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A new marker for early diagnosis of 21-hydroxylase deficiency: 3β,16α,17α-trihydroxy-5α-pregnane-7,20-dione☆,☆☆

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ABSTRACT

In neonates with 21-hydroxylase deficiency the specific marker 11-oxo-pregnanetriol is at low levels in the first days of life and this drives the search for alternatives. We describe the structural characterisation of a new early marker, 3β,16α,17α-trihydroxy-5α-pregnane-7,20-dione.

Urine samples from 87 untreated and 11 recently treated newborns with 21-hydroxylase deficiency (42 males and 56 females) between birth and 40 days of age and control samples from 7 healthy neonates (4 males, 3 females) were compared. Steroids were analyzed as methyloxime-trimethylsilyl ether derivatives by GC–MS and GC–MS/MS, after extraction and enzymatic conjugate hydrolysis. Microchemical methods and deuterated derivatives were used.

The new steroid was identified by comparison with 3β,16α,17α-trihydroxy-preg-5-en-20-one and 3β-hydroxy-5α-pregnane-7,20-dione standards. It was present for the first 4 weeks after birth (with a maximum around day 4) and showed a marked inter-individual variability. No effect of treatment was evident and levels were much higher than for 11-oxo-pregnanetriol in the first days of life. Only traces were found in controls.

The likely involvement of oxysterol 7α-hydroxylase (CYP7B1) from the 'acidic' pathway of bile acid synthesis and 11β-hydroxysteroid dehydrogenase-1 in the generation of the 7-oxo group is discussed.

We conclude that this steroid is a useful early marker of 21-hydroxylase deficiency.

1. Introduction

The most common cause of congenital adrenal hyperplasia is 21-hydroxylase deficiency. It is characterized by increased serum 17α-hydroxyprogesterone and 21-deoxycortisol, which are excreted in urine as numerous metabolites. These are mostly excreted unconjugated or as glucuronides. Urinary steroid profiling is a valuable tool for diagnosis. Interpretation is relatively straightforward in adults and children [1–3], where there are only three major metabolites – 17α-hydroxypregnanolone, pregnanetriol and 11-oxo-pregnanetriol. Of these, 11-oxo-pregnanetriol is the most specific since it is a metabolite of 21-deoxycortisol, a compound derived by 11β-hydroxylation of 17α-hydroxyprogesterone within the adrenal. However, all three metabolites are relatively minor in neonates, where diagnosis often presents a challenge, particularly in the first days of life [2,4] and this drives the search for early markers of this disorder. The usefulness of 15β-hydroxylated compounds as additional markers [5] has been widely accepted [1,6] but they can be also observed in other disorders [7].

We have noted that the steroid sulphate fraction of urine from affected neonates consistently contains a late-eluting, previously undescribed, steroid. This paper sets out the structural characterisation of this early marker of 21-hydroxylase deficiency (referred to here as Compound 607, named after the base peak at *m*/*z* 607), based on a combined GC–MS and GC–MS/MS approach assisted with microchemistry methods. It also examines factors that may influence the level of this compound (age, treatment etc.) and discusses its possible origin.

2. Experimental

2.1. Materials

All solvents were purchased from Rathburn, UK. Other reagents were supplied as follows: *Helix pomatia* juice (SHP) – Biosepra, Cergy, France and also kindly given by Dr. AOK Chan; sodium ascorbate – Sigma–Aldrich, UK; sodium borohydride – VWR Inter-

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national, UK; methoxyamine hydrochloride (MO) – Fluka, via Sigma–Aldrich, UK; trimethylsilylimidazole (TSIM) – Perbio Science, UK.

Sep-Pak cartridges originated from Waters, UK, the capillary column for GC–MS from Varian, UK and for GC–MS/MS from Agilent, UK.

Steroid standards were kindly provided as personal gifts by Prof. HLJ Makin (3β-hydroxy-5α-pregnane-7,20-dione), Prof. CHL Shackleton (3β,16α,17α-trihydroxy-pregn-5-en-20-one), Dr. RA Harkness (3β-hydroxy-5α-pregnane-6,20-dione) or originated from the MRC Steroid Reference Collection (5β-pregnane-3β,20α-diol and 5β-pregnane-3β,20β-diol). Deuterated reagents (d_3MO and d_9-TSIM) were purchased from CDN Isotopes via QM_x Laboratories, UK.

2.2. Urine samples

Urine samples from 98 first presenting newborns with 21-hydroxylase deficiency between birth and 40 days of age were sent by various centres to our referral service as a part of their medical investigation. They were obtained using polythene collecting bags, so fecal contamination was avoided. The presence of free cortisol in 11 samples of patients older than 5 days indicated that recent treatment had been given, while in the remaining 87 samples there was no indication of treatment. The age and sex distribution of the samples was as follows – 56 females aged 6 ± 6 days (mean ± standard deviation) and 42 males aged 15 ± 7 days. Gestational age at birth was available only for 10 infants, of which 6 were term (over 36 weeks) and 4 pre-term. Control samples (total of 21) from 7 healthy term neonates (4 males, 3 females) were collected on disposable gel-containing nappies (diapers) at ages 0–5 days, 9 and 29 days, with written parental permission.

Follow-up samples were available for 14 patients (10 female and 4 male). For 12 of them at least one of the samples was collected during treatment.

2.3. Steroid analysis

Steroid metabolites in urine were analyzed by urinary steroid profiling as described [8] except that the chromatographic separation of glucuronide and sulfate conjugates prior to hydrolysis was omitted. The major steps are solid phase extraction, enzymic conjugate hydrolysis and formation of MO-TMS derivatives. This method includes the use of sodium ascorbate during hydrolysis in order to prevent the formation of artifacts of 3β-hydroxy-5-ene steroids [9].

Urine collected on nappies (diapers) was extracted after equilibration with 4% sodium chloride solution [10] and analyzed as above.

2.4. Microchemistry

Borohydride reduction was used to convert oxo into hydroxy groups, according to a published method [11] except that the extraction of the products from the reaction mixture used Sep-Pak columns after dilution with 20 ml rather than 50 ml deionised water. Acetonide formation [12] was used to demonstrate a syn-position of neighboring hydroxyls.

Cholesterol oxidase activity present in Helix pomatia juice was used to convert 3β-hydroxylated steroids into the 3-oxo form and 3β-hydroxy-5-ene steroids into 3-oxo-4-ene steroids with an additional 6-oxo group. We have previously studied this property of Helix pomatia juice and shown that ascorbate abolishes it [9]. Incubation was carried out with 100 μl of Helix pomatia juice in 1 ml 0.5 M acetate buffer pH 5.0 in the absence of ascorbate, for 1 h at 55 °C.

2.5. GC–MS

GC–MS was performed on a Perkin Elmer Clarus 500 single quadrupole mass spectrometer with a 20 m capillary column, 0.32 mm internal diameter, wall-coated with VF-1 ms (100% dimethylpolysiloxane), film thickness 0.10 μm. The temperature program was: 100 °C (3 μl injection volume), hold for 1 min, then 20°/min to 220 °C, then 4°/min to 270 °C, hold for 4 min. Electron ionization mode was used at 70 eV. The mass range was 100–900 a.m.u. The temperature of the ion source was 220 °C.

2.6. Quantification

Amounts were calculated against the internal standard 5α-androstane-3α,17α-diol using the peak heights of selected ion plots for an ion specific for each steroid and a response factor for a total ion current (TIC) plot, due to lack of synthesised standard for Compound 607. Response factors were calculated as a mean of the ratio between the TIC and the specific ion plot peak heights for the thirty highest peaks of each compound. The specific ions and response factors are as follows: Compound 607 – ion at m/z 607 ($[M-31]^+$), response factor 18.3 (±2.1) (mean (±standard deviation)); 11-oxo-pregnanetriol – ion at m/z 449 ($[M-117]^+$), factor 17.3 (±2.1); 3α,17α-dihydroxy-5β-pregnane-20-one and its 5α epimer – ion at m/z 476 ($[M-31]^+$), response factor 17.8 (±1.9); 3α,15β,17α-trihydroxy-5β-pregnane-20-one and its 5α epimer – ion at m/z 564 ($[M-31]^+$), response factor 48.1 (±3.5); 3β,15β,17α-trihydroxy-5α-pregnane-20-one – ion at m/z 258 (D-ring cleavage ion used due to interference in the measurement of the ion at m/z 564 ($[M-31]^+$)), response factor 11.3 (±0.8); internal standard – ion at m/z 241 ($[M-15-2\times90]^+$), response factor 17.6 (±1.4). All calculated values were expressed as μg/mmol creatinine, to compensate for the urine collections being untimed.

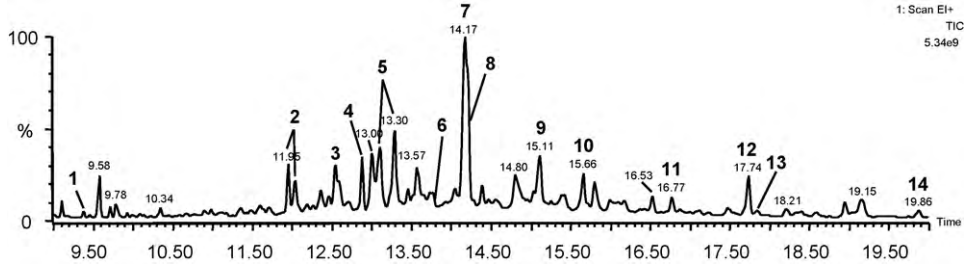

Fig. 1. Total ion current chromatogram obtained by GC–MS of a 3 day old untreated female with 21-hydroxylase deficiency. Endogenous compounds: **2** – 3β,16α-dihydroxy-androst-5-en-17-one (MU 27.61 and 27.71); **3** – 3β,16β-dihydroxy-androst-5-en-17-one (MU 28.19); **4** – 3β,17α-dihydroxy-androst-5-en-16-one (MU 28.5), **5** – 3β,16α,18-trihydroxy-androst-5-en-17-one (MU 28.71 and 28.89); **6** – 3α,17α,20α-trihydroxy-5β-pregnan-11-one (11-oxo-pregnanetriol, MU 29.34); **7** – 3β,16α-dihydroxy-pregn-5-en-20-one (MU 29.65); **8** – androst-5-ene-3β,15β,16α,17β-tetrol (MU 29.70); **9** – androst-5-ene-3β,15α,16β,17β-tetrol (MU 30.45); **10** – pregn-5-ene-3β,20α,21-triol (MU 30.93); **11** – pregn-5-ene-3β,16α,20α,21-tetrol (MU 31.81) and **12** – Compound 607 (MU 32.58). Internal standards: **1** – 5α-androstane-3α,17α-diol (MU 24.67); **13** – stigmasterol (MU 32.67) and **14** – cholesterol butyrate (MU 34.30).

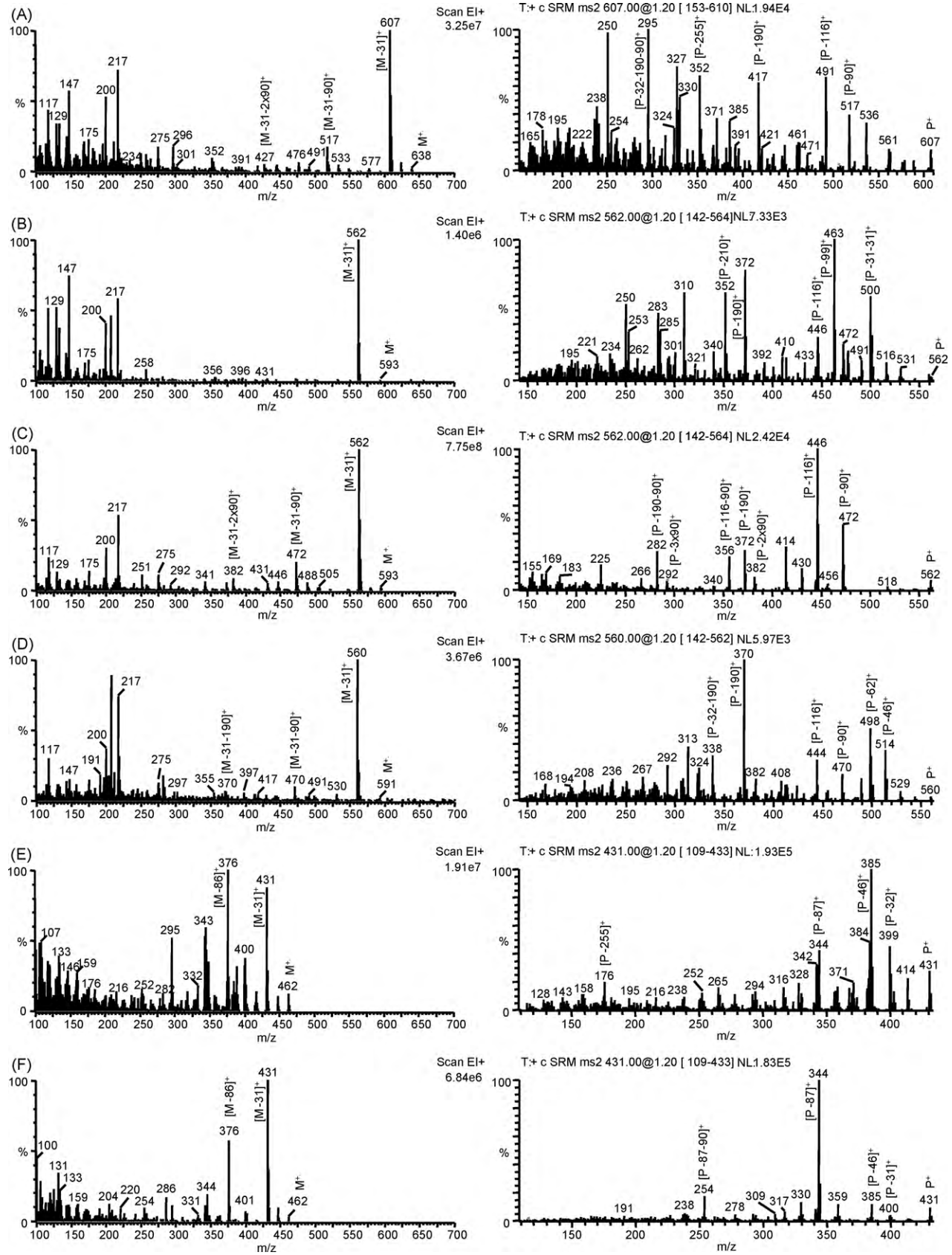

Fig. 2. GC–MS (left) and GC–MS/MS (right) spectra of MO-TMS derivatives of Compound 607, together with 20-oxo standards that are structurally related to it and their transformation products formed by incubation with Helix pomatia in the absence of ascorbate. (A) Compound 607 (identified as 3β,16α,17α-trihydroxy-5α-pregnane-7,20-dione), (B) 16α,17α-dihydroxy-5α-pregnane-3,7,20-trione (peak one) – derived by Helix pomatia transformation of Compound 607, (C) 3β,16α,17α-trihydroxy-pregn-5-en-20-one standard, (D) 16α,17α-dihydroxy-pregn-4-ene-3,6,20-trione (peak four) derived by Helix pomatia transformation of the above standard, (E) 3β-hydroxy-5α-pregnane-7,20-dione standard and (F) 3β-hydroxy-5α-pregnane-6,20-dione standard. $P^+ = [M-31]^+$.

2.7. GC–MS/MS

GC–MS/MS was performed on a Finnigan MAT GCQ ion-trap system fitted with a 25 m capillary column 0.2 mm internal diameter, wall-coated with HP-1 (100% dimethylpolysiloxane), film thickness 0.11 μm. The temperature program was as for the GC–MS above. The temperature of the ion source was 250 °C and 1 μl was injected. Electron ionization mode was used. The MS/MS parameters were as follows: isolation width 3 a.m.u., isolation time 12 ms, collision time 15 ms, excitation voltage 1.2 V. The precursor ion chosen in all cases was $P^+ = [M-31]^+$ (loss of methoxyl radical from a methoximated 20-oxo group). Throughout the text, all ion derivations are represented in square brackets with a plus sign to indicate that positive ion mode was used. It is not intended to indicate whether an odd or even electron species is formed.

2.8. Retention times

Retention times on GC–MS are expressed as methylene units (MU), calculated relative to a mixture of even and odd numbered hydrocarbons containing all members in the series between C_{26} and C_{36} and based on a linear interpolation between the retention times of neighboring hydrocarbons.

3. Results

3.1. Structure of Compound 607

Compound 607 gives a single peak (peak 12) as an MO-TMS derivative with MU 32.58 (Fig. 1). The GC–MS spectrum (Fig. 2A) contains a molecular ion at *m/z* 638 which shifts to 644 (2× 3 a.m.u.) in the d_3MO-TMS and to 665 (3× 9 a.m.u.) in the MO-d_9TMS derivatives, indicating a fully derivatised trihydroxy-pregnanedione structure.

3.1.1. 16α,17α-Dihydroxy-20-oxo structure

The $[M-31]^+$ ion in the MO-TMS derivative, which is the base peak at *m/z* 607, becomes $[M-34]^+$ in the d_3MO-TMS. This corresponds to a loss of a methoxyl group at $C_{20,}$ indicating a 20-oxo structure. Further, the D-ring cleavage ions with *m/z* 200, 217 and 275 in the GC–MS spectrum and $[P-116]^+$, $[P-190]^+$ and $[P-190-90]^+$ in the GC–MS/MS spectrum (Fig. 2A) are shared with 3β,16α,17α-trihydroxy-pregn-5-en-20-one standard (Fig. 2C). Also shared is the ion at *m/z* 319 in the borohydride reduction product of Compound 607 (Fig. 3A) and of the 16,17-dihydroxylated standard (Fig. 3B). These together indicate the presence of a 16,17-dihydroxy-20-oxo structure. The derivation of the above ions is described in a separate communication [13].

Compound 607 formed an acetonide, demonstrating that the 16-hydroxyl is in the alpha position, since all biological pregnanes have a 17α-hydroxyl. Formation of the acetonide was established on the basis of the disappearance after incubation in dry acetone of Compound 607 along with other endogenous steroids with known structure containing cis-diols such as pregn-5-ene-3β,16α,20α,21-tetrol and androst-5-ene-3β,15α,16β,17β-tetrol. No products could be identified for any of these compounds. Endogenous steroids lacking cis-diols such as 3β,16α-dihydroxy-pregn-5-ene-20-one and androst-5-ene-3β,15β,16α,17β-tetrol were not affected, indicating that indeed acetonide formation rather than another transformation had taken place.

3.1.2. 3β-Hydroxy-5α-reduced structure

Compound 607 is found entirely in the sulphate fraction when conjugate separation is performed according to our original method [8]. It is transformed by the cholesterol oxidase activity of Helix pomatia juice in the absence of sodium ascorbate to a 3-oxo product (Fig. 2B). These features indicate that it is 3β-hydroxylated and the second oxo group is not at C_3.

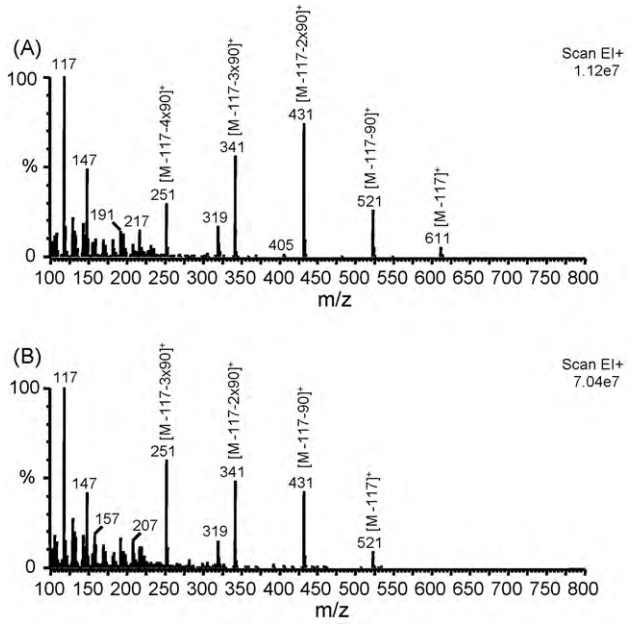

Fig. 3. GC–MS spectra of the MO-TMS derivatives of 20-hydroxy compounds related to Compound 607. (A) 5α-pregnane-3β,7ξ,16α,17α,20ξ-pentol – derived by borohydride reduction of Compound 607, (B) pregn-5-ene-3β,16α,17α,20ξ-tetrol – derived by borohydride reduction of 3β,16α,17α-trihydroxy-pregn-5-en-20-one standard.

We previously established that 3β-hydroxy-5α-pregnanes were converted to 3-oxo-5α-products [9] but did not examine conversion of 3β-hydroxy-5β-pregnanes. For the purpose of this study 5β-pregnane-3β,20α-diol and 5β-pregnane-3β,20β-diol standards were incubated with Helix pomatia juice in the absence of ascorbate. No 3-oxo products were obtained. We therefore conclude that Compound 607 is 5α-reduced.

Levels of known 3α-hydroxy-5α-reduced compounds were found to be strongly related, eg: 3α,17α-dihydroxy-5α-pregnane-20-one (allo 17α-hydroxypregnanolone) vs. 3α,15β,17α-trihydroxy-5α-pregnane-20-one ($r = 0.921$). Compound 607 showed no correlation with the above compounds ($r = -0.081$ and $r = -0.017$ respectively), nor with 3β,15β,17α-trihydroxy-5α-pregnane-20-one ($r = 0.031$) or the 5β/5α ratio of 17α-hydroxypregnanolone epimers ($r = -0.129$). Correlation comparisons were carried out between the ratio over creatinine of all compounds in order to avoid influence of the sample concentration.

3.1.3. 7-Oxo structure

Our comparisons of standards and their transformation products have shown that in 20-oxo pregnanes additional oxo groups give rise to a single peak as MO-TMS derivatives only in the following positions – at C_6 in 5α-reduced but not in 5β-reduced pregnanes nor in pregn-4-enes, at C_7 in 5α-reduced pregnanes and at C_{19} (aldehyde). Of these, position C_{19} is refuted, because the borohydride reduction product of Compound 607 (Fig. 3A) does not have an ion at *m/z* 103 on GC–MS, indicative of a primary hydroxyl group, which would have been formed at C_{19} from the aldehyde. Thus, the C_6 and C_7 alternatives were further considered.

Fig. 2 demonstrates that a prominent fragment $[P-255]^+$ in the GC–MS/MS spectrum of Compound 607 (the ion at *m/z* 352, Fig. 2A) is shared by 3β-hydroxy-5α-pregnane-7,20-dione standard (the ion at *m/z* 176, Fig. 2E). This loss increases by 3 a.m.u. in the d_3MO-TMS derivative ($[P-258]^+$) and by 9 a.m.u in the MO-d_9TMS ($[P-264]^+$) of Compound 607, indicating that the lost fragment con-

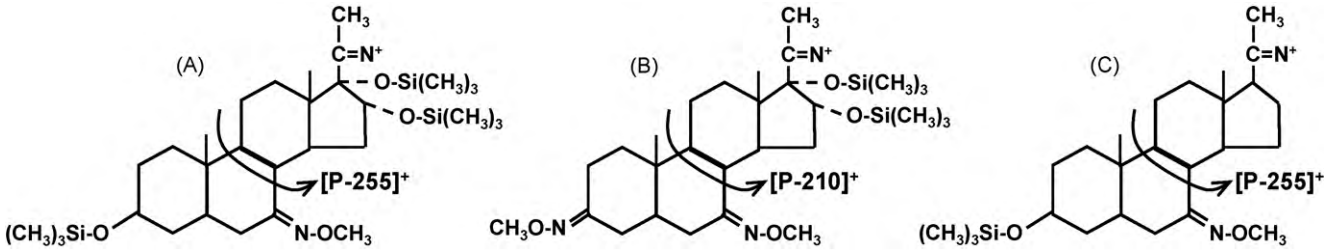

Fig. 4. Schematic presentation of the B-ring cleavage of 7-oxo compounds. (A) Compound 607, (B) 3-oxo product of Compound 607 (derived by incubation with Helix pomatia in the absence of ascorbate) and (C) 3β-hydroxy-5α-pregnane-7,20-dione standard.

tains one hydroxyl and one oxo group. Further, the 3-oxo product of Compound 607 (Fig. 2B), derived by Helix pomatia transformation, contains the same ion at *m*/*z* 352 in its GC–MS/MS spectrum with similar intensity, which represents a $[P-210]^+$ fragment. The 45 a.m.u. difference between the losses in Compound 607 and its 3-oxo variant corresponds to the replacement of the derivatised 3β-hydroxy group (89 a.m.u.) and one hydrogen with a derivatised 3-oxo group (45 a.m.u.). The above suggests that the mechanism of formation of the ion at *m*/*z* 352 involves loss of the A-ring with the 3β-hydroxy group attached to it and the second oxo group. Loss of the A- and B-rings by cleavage of the bonds between C_{7-8} and C_{9-10} would explain the formation of this ion (Fig. 4). This is most likely triggered by a neighbouring 7-oxo rather than a 6-oxo group, because the analogous ions are absent or at very low levels in the GC–MS/MS spectra of 6-oxo compounds – namely an ion at *m*/*z* 352 ($[P-208]^+$) in 16α,17α-dihydroxy-preg-4-ene-3,6,20-trione, a Helix pomatia transformation product of 3β,16α,17α-trihydroxy-pregn-5-en-20-one standard (Fig. 2D) and at *m*/*z* 176 ($[P-255]^+$) in 3β-hydroxy-5α-pregnane-6,20-dione standard (Fig. 2F).

Based on the above considerations we conclude that Compound 607 is 3β,16α,17α-trihydroxy-5α-pregnane-7,20-dione.

3.2. Factors potentially influencing the levels of Compound 607

3.2.1. Post-natal age

Compound 607 was found to be present for the first 3–4 weeks after birth with a maximum around day 4 of life. Levels were subject to great inter-individual variability (Fig. 5) and were not affected by recent treatment. Whilst there were traces in the first 2 or 3 days of life in normal neonates, the lowest levels at that age in affected neonates were over six times higher. Levels in normal neonates were not affected by collection on nappies (unpublished data).

Comparison with 11-oxo-pregnanetriol shows that in the first days of life Compound 607 has much higher values (Fig. 6).

3.2.2. Post-conceptional age

Levels of Compound 607 in pre-term affected neonates 613–882 (median 727 μg/mmol creatinine) were well within the range of, the affected neonates born at term: 45–2079 (median 946 μg/mmol creatinine). This study did not include pre-term unaffected neonates but we have not found increases of this compound in pre-term neonates that have been submitted to our service and did not show inborn errors of steroid metabolism.

3.2.3. Severity of the disorder

For 38 patients there was sufficient information to identify salt-wasting. Since electrolyte disturbances do not present within the first week of life, the two groups of patients – known salt-wasters and those without information on electrolyte status – were further subdivided into groups: up to 7 days and over 7 days old. The levels of Compound 607 in the up to 7 day group subsequently found to be salt-wasters (range 433–2196 (median 882) μg/mmol creatinine) were well within the range of those for whom no information was available: 138–10214 (890) μg/mmol creatinine. However, salt-wasters in the older group had somewhat higher levels: 12–1528 (395) vs. 12–1482 (272) μg/mmol creatinine.

For 45 patients, numerical values for serum 17α-hydroxyprogesterone were available to us. However, only 10 of them were measurable while the rest were above the upper limit of the assay. For the ten measurable values there was a significant correlation between the levels of Compound 607 in urine and serum 17α-hydroxyprogesterone ($r = 0.895$).

3.2.4. Treatment

Evolution of Compound 607 with treatment is shown in Fig. 7. All boys presented with salt-wasting crisis and in all of them there were indications of acute hydrocortisone treatment (high dose intravenous hydrocortisone) given in the first submitted urine sample (indicated by high levels of free cortisol with absent or relatively low tetrahydrocortisone). In two of the girls no treatment was given (the samples were 2 and 25 days apart respectively). Established treatment involved oral administration of hydrocortisone and was indicated by the presence of high levels of tetrahydrocor-

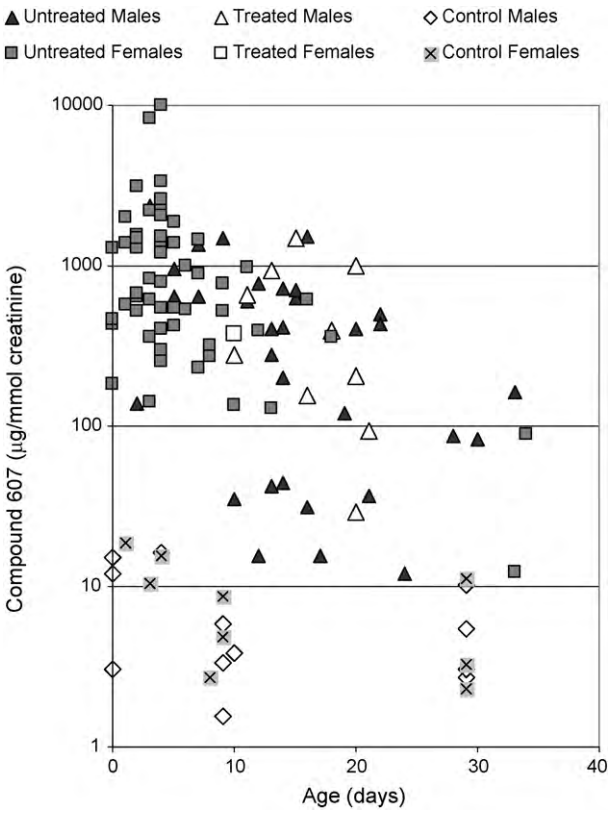

Fig. 5. Evolution of Compound 607 with post-natal age.

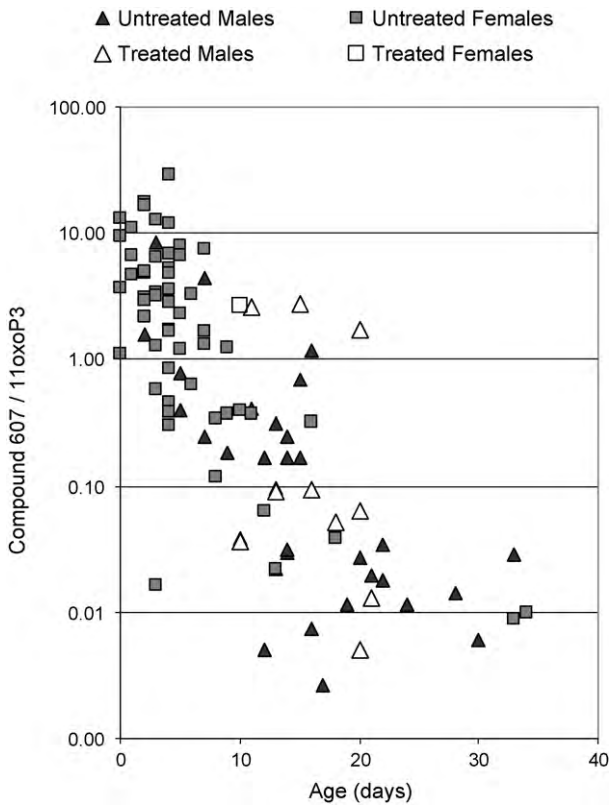

Fig. 6. Evolution with post-natal age of the ratio between Compound 607 and the specific marker of 21-hydroxylase deficiency 11-oxo-pregnanetriol (11oxoP3).

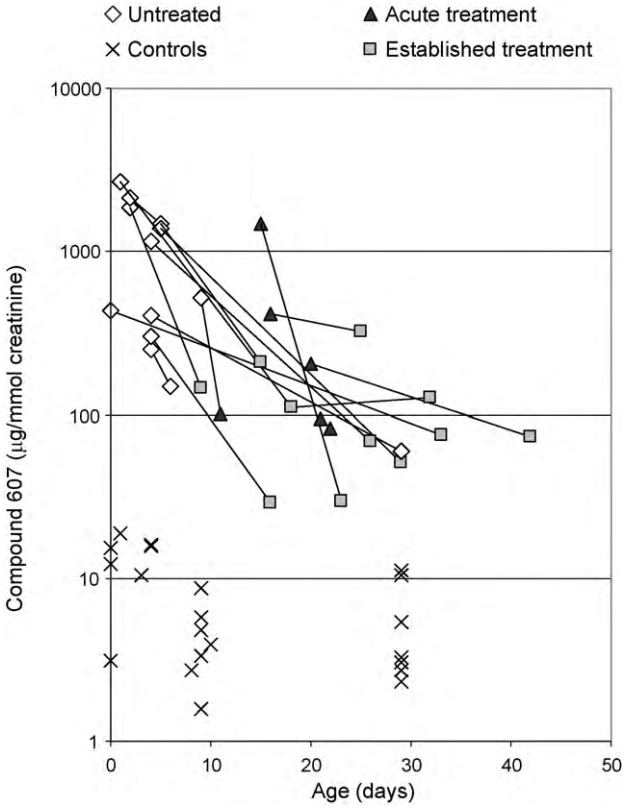

Fig. 7. Evolution of Compound 607 with treatment. Pairs of samples from 2 untreated patients are given for comparison. Acute treatment involved high doses of intravenous hydrocortisone. Established treatment involved oral hydrocortisone.

tisone in urine in the absence of free cortisol. For the cases where the follow-up sample was collected during established treatment, hydrocortisone had been given for 11.3 ± 5.7 days.

3.2.5. Stability

Stability of Compound 607 under storage was checked in 20 samples for which the GC–MS data on the initial analysis were available. After first analysis, samples had been stored for up to 3 weeks at 4 °C and then at −18 °C. Re-analysis took place 3.1 ± 1.8 years (mean ± standard deviation) later. No difference was found: initial analysis, 7.88 ± 8.03; re-analysis, 7.49 ± 8.49 μg/mmol creatinine ($p = 0.31$).

3.3. Compound 607 in other steroid disorders

Compound 607 was undetectable in a 17 day old boy with cytochrome P450 oxidoreductase deficiency on established treatment, while traces were present in a 9 day old girl with 11β-hydroxylase deficiency on established treatment and in a 15 day old boy with 3β-hydroxysteroid dehydrogenase (3β-HSD) deficiency on acute treatment.

4. Discussion

4.1. Structural considerations

4.1.1. 7-Oxo group, D-ring and side chain

The current paper represents part of a comprehensive project to systematically characterize urinary steroid metabolites in 21-hydroxylase deficiency in the neonate. We have already demonstrated that the combination of GC–MS and GC–MS/MS is very useful for structure determination of the D-ring and side chain [13]. D-ring fragmentations are very influential in GC–MS spectra of both non-derivatised [14] and derivatised pregnanes [5,12,15,16] and account for the specificity of the GC–MS/MS spectra [13]. Thus the similarity of the GC–MS and GC–MS/MS spectra of Compound 607 (Fig. 2A) and 3β,16α,17α-trihydroxy-pregn-5-en-20-one standard (Fig. 2C) indicate a shared D-ring and side chain structure. In contrast, there are large differences between the GC–MS spectra of Compound 607 (Fig. 2A) and 3β-hydroxy-5α-pregnane-7,20-dione standard (Fig. 2E), which share structure in rings A–C but have different D-ring structure.

Cleavages in other rings of the steroid nucleus are determined by the structure of rings A–C but the stability of the fragments retaining the D-ring most likely depends on the structure of the D-ring and the side chain. Thus a B-ring cleavage involving the C_{7-8} and C_{9-10} bonds (Fig. 4), which we believe indicates the presence of a 7-oxo group, has been demonstrated for non-derivatised 5α-androstan-7-one [17] in GC–MS. The lower intensity of the GC–MS/MS ion $[P-255]^+$ in 3β-hydroxy-5α-pregnane-7,20-dione standard (Fig. 2E) compared with Compound 607 (Fig. 2A) could be accounted for by the presence of the influential C_{16} and C_{17} hydroxyls on the D-ring of Compound 607.

4.1.2. 3β-Hydroxy-5α-reduced structure

The common 3α-hydroxy-5β-reduced pregnanes that are excreted free or glucuronidated in urine of neonates affected with 21-hydroxylase deficiency are at higher levels than the respective 5α-epimers. The only known 3β-hydroxy-5-reduced pregnane [18] that we commonly observe in this disorder, 3β,15β,17α-trihydroxy-5α-pregnan-20-one, is a 5α-epimer. The failure of cholesterol oxidase activity in Helix pomatia juice to convert 5β-epimers is not unexpected in the light of the large conformational difference between 5α- and 5β-reduced epimers. The former, like 3β-hydroxy-5-enes, have a planar and the latter have an angular configuration at the junction between the A- and B-rings. The above is consistent with our conclusion that Compound 607 is

5α-reduced. The lack of correlation with known 5α-reduced compounds does not preclude a 5α-configuration but rather suggests that the rate limiting step in its synthesis is elsewhere. Hydroxylation at $C_{15\beta}$ is common in unaffected neonates (eg: there is significant excretion of 3β,15β,17α-trihydroxy-pregn-5-ene-20-one) and thus is unlikely to be a rate limiting factor in the synthesis of 3α,15β,17α-trihydroxy-5α-pregnane-20-one, thus leading to the conclusion that the levels of the latter will be determined only by the availability of its precursor – allo 17α-hydroxypregnanolone, resulting in a high correlation between those two compounds. Generation of Compound 607, in contrast, would be dependent on the availability of a 16α-hydroxylating activity or the generation of a 7-oxo group.

4.2. Biological considerations

4.2.1. Link with the 'acidic' bile acid synthesis pathway

The presence of a 7-oxo group in a steroid would require a prior 7-hydroxylation. Hydroxylation in the $C_{7\alpha}$ position is obligatory for the metabolism of bile acids. Bile acids are synthesised from cholesterol via two pathways [19]. The first and rate limiting step in the classical (or 'neutral') pathway is the 7α-hydroxylation of cholesterol by the substrate-specific microsomal cholesterol 7α-hydroxylase (CYP7A1), localised exclusively in the liver. More relevant to pregnane metabolism is the alternative (or 'acidic') pathway, which involves a C_{27}-hydroxylation of cholesterol followed by hydroxylation at $C_{7\alpha}$ by the microsomal oxysterol 7α-hydroxylase (CYP7B1). This enzyme has a wider substrate range, including the 3β-hydroxy-5-ene steroids such as dehydroepiandrosterone, and is found in various tissues involved in steroid synthesis as well as the liver [20]. There is considerable interest in 7α-hydroxylated 3β-hydroxy-5-ene steroids in relation to brain function and immunity [21]. Although considered a compensatory route, the 'acidic' pathway of bile acid synthesis must be of particular importance in the foetus and the neonate, since a defect of the oxysterol 7α-hydroxylase gene in the neonate results in severe liver damage [22]. Conversion of 7α-hydroxylated steroids into 7-oxo steroids is catalysed by 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) [23], which has a similar tissue distribution to CYP7B1 [24]. Further, while adult-type bile acids have a 5β-configuration, their 5α-epimers (so called 'allo bile acids') are found in the foetus.

Since cholesterol as a bile acid precursor has 3β-hydroxy-5-ene structure and Compound 607 is 3β-hydroxylated, the latter probably arises from a 3β-hydroxy-pregn-5-ene precursor. We propose the following metabolic pathway: 3β,17α-dihydroxy-pregn-5-en-20-one (17α-hydroxypregnenolone) is first 16α-hydroxylated to form 3β,16α,17α-trihydroxy-pregn-5-en-20-one, which is then 7α-hydroxylated by CYP7B1 to form 3β,7α,16α,17α-tetrahydroxy-pregn-5-en-20-one and finally the 7α-hydroxyl is converted to 7-oxo by 11β-HSD1. The most likely location of this process is the liver. We have found high levels of pregnanes and pregnenes with 16,17-dihydroxy structure in neonates with 21-hydroxylase deficiency, including 3β,16α,17α-trihydroxy-pregn-5-en-20-one and some of them are present even in children and adults affected by this disorder [13]. The putative 7α-hydroxylated precursor was not found, probably due to a lack of specific MS or MS/MS markers of 7-hydroxylation.

In 21-hydroxylase deficiency the foetus develops in conditions of increased synthesis of steroids with a pressure for clearance of large amounts of steroid metabolites. Thus it is not surprising that minor metabolic pathways become more prominent. Pregnanes [25,26] and bile acids [27] share additional hydroxylations in positions 1β and 6α of the steroid nucleus in the foetus and neonate and Compound 607 may represent a further link between the two metabolic pathways.

4.2.2. Factors potentially determining variability

We have demonstrated that 16,17-dihydroxylated compounds are common in 21-hydroxylase deficiency [13] and thus 16α-hydroxylation is unlikely to be a rate limiting factor in the formation of Compound 607. While it is still possible that the variability in its levels is related to an inter-individual variability of the 5α-reductase activity, it is more likely that the formation of the 7-oxo group is the major source of variation. The latter process would be dependent on the expression of the 'acidic' pathway of bile acid formation. A further source of variability could be a difference in the activities of the adrenal and extra-adrenal 3β-HSD enzymes and the efficiency with which the precursor 17α-hydroxypregnenolone is converted to 17α-hydroxyprogesterone and thus diverted from the Compound 607 pathway.

Since Compound 607 is found up to 3 weeks post-partum, while compounds from maternal or placental origin are cleared within the first 4–5 days of life, it must be synthesised by the neonate.

4.3. Diagnostic considerations

4.3.1. Severity of the disorder

Since Compound 607 originates from a precursor along the cortisol synthesis pathway, we do not expect it to be predictive marker of salt-wasting. Thus, it is not surprising that its levels in patients with salt-wasting do not differ from those for whom no sufficient information was available to determine the status of the electrolyte balance within the first week of life, when no clinical symptoms are present (Section 3.2). The slightly higher levels observed in the salt-wasting group older than 7 days is most likely related to the fact that those infants would be dehydrated and distressed and in them the drive for cortisol synthesis would lead to higher levels of its precursors and their metabolic products. Thus any stressor factors that trigger the cortisol synthesis pathways could be expected to result in higher levels of Compound 607.

4.3.2. Treatment

Whilst only two samples were available for the majority of the patients it is noticeable (Fig. 7) that the levels of Compound 607 decrease relatively fast only in those patients for whom the second sample was collected within the first 3 weeks of life, independently of the presence or absence of treatment. Those, in whom the second sample was collected later demonstrate slower decrease. This suggests that there are at least two different factors determining the levels of Compound 607 and one of them disappears after day 20, while the other remains active later. Results for the only patient for whom one pre-treatment and two post-treatment samples were available, support this suggestion. The exact time at which the switching off occurs could be subject to inter-individual variability. The trend of decrease of Compound 607 during treatment (Fig. 7) follows the general trend of decrease (Fig. 5), thus suggesting that endogenous biochemical factors, rather than treatment determine its evolution.

4.3.3. Specificity

Conclusion on the specificity of this marker will require further comparisons with the rarer forms of CAH. However, an increase would be expected only in those forms where there is an overwhelming increase of 17α-hydroxypregnenolone and/or 17α-hydroxyprogesterone throughout pregnancy. This makes 3β-HSD deficiency a potential and 11β-hydroxylase deficiency a less likely condition for significantly increased Compound 607. Since in cytochrome P450 oxidoductase deficiency multiple cytochromes are affected, the presence of metabolites involving multiple hydroxylation steps in their synthesis is unlikely but may depend on the degree to which the activity of different cytochromes is affected. The high levels of 17α-hydroxypregnenolone in pre-term

neonates are probably within the physiological ranges for their gestational age and thus below the levels required to trigger the additional metabolic pathways that give rise to Compound 607.

5. Conclusions

Compound 607 is present in the first days of life in all patients with 21-hydroxylase deficiency (Fig. 5) in levels higher than that of the specific marker 11-oxo-pregnanetriol (Fig. 6) and is not affected by treatment (Fig. 7). It is stable on storage and is not increased in other forms of CAH. Thus it can be considered a useful early marker of the disorder in a period when the classical markers are at low levels [4].

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