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Steroids excreted in urine by neonates with 21-hydroxylase deficiency. 3. Characterization, using GC–MS and GC–MS/MS, of androstanes and androstenes

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ABSTRACT

Urine from neonates with 21-hydroxylase deficiency contains a large range of androstane(ene)s, many of which have not been previously described. We present their characterization as the third part of a comprehensive study of urinary steroids, aiming to enhance the diagnosis of this disorder and to further elucidate steroid metabolism in neonates.

Steroids were analyzed, after extraction and enzymatic conjugate hydrolysis, as methyloxime-trimethylsilyl ether derivatives on gas-chromatographs coupled to quadrupole and ion-trap mass-spectrometers. GC–MS and GC–MS/MS spectra were used together to determine the structure of hitherto undescribed androstane(ene)s.

GC–MS/MS was pivotal for the structural characterization of 2-hydroxylated androstenediones but GC–MS was generally more informative for androstane(ene)s, in contrast to 17-hydroxylated pregnane(ene)s. Parallels were found between the GC–MS and GC–MS/MS characteristics of structurally similar androstenediones and progesterones without a substituent on the D-ring, but not with those of 17-hydroxylated progesterones. Assignment of 5α(β) orientation, based on GC–MS characteristics, was possible for 11-oxo-androstanes.

The major endogenous 3β-hydroxy-5-enes in 21-hydroxylase deficiency did not differ from those in unaffected neonates. The key qualitative and quantitative differences encompassed 5α(β)-androstanes and 3-oxo-androst-4-enes. Major positions of hydroxylation in these were C_2, C_6, C_{11}, C_{16} and C_{18}. Additional oxo-groups were common at C_6, C_{11} and C_{16}.

We conclude that the presence of multiple further oxygenated metabolites of androstenedione in urine from neonates with 21-hydroxylase deficiency and their pattern indicate a predominance of the classical pathway of androgen synthesis and reflect an increased demand for clearance. The positions of oxygenation in androstane(ene)s are dependent on the configuration at C_3–C_5.

1. Introduction

Deficiency of 21-hydroxylase is the commonest cause of congenital adrenal hyperplasia (CAH). It is characterized by increased serum androstenedione and to a lesser extent testosterone as well as 17-hydroxyprogesterone and 21-deoxycortisol, which in adults and older children are excreted in urine as a few familiar metabolites [1]. In neonates, however, the pattern is incomparably more complex, with a vast range of additionally hydroxylated metabolites, many of which have not been previously described, providing an unparalleled opportunity to explore less familiar steroid structures and pathways relevant to neonatal steroid metabolism. This disorder could more generally be seen as an *in vivo* model of substrate-driven induction of phase I drug metabolism, because the CYP450 enzymes involved in catabolic steroid hydroxylation, unlike those responsible for steroid synthesis, are non-specific and have a wide range of substrates, including bile acids and medicines.

The presented study forms the third part of a comprehensive steroidomic project, aiming to characterize and identify steroids excreted in urine by affected neonates. To achieve this, a combination of GC–MS, GC–MS/MS and microchemistry after partial fractionation has been utilized. This approach is more practical and easily accessible than the classical methods, which rely on purification of individual components. We have so far examined discrimination of various D-ring and side chain configurations in C_{21} steroids without oxo groups other than at C_{20} [2] and C_{21} steroids with oxo groups on the A- or B-ring [3]. C_{21} metabolites containing the non-derivatizable 11-oxo group will be described separately. This part covers all observed structures of endogenous androstanes and androstenes (collectively termed androstane(ene)s). Since for

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C_{21} steroids, the structures of the D-ring and side chain are among the key factors directing fragmentation of steroid methyloxime-trimethylsilyl ethers on electron impact ionization, C_{19} steroid metabolites form a distinct group due to the lack of a side chain. They are also of particular interest from a functional point of view, being derived from the increased serum androgens and androgen precursors [4], which are responsible for one of the key clinical features of 21-hydroxylase deficiency – virilisation in females. The pattern of C_{19} steroid metabolites in neonates with CAH has also relevance to the hypothesized alternative 'backdoor pathway' of generation of dihydrotestosterone via androsterone [5].

Although for many of the configurations presented in this paper, commercial standards were not available, interpretation of the fragmentations observed in the urinary metabolites was possible using comparison with available standards or their transformation products with the closest structure.

While this project primarily aims to enable improvement in diagnosis and monitoring of 21-hydroxylase deficiency, it should also contribute to the general knowledge of neonatal types of steroid metabolism and induction of drug metabolizing enzymes. Characterization of markers for steroid secreting-tumors, where unusual metabolites are frequent, will also be aided.

2. Experimental

2.1. Materials

All materials were supplied as previously described [2,3].

2.2. Urine samples

Urine samples from 98 newborns presenting with 21-hydroxylase deficiency between birth and 40 days of age, as previously described [3], were used. 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.

2.3. Steroid analysis

Steroid metabolites in urine were analyzed and methyloxime-trimethylsilyl (MO–TMS) derivatives were prepared by the well-established method of urinary steroid profiling [6] but modified by omitting the separation of steroid conjugates prior to deconjugation, as previously described [2]. Urine collected on nappies (diapers) was extracted after equilibration with 4% sodium chloride solution [7] and analyzed as above. Deuterated derivatives with d_3-MO or with d_9-TSIM and derivatized deuterated pregn-4-ene-3,20-dione-2,2,4,6,6,17,21,21,21-d_9 (d_9Prog) standard were used to help establish the origin of the fragmentation ions. For TMS-only derivatives, the methoxymation step was omitted. The dried sample was dissolved in 125 μl pyridine and persilylation was carried out with 85 μl TSIM, as for the MO–TMS derivatives.

2.4. Quantification

Urine steroid quantification was based on extracted ion chromatograms of an ion characteristic of each compound. Since matching synthetic standards were lacking for the majority of the endogenous metabolites, an available standard with as closely relevant structure as possible or a qualitatively similar spectrum was selected for each metabolite and its calibration curve was used for quantification. The ions and calibrants selected for each endogenous metabolite are listed in Supplementary data. Calibration curves were prepared for the ratios between the peak height of the ion specific for the standard and the ion at m/z 241 for an internal standard 5α-androstane-3α,17α-diol vs. the ratio between the amount of steroid standard and the internal standard. Due to lack of linearity, two curves were fitted to the calibration points individually for each standard – a power function for the low concentrations and a linear regression line for the high concentrations. The amounts of steroids were related to the amount of creatinine (μg/mmol creatinine). Values for nappies were adjusted by taking into account the amount of sodium chloride solution used for the extraction.

Quantified levels were compared between untreated patients and controls with a t-test for independent samples in SPSS 17.0.

2.5. Microchemistry

2.5.1. Chromate oxidation

Chromate oxidation in pyridine, as previously described [3], was used to convert hydroxyl groups at C3, C6 and C11 into oxo groups

2.5.2. Borohydride reduction

Reduction with borohydride, as previously described [2], was used to convert oxo groups at C_3, C_6, C_{17} (but not C_{11}) into hydroxyl groups, usually with a preference to one of the alternative orientations (e.g. 16β from a 16-oxo and 17β from a 17-oxo group) without affecting skeletal unsaturated bonds.

2.5.3. Helix pomatia juice transformation

Cholesterol oxidase activity present in *H. pomatia* juice was used, as previously described [3], to convert 3β-hydroxy-5-ene steroids into their 3-oxo-4-ene equivalents. We have previously studied this property of *H. pomatia* juice and have shown that ascorbate abolishes it [8].

2.6. GC–MS and GC–MS/MS

GC–MS was performed on a Perkin Elmer Clarus 500 single quadrupole instrument as previously described [2]. GC–MS/MS was performed on a Thermo ITQ 700 ion-trap system as previously described [3]. The precursor ion chosen was $P^+ = [M\text{-}31]^+$, corresponding to a loss of a methoxyl from a derivatized oxo group.

Retention times are based on chromatography on the GC–MS system and are expressed as methylene units (MU) as previously described [2].

2.7. Abbreviations

For chemical derivatives, MO represents a methyloxime and TMS represents a trimethylsilyl ether. For the results and discussion sections, GC–MS and GC–MS/MS are further abbreviated as MS and MS/MS respectively (with the exception of the subheadings and captions of Tables and Figures).

The names of steroids are abbreviated using the following stems: A for androstanolone (3x-hydroxy-5x-androstan-17-one), where x is either α, β or ξ (if orientation is unknown); A2 for androstanediol (5x-androstane-3x,17x-diol); ΔA for androstenolone (3x-hydroxy-androst-5-en-17-one); ΔA2 for androstenediol (androst-5-ene-3x,17x-diol); $\Delta_{3\alpha,5}$A for 3α,5-cyclo-5α-androstane-17-one; $\Delta_{3\alpha,5}$A2 for 3α,5-cyclo-5α-androstane-17x-ol; AD for androstenedione (androst-4-ene-3,17-dione); T for testosterone (17β-hydroxy-androst-4-en-3-one); Prog for progesterone (pregn-4-ene-3,20-dione). If orientation at C_3, C_5 or C_{17} as relevant is established, it is specified in parentheses after the stem (e.g. A(3α,5α) for androsterone (3α-hydroxy-5α-androstan-17-one). The positions of additional oxo and hydroxyl groups prefix the stem, with X designating an unassigned position. Epimers of