated excretions of 2.5 and 5.7, 0.9 and 2.0 μmol/24 h, in patients I and II respectively. No cortisol metabolites were detected by routine GC or GC/MS as the low amounts excreted co-eluted with the relatively abundant corticosterone metabolites.

## INTRODUCTION

Deficiency of 17α-hydroxylase leads to a reduced synthesis of cortisol, androgens and estrogens. ACTH secretion is increased and the adrenal cortex over produces progesterone, deoxycorticosterone and corticosterone because normal cortisol synthesis is not possible. The over production of these steroids also causes an increase in excretion of their metabolites in urine, and has been reported in studies using gas chromatography (GC) [1–6].

The cortisol production (CPR) rate in patients with this disorder has been measured using tritiated or $^{14}$C-labelled cortisol [7–13]. The method used to measure the CPR is that developed by Cope and Black [14, 15], and consists of administering the labelled cortisol to the patient and then measuring the isotope dilution in the isolated urinary cortisol metabolites, when excretion of the tracer is complete.

The present report describes the measurement of the CPR in two sisters aged 11 and 3 yr (patients I and II respectively) suffering from 17α-hydroxylase deficiency using stable non-radioactive labelled cortisol. [1,2,3,4-$^{13}$C]cortisol [16] was i.v. administered to the two patients and urine collected for the following 3 days. The mass spectrometric technique used to measure the isotope dilution in the cortisol metabolites was recently developed [17]. Following extraction, enzymic hydrolysis and fractionation of the urinary cortisol metabolites by high-performance liquid chromatography (HPLC), tetrahydrocortisone (THE), tetrahydrocortisol (THF), α- and β-cortolone are separated, and oxidized to

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the common product 11-oxo-aetiocholanolone (11-oxo-Et), and their corresponding 5α-tetrahydro metabolites to 11-oxo-androsterone (11-oxo-An). The methyloxime tert-butyldimethylsilyl ether derivative (MO-TBDMS) was analyzed by gas chromatography/mass spectrometry (GC/MS), and quantitation of the isotope dilution carried out by selective ion monitoring (SIM). We successfully measured the isotope dilution in the exceedingly small amounts of some cortisol metabolites excreted, despite the relatively large amount of corticosterone metabolites present in the urine of these two patients.

In addition GC and GC/MS urinary steroid profiling was carried out of the two affected sisters and their non-affected parents and sister.

## EXPERIMENTAL

### *Subjects*

The eldest sister age 11 yr (patient I) was referred to us because during a routine physical examination at school she was found to be hypertensive. She had a low plasma concentrations of cortisol, 17β-estradiol, PRA and aldosterone but high plasma concentrations of progesterone, ACTH and also of FSH and LH. Urinary steroid profiling by gas chromatography revealed elevated excretions of tetrahydrocorticosterone 2.5 μmol/24 h and 5α-tetrahydrocorticosterone 5.7 μmol/24 h and other corticosterone metabolites, and absence of any cortisol metabolites [8] (Table 1). Identity of the urinary steroids was confirmed by GC/MS (Fig. 1a) and gave unambiguous evidence of 17α-hydroxylase deficiency. Chromosomal analysis showed a normal female karyotype 46, XX. Trivial and non-standard steroid abbreviations are given in the legend to Fig. 1.

Subsequent gas chromatography and GC/MS analysis of urinary steroids of the other family members, two sisters and their parents was carried out (Table 1). One 3-yr old sister (patient II) was also found to be suffering from 17α-hydroxylase deficiency as she too excreted elevated amounts of tetrahydrocorticosterone and 5α-tetrahydrocorticosterone 0.9 and 2.0 μmol/24 h respectively (Table 1). Other corticosterone metabolites were present in urine at elevated concentrations and cortisol metabolites were not observed. (Fig. 1b). She had similar plasma hormone concentrations, except for FSH and LH. Chromosomal analysis showed a normal female karyotype 46, XX. She was not hypertensive.

The urinary steroid profiles of the parents and third sister were normal (Table 1) and none suffered from hypertension.

### *Labelled compounds and chemicals*

The [1,2,3,4-$^{13}$C]cortisol administered to the patients to measure the CPR was synthesized as described [16]. Sep-Pak $C^{18}$ Cartridges were purchased from Water Associates Inc. (Milford, Mass, U.S.A.). *Helix pomatia* digestive juice was supplied by Reactifs IBF (Clichy, France). Methoxyamine hydrochloride and tert-butyl dimethyl chlorosilane/imidazole were obtained

Table 1. Urinary steroid excretion in two sisters (patients I and II) with 17α-hydroxylase deficiency and three other family members

| Steroid | Patient I | Normal 11 yr | Patient II | Normal 3 yr | Sister | Normal 15 yr | Mother | Normal adult | Father | Normal adult |
|---|---|---|---|---|---|---|---|---|---|---|
| Pregnanediol | 5.4 | 0.8 | 0.5 | 0.2 | 1.8 | 1.7 | 6.5 | 2.0 | 3.0 | 1.8 |
| Corticosterone metabolites | | | | | | | | | | |
| THA | 3.1 | 1.3[a] | 0.3 | 0.2[b] | 0.9 | 1.3[a] | 1.4 | 1.6 | 2.0 | 2.3 |
| THB | 2.5 | 0.8[a] | 0.9 | 0.1[b] | 2.3 | 0.8[a] | 1.0 | 0.8 | 2.2 | 1.4 |
| 5α-THB | 5.7 | 1.4[a] | 2.0 | 0.6[b] | 0.7 | 1.4[a] | 1.6 | 1.2 | 1.5 | 1.2 |
| Cortisol metabolites | | | | | | | | | | |
| THE | 0 | 3.8 | 0 | 1.2 | 4.5 | 4.7 | 5.4 | 8.4 | 6.1 | 11.7 |
| THF | 0 | 1.1 | 0 | 0.6 | 3.0 | 1.9 | 4.4 | 4.5 | 5.8 | 6.6 |
| 5α-THF | 0 | 1.9[a] | 0 | [c] | 2.0 | 1.4 | 2.3 | 2.2 | 3.7 | 3.9 |
| α-cortolone | 0 | 2.2[a] | 0 | [c] | 2.2 | 2.2[a] | 2.6 | 2.9 | 2.5 | 3.6 |
| β-cortolone | 0 | 1.1[a] | 0 | [c] | 1.8 | 1.1[a] | 2.6 | 2.1 | 2.2 | 3.3 |
| α-cortol | 0 | 0.7[a] | 0 | [c] | 1.0 | 0.7[a] | 1.8 | 0.4 | 1.2 | 1.1 |
| Total cortisol metabolites | 0 | 10.8 | 0 | 1.8 | 14.5 | 12.0 | 19.1 | 20.5 | 21.5 | 30.2 |
| Total corticosterone metabolites | 11.3 | 3.5 | 3.2 | 0.9 | 3.9 | 3.5 | 4.0 | 3.6 | 5.7 | 4.9 |
| Ratio of corticosterone: cortisol metabolites | ∞ | 0.3 | ∞ | 0.5 | 0.3 | 0.3 | 0.2 | 0.2 | 0.3 | 0.2 |

Results are expressed as μmol per 24 h.
The normal values given are those established in our group.
[a]No normal values were available for this age, the values given are for a 18–20 yr old.
[b]The normal values given are from patients aged between 4.5 months and 2.5 yr [4].
[c]No normal values were available for this age.

from Alltech Ass. Inc. (Applied Science Labs., Ill., U.S.A.). *N*-trimethylsilyl-imidazole (TSIM) was purchased from Pierce (Rockford, Ill., U.S.A.. Unlabelled steroids were obtained from Sigma Chemical Co., (St Louis, Mo., U.S.A.). Labelled standard mixtures of 11-oxo-aetiocholanolone (11-oxo-Et) were synthesized as reported [17]. All other reagents and solvents were of analytical grade and purchased for Merck Co. (Darmstadt, Germany).

*Steroid profiling*

Quantitative analysis of urinary steroid metabolites of all the family members was carried out by GC, and identity of all major components in the samples verified by GC/MS using the method previously reported [20]. 1 ml urine samples from the 24 h collections were used. The accuracy and precision of the GC analyses is estimated to be about 10%, based upon a quality control research project [32].

*Measurement of the CPR*

*(a) Administration of the tracer.* The [1,2,3,4-$^{13}$C]cortisol was dissolved in 25 ml of 5% glucose solution and diluted with 25 ml of 0.9% NaCl solution. 44 ml of the mixture was i.v. administered in 10 min. To the remaining 6 ml of

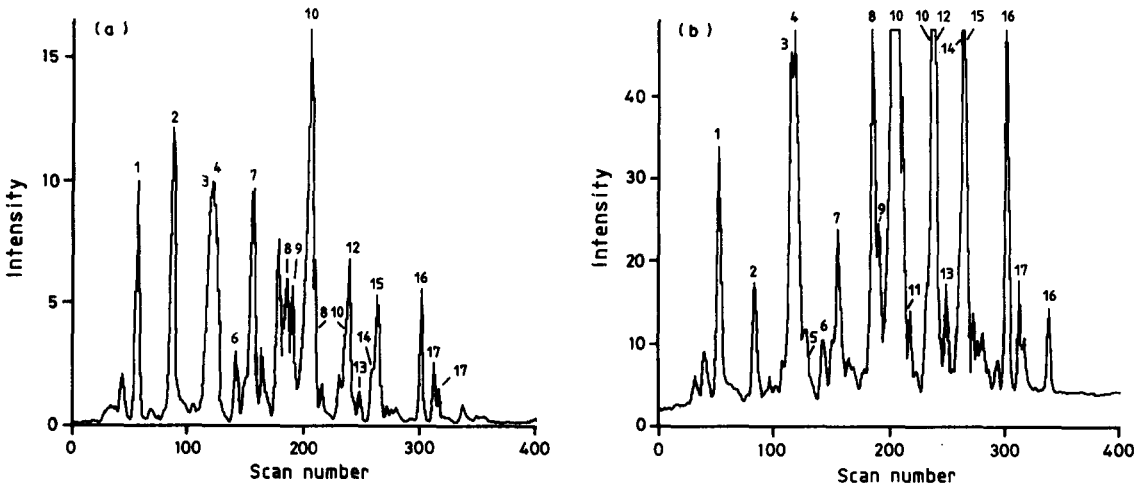


Fig. 1. Urinary steroid profiles (GC/MS reconstructed total ion current recording) of urine extracts from patients I and II with 17α-hydroxylase deficiency. The major steroid MO-TMS derivatives are labelled in order of elution from a 25 m CP Sil 5 CB capillary column.

| No. | Steroid (trivial name, abbreviation and systematic name) | |
|---|---|---|
| 1. | Pregnanediol | 5β-Pregnane-3α,20α-diol |
| 2. | 5-Pregnene-3β,20α-diol | |
| 3. | 5β-Pregnane-3α,16α,20α-triol | |
| 4. | 11-Keto-pregnanediol | 5α-Pregnane-3α,20α-diol-11-one |
| 5.* | 5β-Pregnane-3α,11β,20α-triol | |
| 6. | 5α-Pregnane-3α,11β,20α-triol | |
| 7. | 5-Pregnene-3β,16α-diol-20-one | |
| 8.† | Tetrahydro compound A (THA) | 5β-Pregnane-3α,21-diol-11,20-dione |
| 9. | Tetrahydrocorticosterone (THB) | 5β-Pregnane-3α,11β,21-triol-20-one |
| 10.† | 5α-Tetrahydrocorticosterone (5αTHB) | 5α-Pregnane-3α,11β,21-triol-20-one |
| 11. | 5α-Pregnane-1β,3α,20α-triol-11-one | |
| 12. | Hexahydro compound A | 5β-Pregnane-3α,20α,21-triol-11-one |
| 13. | 5α-Hexahydro compound A | 5α-Pregnane-3α,20α,21-triol-11-one |
| 14. | Hexahydrocorticosterone | 5β-Pregnane-3α,11β,20α,21-tetrol |
| 15. | 5α-Hexahydrocorticosterone | 5α-Pregnane-3α,11β,20α,21-tetrol |
| 16.† | 6α-Hydroxytetrahydrocorticosterone | 6α-OH THB |
| 17.† | Corticosterone | |

* Steroid found only in urine of patient II.
† Some steroids are present as two peaks, the α-ketol side chain gives rise to two derivatives in the *syn*- and *anti*-forms.

Nomenclature of other steroids included in the study. 11-oxo-aetiocholanolone (11-oxo-Et) 5β-androstane-3α-hydroxy-11,17-dione; 11-oxo-androsterone (11-oxo-An) 5α-androstane-3α-hydroxy-11,17-dione; tetrahydrocortisone (THE) 5β-pregnane-3α,17α,21-triol-11-20-dione; 5α-tetrahydrocortisone (5α-THE) 5α-pregnane-3α,17α,21-triol-11-20-dione; tetrahydrocortisol (THF) 5β-pregnane-3α,11β,17α,21-tetrol-20-one; α-cortolone 5β-pregnane, 3α,17α,20α,21-tetrol-11-one; β-cortolone 5β-pregnane, 3α,17α,20β,21-tetrol-11-one; α-cortol 5β-pregnane, 3α,11β,17α,20α,21-pentol.

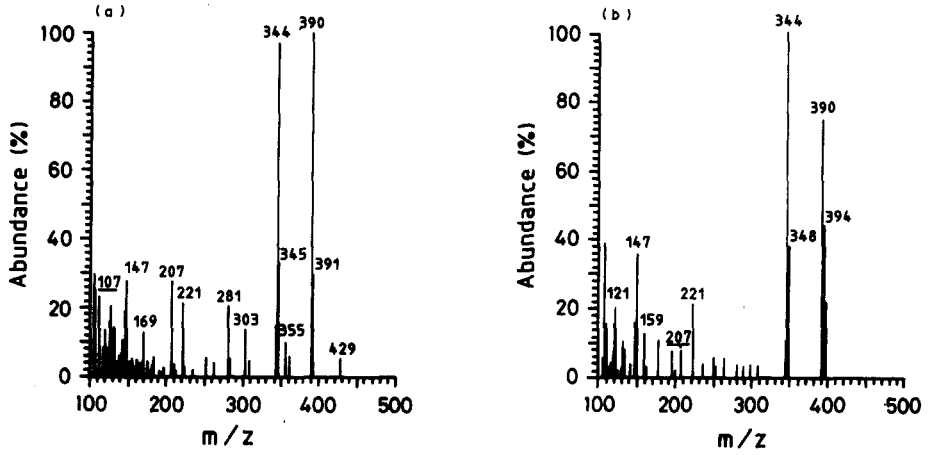


Fig. 2. Mass spectra of 11-oxo-Et MO-TBDMS derivatives. (a) Reference spectrum. (b) 11-oxo-Et oxidized from α- and β-cortolone fraction obtained from patient I from the 0.5 day urine collection. The presence of the $^{13}C_4$-labelled tracer is clearly evident.

solution prednisolon was added as internal standard, the two steroids were then extracted with a dichloromethane/ethyl acetate mixture and the dry residue was dissolved in HPLC eluents, and their concentrations were determined by HPLC, using solvent system 3. [19]. The amount of tracer given to patients I and II was 60 and 2.4 μg (with a relative SD of < 5%).

Administration of [1,2,3,4-$^{13}$C]cortisol for the measurement of the CPR was approved by the local medical ethical committee and informed consent was obtained from the parents.

*(b) Urine collection.* Urine was collected for 3 days after i.v. administration of the label and times and volume of voided urine noted. Urines were stored at −20°C until analysis.

*(c) Steroid extraction.* Extraction and hydrolysis of cortisol metabolites was carried out as previously published [17, 19, 20].

*(d) HPLC.* Isolation of the cortisol metabolites from the other steroids including tetrahydro corticosterone metabolites was carried out by HPLC as previously reported [17, 19, 20].

*(e) Oxidation of cortisol metabolites.* Oxidation of the steroids in the HPLC fractions which eluted at retention times corresponding to cortisol metabolites [19] was carried out for reasons previously discussed [17]. Oxidation of THE, THF and α- and β-cortolone yields 11-oxo-Et, and of 5α-THF and 5α-THE yields 11-oxo-An.

*(f) Determination of $^{13}C_4$-enrichment (isotope dilution) in the oxidized cortisol metabolites.* Derivatization of 11-oxo-Et and 11-oxo-An with methoxyamine hydrochloride and tert-butyl dimethyl chlorosilane/imidazole gives rise to MO-TBDMS ethers of similar spectra [17]. The $^{13}$C-enrichment (isotope dilution) in the 11-oxo-Et derivatives were measured by selected ion monitoring (SIM) at $m/z$ 344 $[M-103]^+$ and $m/z$ 348 for natural and labelled 11-oxo-Et, respectively. With the 11-oxo-An derivatives the $^{13}$C-enrichment was measured by SIM at $m/z$ 390 $[M-57]^+$ and $m/z$ 394 for natural and labelled material, respectively as previously reported [17]. The spectrum of 11-oxo-Et is given in Fig. 2a.

*(g) Calculation of the CPR.* The method used to measure the CPR is that developed by Cope and Black [14, 15]. Labelled cortisol is administered to the subject and the isotope dilution measured in the urinary cortisol metabolites.

Table 2. $^{13}C_4$-enrichments in cortisol metabolites and cortisol production rates in patient I with 17α-hydroxylase deficiency

| Time (days) | Cumulative urine excretion (ml) | Volume used for measurement (ml) | Tetrahydrocortisone | | α- and β-cortolone | |
|---|---|---|---|---|---|---|
| | | | Isotope dilution | CPR (nmol/day) | Isotope dilution | CPR (nmol/day) |
| 0.50 | 747 | 149 | 0.30 ± 0.008 | 1110 | 0.509 ± 0.028 | 656 |
| 0.92 | 846 | 85 | 0.184 ± 0.006 | 980 | 0.230 ± 0.005 | 784 |
| 1.50 | 1459 | 97 | 0.122 ± 0.003 | 907 | 0.190 ± 0.005 | 583 |
| 2.00 | 1724 | 86 | 0.086 ± 0.001 | 965 | 0.148 ± 0.004 | 561 |
| 2.48 | 2436 | 97 | 0.088 ± 0.001* | 765 | 0.114 ± 0.001 | 587 |
| 2.95 | 2692 | 90 | NA | NA | 0.150 ± 0.003 | 536 |

The isotope dilution is given as a mean ± SD.
$n = 4$ unless otherwise specified; *indicates $n = 3$; NA = not assayed.

Substitution of the measured isotope dilution for each metabolite gives the CPR according to the equation:

$$CPR = R/(ID \cdot t);$$

where $R$ is the dose administered in nmol, ID the isotope dilution of $^{13}C_4$-labelled metabolite, $t$ the time of the urine collection used in days and CPR the cortisol production rate in nmol/day.

*Statistics*

All statistics was carried out using the paired $t$-test.

## RESULTS

*CPR in patient I*

The CPR calculated from THE using the $2\frac{1}{2}$ day urine collection was 765 nmol/day which was significantly lower than the apparent CPR at 1 day of 989 nmol/day (Table 2). The apparent CPR calculated from α- and β-cortolone at 1 day was 784 nmol/day (Table 1) which was significantly higher than that at 2 or 3 days of 561 and 536 nmol/day respectively. The CPR calculated from the cortolones was always ~40% lower than the CPR calculated from THE (except at 0.5 day).

The spectrum of 11-oxo-Et MO-TBDMS obtained from α- and β-cortolone isolated from the 0.5 day urine collection is given in Fig. 2b, which is similar to the reference compound spectrum shown in Fig. 2a except for the $^{13}C_4$-isotope enrichment at $m/z$ 348 and 394. Selected ion monitoring recordings at $m/z$ 344 and 348 are given in Fig. 3a of the same sample. The SIM recordings of 11-oxo-Et MO-TBDMS derivative derived from THE isolated from the 0.5 day urine collection is given in Fig. 3b.

Table 3. $^{13}C_4$-enrichments in tetrahydrocortisone and cortisol production rates in patient II with 17α-hydroxylase deficiency

| Time (days) | Cumulative urine excretion (ml) | Volume used for measurement (ml) | Isotope dilution | CPR (nmol/day) |
|---|---|---|---|---|
| 0.49 | 57 | 6 | 0.316 ± 0.005* | 43 |
| 1.00 | 202 | 20 | 0.152 | 44 |
| 1.50 | 536 | 54 | 0.099 ± 0.002† | 44 |
| 1.99 | 678 | 68 | 0.056 | 59 |
| 2.99 | 1110 | 111 | 0.036 | 62 |

$n = 2$. * indicates $n = 4$; † indicates $n = 3$.

*CPR in patient II*

The CPR calculated from THE using the 3-day urine collection was 62 nmol/day, which was not significantly different from the CPR calculated at any other urine collection times (Table 3). The increase in apparent CPR with time up until excretion of the label is complete is contrary to that normally observed (see Table 2 for patient I). This may be due to a very low CPR on day one of urine collection, compared to days 2 and 3 in this study. The SIM recordings of 11-oxo-Et MO-TBDMS derived from THE isolated from the 0.5 day urine collection are given in Fig. 3c.

The CPR could not be calculated from all the

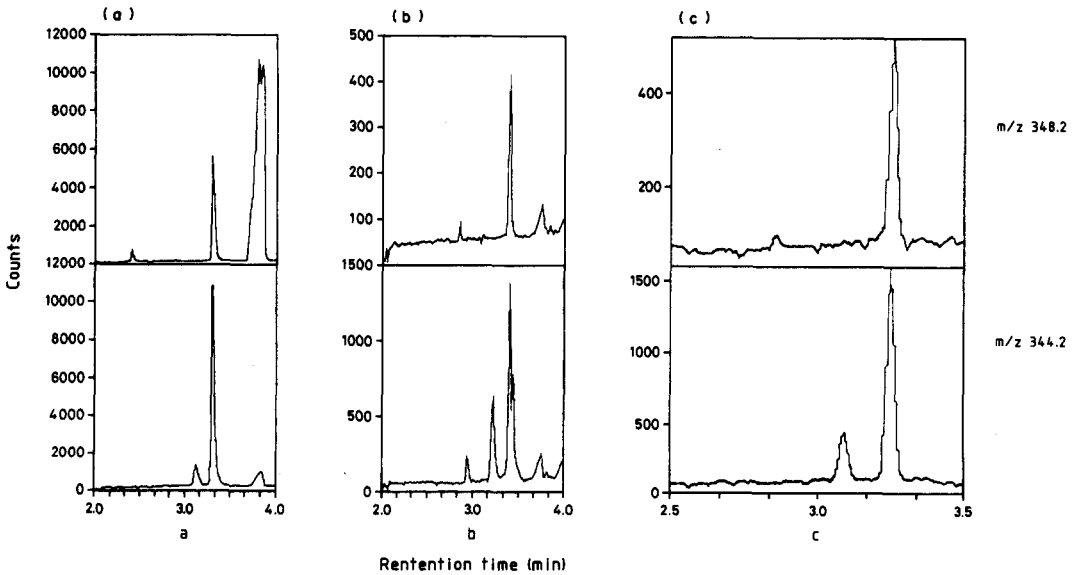


Fig. 3. Selected ion monitoring recordings of 11-oxo-Et MO-TBDMS derivatives measured at $m/z$ 344.2 and $m/z$ 348.2 prepared from cortisol metabolites isolated from 0.5 day urine collections. (a) α- and β-cortolone from patient I (b) THE from patient I and (c) THE from patient II.

urinary cortisol metabolites in these patients. This was due to interfering compounds co-eluting with the oxidized and derivatized cortisol metabolites when using GC/MS SIM for measurement of the $^{13}$C-isotope dilution, or with the 5α-THE and 5α-THF as insufficient mass was found.

*Urinary steroid profiling*

Metabolites assayed in urine by gas chromatography of all the family members are listed in Table 2. The concentrations of both 5-pregnene-3β,11β,20α-diol and 5-pregnene-3β,11β,20α-triol (Fig. 1) legend no's 2 and 7, respectively may be reduced as a consequence of hydrolysis with *Helix pomatia* digestive juice. It has been reported that *Helix pomatia* digestive juice converts these steroids into the corresponding 3β-hydroxy-5-ene steroids [33].

The reconstructed total ion current chromatograms of urinary steroid profiles of patients I and II, both suffering from 17α-hydroxylase deficiency are presented in Figs 1a and b. The metabolites were identified according to their retention times and mass spectra. The reference spectra used for comparison are those from the literature [1, 2, 6] or cited therein.

Some peaks were observed by gas chromatography eluting at retention times corresponding to cortisol metabolites, but subsequent GC/MS analysis revealed them to be corticosterone metabolites.

The gas chromatographic urinary steroid profiles of both parents and the third daughter were normal, and quantitation of pregnanediol, cortisol and tetrahydro-corticosterone metabolites were all within the normal range (Table 1).

The ratio of the sum of the major metabolites of corticosterone to the sum of the metabolites of cortisol were abnormally high in patients I and II due to reduced cortisol production. This ratio was normal for both parents and third daughter.

## DISCUSSION

This study demonstrates that our recently developed stable isotope mass spectrometric method [17] using [1,2,3,4-$^{13}$C]cortisol [16], can be successfully applied to measure the very low CPR in patients with 17α-hydroxylase deficiency.

*The CPR in 17α-hydroxylase deficiency*

Previous studies measuring the CPR in patients with 17α-hydroxylase deficiency using radioactive labelled cortisol observed values ranging from 1.4 to 18.5 μmol/day [8–13]. One study using radioactively labelled cortisol [7] even reported that no cortisol was produced by the patient. The other studies [1, 3, 4, 21] which measured the CPR indirectly by measuring the mass of cortisol metabolites excreted, either by GC or GC/MS found values between 36 and 185 nmol/day. Our patient II was in the extreme low range producing 62 nmol/day, and patient I having a CPR of ~600 nmol/day was also low compared to previous measurements using radioactivity labelled cortisol. The much higher values observed by other investigators may be due to the analytical methods employed [8–13].

With patient II, the CPR calculated from the 3-day urine collection was not different than the 1 day collection. Normally the longer collection time period is more reliable. After 2 days excretion of the label is considered complete by some groups [14, 22–25] (4–20% of the administered dose is excreted in the second day). Other groups [26] prefer to wait for 3 days. Calculation of the CPR can only be carried out correctly when excretion of the label is complete [15, 27].

Recently two groups [28, 29] besides ourselves [20] have published work using HPLC to separate and quantitate radioactively labelled cortisol metabolites in CPR studies. Only one other group is using stable labelled cortisol to measure the CPR [30]. It is hoped that there will be a renewal of interest in this clinical test as the measurement of the CPR is considered one of the major assays to assess adrenocorticol function [24, 31].

*Analytical aspects*

None of the HPLC chromatograms of the steroid extracts from the urine contained observable peaks eluting at retention times corresponding to cortisol metabolites. All HPLC fractions potentially containing cortisol metabolites were examined by GC/MS SIM.

With the HPLC fractions from urine collection abstracts from patient I containing 5α-THF and THF interfering co-eluting peaks prohibited isotope ratio measurements.

In patients II the CPR could only be determined from THE. With THF and cortolone co-eluting peaks prevented measurement, and the 5α-metabolites were not present in sufficient amounts even with up to 111 ml of urine to enable measurement.

Further HPLC of the cortisol metabolites, and of the oxidation products themselves may have separated the 11-oxo-Et and 11-oxo-An from interfering co-eluting compounds to have enabled GC/MS SIM to be carried out.

This is the first report using stable labelled cortisol and the urinary method for measuring the CPR in patients with 17α-hydroxylase deficiency. Only even after HPLC purification, specific oxidation, derivatization and GC/MS selective ion monitoring, could the isotope dilution and therefore the CPR be determined in these patients. Even then the CPR could only be calculated from THE and the cortolones in patient I and THE in patient II. If non stable labelled isotopes were used it would have been difficult if not impossible to measure the CPR in patients with 17α-hydroxylase deficiency with present day techniques. The use of stable isotopes enables a more sensitive, specific and thus more accurate GC/MS method to be used than one employing radioactively labelled cortisol.

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A preliminary account of the results were presented at the *25th European Society for Pediatric Endocrinology*, Zurich, Switzerland (1986) [18].

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