THEMATIC RESEARCH

SULFATION PATHWAYS

Alternate steroid sulfation pathways targeted by LC–MS/MS analysis of disulfates: application to prenatal diagnosis of steroid synthesis disorders

Oscar J Pozo¹, Josep Marcos²–³, Olha Khymenets¹, Andy Pranata⁴, Christopher C Fitzgerald⁴, Malcolm D McLeod⁴ and Cedric Shackleton⁵,⁶

¹Integrative Pharmacology and Systems Neuroscience Group, IMIM, Hospital del Mar, Barcelona, Spain
²Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain
³Cerba Internacional, Barcelona, Spain
⁴Research School of Chemistry, Australian National University, Canberra, Australia
⁵Institute of Metabolism and Systems Research (IMSR), University of Birmingham, College of Medical and Dental Sciences, Birmingham, UK
⁶UCSF Benioff Children's Hospital Oakland Research Institute, Oakland, California, USA

Correspondence should be addressed to O J Pozo: opozo@imim.es

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Abstract

The steroid disulfates (aka bis-sulfates) are a significant but minor fraction of the urinary steroid metabolome that have not been widely studied because major components are not hydrolyzed by the commercial sulfatases commonly used in steroid metabolomics. In early studies, conjugate fractionation followed by hydrolysis using acidified solvent (solvolysis) was used for the indirect detection of this fraction by GC–MS. This paper describes the application of a specific LC–MS/MS method for the direct identification of disulfates in urine, and their use as markers for the prenatal diagnosis of disorders causing reduced estriol production: STSD (steroid sulfatase deficiency), SLOS (Smith-Lemli-Opitz syndrome) and PORD (P450 oxidoreductase deficiency). Disulfates were detected by monitoring a constant ion loss (CIL) from the molecular di-anion. While focused on disulfates, our methodology included an analysis of intact steroid glucuronides and monosulfates because steroidogenic disorder diagnosis usually requires an examination of the complete steroid profile. In the disorders studied, a few individual steroids (as disulfates) were found particularly informative: pregn-5-ene-3β,20S-diol, pregn-5-ene-3β,21-diol (STSD, neonatal PORD) and 5α-pregnane-3β,20S-diol (pregnancy PORD). Authentic steroid disulfates were synthesized for use in this study as aid to characterization. Tentative identification of 5β-preg-7-ene-3β,20S-diol and 5β-preg-7-ene-3β,17,20S-triol disulfates was also obtained in samples from SLOS affected pregnancies. Seven ratios between the detected metabolites were applied to distinguish the three selected disorders from control samples. Our results show the potential of the direct detection of steroid conjugates in the diagnosis of pathologies related with steroid biosynthesis.

Key Words
- steroid bis-sulfates
- steroid disulfates
- steroid sulfation
- prenatal diagnosis
- LC–MS/MS
- steroid metabolomics
Introduction

From the earliest days of steroid metabolomics, the principal conjugated forms of steroids (sulfates and glucuronides) have been hydrolyzed prior to analysis, and for decades, the instrument of choice for steroid separation and measurement has been GC–MS (Shackleton & Marcos 2006). While this technique remains the gold standard for steroid profiling, LC–MS/MS has been increasingly adopted because of the simplified sample preparation and speed of analysis, mainly provided by absence of a derivatization step. This is in spite of the poor ionization for fully reduced steroids by electrospray (ESI) (Pozo et al. 2007). While an advance, this methodology still retains the most time-consuming step of sample preparation, the enzymatic or chemical hydrolysis of conjugates (Gomes et al. 2009). Hydrolysis itself can take several hours and requires a further solid-phase extraction (SPE). Necessary chemical derivatization for GC–MS can also take hours. Intact steroid conjugates have been analyzed by mass spectrometry since the introduction of particle beam ionization (e.g. Fast Atom Bombardment, FAB) in the 1980s (Shackleton & Straub 1982, Shackleton 1983). Their spectra have dominant deprotonated molecules [M−H]− in negative ion mode allowing ease of mass determination. Conjugate analysis was simplified with the introduction of electrospray ionization (ESI) and incorporation of HPLC and MS/MS. Glucuronides can be analyzed in both positive and negative ionization modes by monitoring [M+NH4]⁺ and [M−H]− respectively (Fabraget et al. 2013). In the case of monosulfates, collision-induced dissociation (CID) of the strong [M−H]− ions shows a distinctive hydrogen sulfate (HSO₄⁻) fragment at m/z 97 (Shackleton 1983, Galuska et al. 2013). Direct detection of steroid conjugates also circumvents the ionization problems of reduced steroids (Pozo et al. 2007) as phase II metabolites have readily ionized functionality (i.e. a carboxylic acid in glucuronides and an acidic sulfate ester in sulfates).

While monoconjugates dominate the sulfate fraction of urinary steroids, it has been known since the 1960s that sulfates (diS, also referred to as bis-sulfates to distinguish them from compounds containing the disulfate (SO₃²⁻) unit) are significant components of the metabolome (Pasqualini & Jayle 1962, Arcos & Lieberman 1967, Shackleton et al. 1968a,b, Jänne et al. 1969). Early studies by GC–MS of separated conjugate fractions showed that, in addition to the classic 3β-sulfated steroids, hydroxyls at positions 16β, 17α and 17β in androgens and 20- and 21- pregnenes were prone to sulfation (Jänne et al. 1969, Jänne & Vihko 1970, Laatikainen et al. 1973, Meng & Sjövall 1997).

Since these original studies, disulfates have been a largely ignored component of the metabolome that nevertheless had significant potential to expand the understanding of steroid biosynthetic and metabolic pathways. Given this, we sought to develop LC–MS/MS methodology to target this group. It was found that constant ion loss (CIL) of hydrogen sulfate (HSO₄⁻) fragment at m/z 97 from the molecular di-anion [M−2H]²⁻ was the most useful reaction to monitor (McLeod et al. 2017).

The ease of steroid disulfate analysis led us to investigate their use in diagnosis of steroid biosynthetic disorders. One particular area of interest to the authors has been the prenatal diagnosis of single-gene disorders of estriol (E3) synthesis by urine analysis, of which we have studied three conditions by GC–MS, viz., steroid sulfatase deficiency (STSD), Smith-Lemli-Opitz syndrome (SLOS, 7-dehydrosterol reductase deficiency) and cytochrome P450 oxido-reductase deficiency (PORD) (Arlt et al. 2004, Shackleton et al. 2004a,b, 2007, Marcos et al. 2009, Reisch et al. 2013). This communication offers our preliminary observations of the disulfated steroids excreted in these disorders at around mid-pregnancy. While focusing on disulfates, selected monosulfates and glucuronides were also included; evaluating the complete steroid profile is crucial to diagnosing aberrant steroid biosynthesis (Shackleton & Marcos 2006).

Materials and methods

Reagents and chemicals

Steroid starting materials were obtained from Steraloids (Newport, RI, USA). Chemicals and solvents including sulfur trioxide pyridine complex (SO₃-py), N,N-dimethylformamide (DMF) and ammonium formate (HPLC grade) were purchased from Sigma-Aldrich. Aqueous ammonia solution (25%), and acetonitrile and formic acid (LC–MS grade) were from Merck. MilliQ water was obtained using a MilliQ purification system (Millipore Ibérica).

Synthesis of reference steroid disulfates

The qualitative synthesis of steroid disulfates as the ammonium salts was performed as previously described (McLeod et al. 2017) with small modifications. Briefly, 1 mg of each steroid standard was directly dissolved in a freshly
preparation of a solution of SO$_3$.py complex (20 mg, 124 µmol, ~38 eq/steroid or ~19 eq/hydroxyl group) in DMF (100 µL) and incubated at room temperature for 72 h. The success of synthesis was confirmed by analysis of reaction using both LC–MS in scan mode and LC–MS/MS for CID studies. The purification of synthesized disulfates was performed using the method previously described (McLeod et al. 2017).

Steroid disulfate reference materials isolated as the corresponding ammonium salts and used in this study included: 5α-pregnane-3β,20S-diol disulfate, (3βαPD-diS); 3β,21-dihydroxypregn-5-en-20-one disulfate, (21-hydroxypregnanolone disulfate, 21OHPreg-diS); androst-5-ene-3β,17α-diol disulfate, (SAD(17α)-diS); androst-5-ene-3β,17β-diol disulfate, (SAD(17β)-diS); 3β,16α-dihydroxyandrost-5-en-17-one disulfate, (16α-hydroxyandrosterone disulfate, 16αHOAHEA-diS); 3β,16β-dihydroxyandrost-5-en-17-one disulfate (16β-hydroxyandrosterone disulfate, 16βHOAHEA-diS); pregn-5-ene-3β,17α-triol 3,20disulfate, (SPT-diS); pregn-5-ene-3α,20S-diol disulfate, (5PD-diS); 5β-pregnane-3β,20S-diol disulfate; 5β-pregnane-3α,20S-diol disulfate; 5α-pregnane-3α,20S-diol disulfate; 5β-pregnane-3β,20R-diol disulfate; 5α-pregnane-3β,20R-diol disulfate; 5β-pregnane-3α,20R-diol disulfate. In this article, the IUPAC terms for the 20-hydroxyprogrenadiasteroerms are used, S and R, that in some publications are trivialized to α and β, respectively.

Two reference materials (3βαPD-diS and 21OHPreg-diS) were prepared on larger scale and subjected to characterization by spectroscopic methods. Experimental details and characterization data for these new compounds, together with copies of the $^1$H NMR, $^{13}$C NMR and ESI LRMS spectra are available from the authors (MM).

Urine samples

One of our laboratories (Children’s Hospital Oakland, Dr Cedric Shackleton) has been the recipient for urine samples from patients with suspected abnormal steroidogenesis in an attempt to characterize the defects. The studies were approved by the Children’s Hospital Institutional Review Board (IBR#2010-038). Many of the samples used in this study were remnants of those sent to the laboratory for investigation of low pregnancy estriol (generally defined as individuals with serum unconjugated estriol <0.3 MoM, multiples of median). Other samples were from women who had had a previously affected SLOS child or other symptomatic reasons for concern regarding steroidogenesis. The samples have generally been collected between week 16 and 30 of gestation. They have been stored frozen at −20°C. Eleven STSD samples were analyzed, and six samples from SLOS-affected pregnancies. The neonatal FORD samples were collected at 7, 18 and 23 days. Urine samples from unaffected pregnancies were from a collection held by IMIM (Institut Hospital del Mar d’Investigacions Mèdiques, Barcelona). Normal neonatal urine specimens were from a control urine collection at the Institute of Metabolism and Systems Research (IMSR), University of Birmingham UK.

Sample treatment

Urine extraction was by C18 SPE (Oasis HLB, Waters). Generally, a 2 mL aliquot of urine was passed through a pre-conditioned cartridge. After a washing step with 3 mL water, steroid conjugate analytes were eluted using 2 mL of methanol. After evaporation of a 200 µL aliquot of the elution solvent, the extract was reconstituted in 100 µL of water and 5 µL was injected into the UHPLC–MS/MS system. Stably labeled 17-S[18O]$_2$.5α-androstane-3β,17β-diol disulfate and 17-S[18O]$_3$.5α-androstane-3β,17β-diol disulfate were used as internal standards. The labeled sulfate residue was introduced to the steroidal diol monosulfate using labeled 5[18O]$_2$.py generated in situ from labeled sulfuric acid (95% atom) and acetic anhydride in pyridine. Experimental details and characterization data for these internal standards, together with copies of the $^1$H NMR, $^{13}$C NMR and ESI LRMS spectra are available from the authors (MM).

UHPLC–MS/MS analysis

Disulfates

The study was carried out using a triple quadrupole (XEVO TQ-S micro) mass spectrometer equipped with an ESI source and interfaced to an Acquity UPLC system for the chromatographic separation (all from Waters Associates, Milford, MA, USA). Drying gas as well as nebulizing gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h, and the cone gas flow was 50 L/h. A cone voltage of 30 V and a capillary voltage of 0.4 kV were used in negative ionization mode. The nitrogen desolvation temperature was set to 600°C, and the source temperature was 150°C.

The UHPLC separation was performed using an Acquity UPLC CSH Phenyl-Hexyl column (2.1 x 100 mm i.d., 1.7 µm) (Waters Associates), at a flow rate of 300 µL/min. Water and acetonitrile:water (9:1) both with formic acid (0.01% v/v) and ammonium formate (25 mM) were selected as mobile phase solvents. A gradient
program was used; the percentage of organic solvent was linearly changed as follows: 0 min, 15%; 0.5 min, 15%; 25 min, 30%; 26 min, 100%; 27 min, 100%; 28 min, 15%; 30 min, 15%. The total analysis time was 30 min.

For the CIL scan, dwell times of 6 ms and collision energies of 15 eV were selected for each ion transition. Due to the molecular masses of steroid hormones and metabolites (250–400 Da), the precursor ions of disulfates ([M–2H]2–) were restricted to the range from m/z 199 to m/z 274. A selected reaction monitoring (SRM) approach containing 75 preselected transitions was used for the simultaneous detection of steroid disulfates. Among them, the transition 228→359 corresponded to the internal standards used in the analysis.

**Monoconjugates**

While the focus has been on steroid disulfates, we have acquired data on steroid monosulfates and glucuronides previously reported as relevant for the studied disorders. Based on previous studies (Gomez et al. 2014), the product ions at m/z 97 and m/z 75 for sulfates and glucuronides respectively were chosen (Table 1). Exceptions were estriol conjugates due to the influence of the aromatic ring. The neutral loss of the conjugate (80 Da and 176 Da for sulfates and glucuronides respectively were detected).

**Quantification**

For this study, accurate quantitative measurements have not been conducted for two reasons: (1) lack of some authentic compounds prevented the determination of relative responses of analyte transitions to internal standard transitions; (2) the urine samples were random ‘spot’ collections and not accurate 24-h collections. Instead, we have determined ‘diagnostic ratios’ from raw mass spectrometric transition responses. These ratios are of an analyte known to be overproduced to one known to be underproduced in a particular disorder. Such ratios have long been used in GC–MS analysis (Shackleton & Marcos 2006).

**Results and discussion**

**Method development**

This communication applies recent LC–MS/MS studies on steroid disulfate analysis using the CIL from the di-anionic precursor [M–2H]2– (McLeod et al. 2017). The method was developed for untargeted detection and designed for the analysis of a maximum number of natural disulfates. The use of this precursor ion and the fact that the product ion has a higher m/z value is unusual for small molecules. Determination of disulfates under these conditions gives clean chromatograms and the main interferences observed in the chromatograms are due to the relatively high natural abundance of the 34S isotope (4.25%). The transition coming from the m/z 97 loss from an unsaturated (Δ, Δ2, etc.) [34S]1-disulfate isotope is completely indistinguishable from the one coming from an A-ring reduced steroid disulfate at low resolution.

**Table 1** SRM parameters of selected steroids.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Disorder</th>
<th>MW</th>
<th>Retention time (min)</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoconjugates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3-3G</td>
<td>All</td>
<td>464</td>
<td>2.3</td>
<td>463</td>
<td>287</td>
<td>30</td>
</tr>
<tr>
<td>E3-16G</td>
<td>All</td>
<td>464</td>
<td>9.3</td>
<td>463</td>
<td>287</td>
<td>30</td>
</tr>
<tr>
<td>E3-3S</td>
<td>All</td>
<td>368</td>
<td>7.3</td>
<td>367</td>
<td>287</td>
<td>35</td>
</tr>
<tr>
<td>16OHDHEA-S</td>
<td>STSD/PORD*</td>
<td>384</td>
<td>16.0</td>
<td>383</td>
<td>97</td>
<td>40</td>
</tr>
<tr>
<td>DHE3-G</td>
<td>SLOS</td>
<td>462</td>
<td>8.6/9.1</td>
<td>461</td>
<td>285</td>
<td>30</td>
</tr>
<tr>
<td>DHPT-G</td>
<td>SLOS</td>
<td>510</td>
<td>21.3</td>
<td>509</td>
<td>75</td>
<td>30</td>
</tr>
<tr>
<td>Disulfates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5AD(17α)-diS</td>
<td>STSD</td>
<td>450</td>
<td>15.5</td>
<td>224</td>
<td>351</td>
<td>15</td>
</tr>
<tr>
<td>5AD(17β)-diS</td>
<td>STSD</td>
<td>450</td>
<td>13.4</td>
<td>224</td>
<td>351</td>
<td>15</td>
</tr>
<tr>
<td>16αOHDHEA-diS</td>
<td>STSD</td>
<td>464</td>
<td>10.0</td>
<td>231</td>
<td>365</td>
<td>15</td>
</tr>
<tr>
<td>16βOHDHEA-diS</td>
<td>STSD</td>
<td>464</td>
<td>8.2</td>
<td>231</td>
<td>365</td>
<td>15</td>
</tr>
<tr>
<td>5PT-diS</td>
<td>STSD</td>
<td>494</td>
<td>10.5</td>
<td>246</td>
<td>395</td>
<td>15</td>
</tr>
<tr>
<td>210HReg-diS</td>
<td>STSD/PORD</td>
<td>492</td>
<td>18.2</td>
<td>245</td>
<td>393</td>
<td>15</td>
</tr>
<tr>
<td>5PD-diS</td>
<td>STSD/PORD*</td>
<td>478</td>
<td>16.7</td>
<td>238</td>
<td>379</td>
<td>15</td>
</tr>
<tr>
<td>3β5xPD-diS</td>
<td>PORD</td>
<td>480</td>
<td>17.6</td>
<td>239</td>
<td>381</td>
<td>15</td>
</tr>
<tr>
<td>DHSAD-diS</td>
<td>SLOS</td>
<td>448</td>
<td>11.4</td>
<td>223</td>
<td>349</td>
<td>15</td>
</tr>
<tr>
<td>DHPT-diS</td>
<td>SLOS</td>
<td>494</td>
<td>12.6</td>
<td>246</td>
<td>395</td>
<td>15</td>
</tr>
<tr>
<td>DHPD-diS</td>
<td>SLOS</td>
<td>478</td>
<td>15.6</td>
<td>238</td>
<td>379</td>
<td>15</td>
</tr>
</tbody>
</table>

*PORD neonatal.
To maximize isobaric steroid metabolite separation (e.g. pregnenediol disulfates, pregnanediol disulfates and androstenediol disulfates) in this study, a phenyl-hexyl column with a relatively high amount of ammonium formate (25 mM) was required to obtain sharp and well-resolved chromatographic peaks. Column temperature was critical for this purpose with 30°C determined as optimum. Under these conditions, a 25-min gradient from 15% to 30% of organic solvent provided desired separation (Fig. 1A).

Under optimized conditions, the elution order of disulfates was dihydroxyandrostanones < dihydroxypregnanes < androstenediols < pregnanediols. In a specific group, 17β-hydroxysteroid disulfates eluted earlier than their 17α-counterparts and 20S-hydroxysteroid disulfates eluted earlier than their 20R counterparts. Regarding A-ring derivatives, Δ5 steroid disulfates eluted before the fully reduced metabolites, the elution order of the reduced steroids being 3β,5β<3β,5α<3α,5α<3α,5αβ.

The chromatographic conditions were also able to separate the two estriol glucuronide isomers i.e. the 16-glucuronide and 3-glucuronide. Unfortunately, sulfate and glucuronide conjugates of two useful steroids in PORD diagnosis, androsterone and etiocholanolone, could not be separated under the selected conditions even after increasing the gradient to 1 h (Fig. 1B).

Application to prenatal detection of disorders affecting estriol synthesis

We report preliminary studies to determine whether steroid disulfates in urine can be useful markers in the prenatal detection of disorders affecting estriol synthesis; until now only monoconjugates had been used. The background to this study was that unconjugated serum E3 is frequently measured at mid-pregnancy as a marker for Down’s syndrome as part of a test called triple- or quad-marker screening (Haddow et al. 1994). If results are low the question remains as to the reason, and our initial research was directed to diagnosis of SLOS, the clinically most severe cause of low E3 (Shackleton et al. 2007). These studies led to investigation of other causes such as STSD and PORD.

Diagnostic ratios are frequently employed in steroid metabolomics and E3 is frequently used as denominator. Dominant E3 conjugates are 3- and 16-glucuronides (30% and 60%, respectively) with about 2.5% as monosulfate and estriol-3-glucuronide-16-sulfate (6.5%) (Tikkanen et al. 1973). We assessed E3 excretion from the measurement of glucuronide and monosulfate conjugates (Table 1).

**STSD (OMIM, 308100, location, Xp22.31)**

This X-linked disorder prevents the release of steroid from steroid sulfates. A summary of the biosynthetic pathway leading to estriol is shown in Fig. 2, illustrating that inactivity of the enzyme in placenta prevents 16α-OHDHEA-S conversion to E3. This fetal 16α-OHDHEA-S, androst-5-ene-3β,16α,17β-triol sulfate (5AT-S) and other steroid sulfates pass through the placenta and mother to be excreted in urine largely unchanged (Taylor & Shackleton 1979).

STSD urine samples (N=11) and 11 controls were analyzed using the CIL scan method for disulfates complemented with the acquisition of 16α-OHDHEA-S and E3 monoconjugates (Table 1). Among the disulfates measured by the CIL method, we found that the response ratio between six of them, namely 16α-OHDHEA-diS, SAD(17α)-diS, SAD(17β)-diS, 5PT-diS, 21OHreg-diS and
5PD-diS against E3 glucuronide (measured as sum of 3- and 16-glucuronides) was markedly increased in STSD.

Representative chromatograms of normal pregnancy urine and one with an STSD-affected fetus are shown in Fig. 3. The ratio values for our normal and STSD data sets are shown in Fig. 4A and show all analytes clearly distinguish STSD from normal. Additionally, we used the ratios to evaluate the relative efficacy of each analyte in diagnosis. The best steroid discriminatory ratio would show greatest difference between the lowest steroid sulfate/E3-G ratio value in STSD, and the highest ratio found in controls (Fig. 4B). Interestingly, the ratios that gave the greatest differential were Δ₅ pregnenes; 5PD-diS, 5PT-diS and 21OHPreg-diS, not the C₁₉ steroid sulfates on the direct biosynthetic pathway to E3. Combining ratio data can give an even greater separation for normal and affected; note the combined data for 21OHPreg-diS and SPD-diS in Fig. 4C. Such pregnene metabolites should be incorporated in MS-based methodologies for detection of the disorder.

**P450 oxido-reductase deficiency (PORD) (POR OMIM 124015 location: 7q11.23)**

Several pregnant women carrying PORD fetuses have been studied by GC–MS (Shackleton et al. 2004, Reisch et al. 2013), but for only two were samples available for this study. Shackleton et al. (2004a) deduced that the dominant ‘feto-placental’ maternal urinary steroid in PORD pregnancies was 3β5aPD-diS. While this steroid is also present in the disulfate fraction of normal pregnancy urine, it is in much greater amount in PORD-affected
 Pregnancies. From its dominance together with reduced E3, it was concluded that this metabolite is a maternal excretory product of fetal pregnenolone. An intermediate precursor would be fetal steroid 5PD-diS. Excess pregnenolone and its sulfate are the result of an apparent ‘block’ in 17-hydroxylase/C17-20 lyase secondary to attenuated POR activity (Fig. 5). This block, together with suppressed 16α-hydroxylase (also due to PORD), causes reduction of fetal 16αOHDHEA-S production leading to low maternal E3 production and excretion. The precise sequence of reactions from fetal pregnenolone to urinary metabolites in normal and PORD-affected pregnancies.
and neonate are shown in Fig. 5. Evidence suggests the corresponding conversion of pregnenolone sulfate to DHEA-S is not an available pathway (Neunzig et al. 2014, Sánchez-Guijo et al. 2016, Rege et al. 2017).

Besides the increased excretion of 3β5αPD-diS, we also observed an increase in the transitions corresponding to 5PD-diS and 3β,21-dihydroxy-5α-pregn-20-one disulfate (21OHPreg3β5α-diS), the latter in spite of a likely POR requirement by fetal 21-hydroxylase. However, it should be noted that this fetal enzyme differs from that coded by CYP21A2 required in cortisol synthesis (Guerami et al. 1988, Corsan et al. 1997).

In Fig. 6, we illustrate the chromatographic profiles of the 3β5αPD-diS, 5PD-diS and 21OHPreg3β5α-diS and the E3 conjugates in a control (Fig. 6A) and affected pregnancy (Fig. 6B). The dominance of the 3β5αPD-diS in the affected pregnancies is striking. In GC–MS diagnosis of PORD prenatally the ratio of 3β5αPD/E3 was used, i.e. the ratio of principal PORD fetal metabolite to E3, the conventional feto/maternal metabolite. In Fig. 6C are shown ratios for intact conjugates in PORD and controls. For the denominator (E3), we summed the total of both glucuronylated forms.

One of the GC–MS prenatal diagnostic ratios for PORD remains a challenge for LC–MS/MS under conditions developed for this study. With fetal PORD there is increased androsterone production as a result of the ‘alternative pathway’ activity (Arlt et al. 2004) resulting in markedly increased androsterone/etiocholanolone ratio (Shackleton et al. 2004a). That ratio should theoretically be determined by direct analysis of glucuronides and this separation has been already reported by C18 columns both in glucuronides (Pozo et al. 2008) and unconjugated (Marcos & Pozo 2016). Unfortunately, under current chromatographic conditions developed for the disulfates such isobaric monoconjugates (sulfates or glucuronides) could not be resolved.

**Postnatal detection of PORD**

While this paper has focused on prenatal diagnosis of PORD by 3β5αPD-diS measurement, Shackleton et al. (2004b) have shown that its precursor 5PD-diS is a key analyte in diagnosing the condition in the first months of life suggested its inclusion here. In PORD neonatal samples this steroid is dominant, excretory values exceeding the classical major metabolites such as 16αOHDHEA-S and 16α-hydroxy pregnenolone sulfate whose biosynthesis by 16α-hydroxylation is also POR dependent. In the first weeks of life, the fetal zone of the adrenal is still dominant, but diminishing, and is responsible for producing a large amount of 3β-OH-Δ⁵ steroids.

**Figure 7** illustrates the separation of steroid disulfates in an affected PORD infant and normal infant. We have included 16αOHDHEA-S as analyte to act as denominator for a potential diagnostic ratio 5PD-diS/16αOHDHEA-S. This ratio is shown for three affected infants and normal controls in Fig. 7C, clearly defining the condition. Interestingly, one of the first steroid disulfates to be identified in the neonatal period were 5AD(17α and 17β) diS (Shackleton et al. 1968a, Laatikainen et al. 1973), and 16βOHDHEA-diS (Shackleton et al. 1968b, Laatikainen et al. 1973), and these are clearly separated with this methodology (Fig. 7).
Smith-Lemli-Opitz syndrome ‘7-dehydrosterol reductase’ deficiency (SLOS) (OMIM 602858 location: 11q13.4)

This condition is caused by deficiency in 7-dehydrosterol reductase and the notable feature is a build-up of 7- and 8-dehydrocholesterol, which can be used to diagnose the condition when measured in amniotic fluid (Kelley 1995). The affected fetus can use these sterols as steroid precursors, resulting in the appearance in maternal urine of dehydro (DH) versions of common natural steroids. For instance, 5β-pregn-7(8)-ene-3α,17α,20S-triol, (7(8)-DHPT) and an estriol equivalent, principally 8-dehydroestriol (8-DHE3) (Shackleton et al. 1999, Guo et al. 2001). The biosynthesis of steroids in SLOS pregnancy is illustrated in Fig. 8.

SLOS steroids are mainly excreted as glucuronides. Thus, distinct peaks corresponding to different isomers of 8-DHE3-G were found in all SLOS samples (Fig. 9). On the other hand, the detection of 7(8)-DHPT-G provided more difficulties due to endogenous interferences probably coming from other pregnenetriols and dihydroxypregnenolones, which would share the same transition (Fig. 9).

A systematic study has not been made of steroid sulfates in this condition and lack of appropriate authentic compounds has meant that only candidate chromatographic peaks were provisionally identified. Such peaks were chosen by having the expected CIL transitions for steroids with additional unsaturation and to be accepted as SLOS-specific ‘candidate’ analytes these peaks had to be present in all six confirmed SLOS pregnancies and be absent from controls. Two main metabolites were found. Peaks with the expected transitions for DHPT-diS and DHPD-diS were observed in all SLOS samples. Additionally, other minor metabolites such as DH-androstenediol-diS were also found. In Fig. 9, the chromatograms of the proposed steroid disulfate analytes with DH-pregnenetriol glucuronide and
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DHE3-glucuronide are shown. Little information can be stated on stereochemistry of candidate analytes; not only that of 3- and 5-positions but both Δ⁷ and Δ⁸ isomers are likely present. The chromatograms illustrated were from one affected pregnancy and one control. Similar chromatograms were produced for the other five affected pregnancies and controls. Clearly this is the most challenging of the three conditions for conjugate LC–MS/MS analysis although aberrant steroid conjugate peaks definitive for SLOS were detected.

Distinguishing the disorders: summary

This study has focused on the mass spectrometric analysis of steroid disulfates, but steroid monosulfates and glucuronides have been included where required to determine ratios used for diagnosis. In order to evaluate the potential of the approach based on the combined screening of glucuronides, monosulfates and disulfates, we propose a panel of markers able to differentiate between the selected disorders and control samples. We found that using the ratios 16αOHDHEA-S/E3-G, 3β5αPD-diS/E3-G, 5PD-diS/16αOHDHEA-S, 21OHpreg-diS/E3-G, 21OHpreg-diS/16αOHDHEA-S, 8DHE3-G/E3-G and DHPD-diS/E3-G allowed for the successful differentiation between the controls and the different disorders.

Hopefully, the study emphasizes the potential of LC–MS analysis of all conjugate types in future development of steroid metabolomics.

General discussion

The steroid disulfates are a minor fraction of the urinary steroid metabolome, but may provide significant markers of aberrant steroid biosynthesis. As a family, intact steroid disulfates have not been recently subject to detailed study due to a lack of suitable analytical methodology. In the past, studying this family always involved time-consuming fractionation of free and conjugate families followed by solvolysis and GC–MS analysis. Most of the available literature stems from the 60s and 70s, and it was shown early which secondary positions (assuming the primary sulfated position is the 3-hydroxyl) could be sulfated. These were 17 (α- and β), 16β- and 18 in C₁₉ steroids and 20β- and 21- in C₂₁ steroids. During that early research period, the dominant biological materials chosen to study were associated with pregnancy. In that respect, our current studies have followed this lead and the major disulfate components reported here were also noted in the early publications (Shackleton et al. 1968a, b, Jänne et al. 1969, Jänne & Viiko 1970, Laatikainen et al. 1973, Meng & Sjövall 1997).

There is little definitive evidence as to which sulfotransferases are responsible for the secondary sulfation (Mueller et al. 2015) and how disulfates are transported (Grosser et al. 2018). Available sulfation evidence points solely to SULT2A1, which appears to have an active site capable of encompassing a wide variety of steroid substrates (both free and monosulfated) and conduct sulfation at either end of the steroid molecule. Thus, it can sulfate free steroids or steroid monosulfates (Cook et al. 2009).

A question remains as to whether disulfation is purely a catabolic reaction or if such steroids could be transportable reservoirs of active hormone precursors, as is likely the case for DHEA and estrone sulfates.
Guerami et al. (1988) have proposed that 21OHpreg-diS is an 11-deoxycorticosterone (DOC) precursor during pregnancy, particularly since circulating levels of this mineralocorticoid and its sulfate are increased during gestation (Corsan et al. 1997). It is known that the placenta is capable of hydrolyzing 21-sulfates and the enzyme responsible is the usual STS as 21-desulfation does not occur in STS (Guerami et al. 1988). Another possible reservoir for disulfates is 5AD(17β)-diS, potentially a testosterone or estradiol precursor. This steroid is also subject to STS action in mammals. In contrast, it is believed that human sulfatases are inactive on 17α- (C₁₉ steroids) or 20S configured sulfates, a situation shared with the commercial snail and mollusk enzymes used for hydrolysis in steroid analysis (Stevenson et al. 2014).

In summary, we have provided analytical data on the steroid disulfates through their measurement as intact molecules by LC–MS/MS, employing CIL scan monitoring. We have attempted to use these additional members of the steroid metabolome to distinguish fetal disorders of steroid synthesis. To the best of our knowledge, this is the first time that direct analysis of steroid disulfates has proved its value for clinical diagnosis.

The ultimate goal of these studies is the ability to quantify the whole urinary steroid metabolome as unhydrolyzed conjugates, the monosulfates, disulfates, glucuronides and mixed sulfate-glucuronide conjugates. Studies of the plasma steroid metabolome should also be included. To achieve this goal will require the synthesis of a multitude of authentic steroids including appropriate internal standards and an improvement in chromatographic resolution.

Declaration of interest
There is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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