

OPTIMISED DECONJUGATION OF ANDROGENIC STEROID CONJUGATES IN BOVINE URINE

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Abstract

After administration of steroids to animals the steroids are partially metabolised in the liver and kidney to phase 2 metabolites *i.e.* glucuronic acid or sulphate conjugates. During analysis, these conjugated metabolites are normally deconjugated enzymatically with aryl sulphatase and glucuronidase resulting in free steroids in the extract. It is well known that some sulphates are not deconjugated using aryl sulphatase; instead *e.g.* solvolysis can be used for deconjugation of these aliphatic sulphates. Sulphate conjugates of selected steroids were synthesized by reaction with excess of sulphur trioxide/trimethylamine in dimethylformamide. The effectiveness of solvolysis on androgenic steroid sulphates is tested with selected aliphatic steroid sulphates (boldenone sulphate, nortestosterone sulphate and testosterone sulphate). And the method is validated for analysis of androgenic steroids in *bovine* urine using free steroids, steroid sulphates and steroid glucuronides as standards. Glucuronidase and sulphuric acid in ethylacetate are used for deconjugation and the extract is purified by SPE. The final extract is evaporated to dryness, re-dissolved and analysed by LC-MS/MS.

Introduction

Biotransformation of steroids is generally divided into two classes. Phase 1 reactions introduce polar functional groups into the steroid molecule via oxidation, reduction or hydroxylation to inactivate the drug and to increase its tendency to be excreted from the body. In phase 2 reactions, the steroid and/or the phase 1 metabolites are conjugated with *e.g.* glucuronic acid and sulphate forming polar water soluble products, and like phase 1 compounds, leading to an increasing tendency to be excreted in urine (Schänzer 1996). The basic structure of steroids consists of the perhydrocyclopentanophenanthrene ring system (ring A, B, C and D). Functional groups are attached to the rings *e.g.* a hydroxy group on C-3 for sterols or a carbonyl on C-3 and hydroxyl on C-17 for testosterone and boldenone.

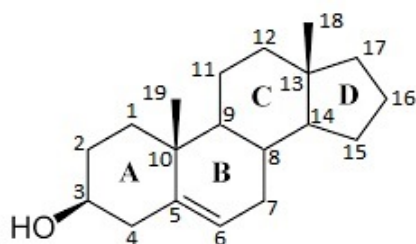


Figure 1. The steroid ring system with the IUPAC approved ring lettering (ABCD) and atom numbering.

As a rule of thumb, the 3α -hydroxy group on ring A (after metabolic reduction of the 3-keto group for *e.g.* testosterone and androsterone) are often conjugated with glucuronic acid and 3β -steroids are often conjugated with sulphate. However, some 3α -hydroxysteroids are also excreted as sulphate (like testosterone). In human, metabolism of testosterone to androsterone is reported and androsterone is excreted as sulphate conjugate (Higashi *et al.*, 2007). And further nortestosterone and a 3β -hydroxy metabolite are excreted as sulphate conjugates (Schänzer 1996; Scarth *et al.*, 2009). Following the administration of nortestosterone laurate to boar, nortestosterone sulphate was the predominant metabolite obtained (Ventura *et al.*, 2008).

Glucuronidation of secondary 17β -hydroxy groups (*e.g.* testosterone, nortestosterone and boldenone), on ring D, is well known and sulphatation is possible and described for *e.g.* testosterone, boldenone and altrenogest in horses (Le Bizec *et al.*, 2006; Pu *et al.*, 2004; Lampinen-Salomonsson *et al.*, 2006) and *bovine* (Scarth *et al.*, 2009). For some tertiary 17β -hydroxy groups this glucuronidation also takes place (Schänzer 1996). In sheep, testosterone is primarily excreted as glucuronide conjugates in urine and sulphate conjugates in bile and it is further reported that in pig plasma conjugated steroids are mainly found as sulphate conjugates (Scarth *et al.*, 2009).

Methods for detection of steroids often includes a deconjugation step with enzymes, to convert the phase 2 metabolites to the free steroid. The digestive juice of *Helix pomatia* is one of the most used enzymatic preparations for this, but this enzyme preparation does not contain the sulphatase activity to hydrolyse all steroid sulphates. It has been published that 3α -hydroxy- 5β -sulphates (*e.g.* androsterone) and C19-steroids sulphated at C17 (*e.g.* testosterone and boldenone) are resistant to

this enzymatic hydrolysis (Cawley *et al.*, 2005; Schackleton 1986). Moreover, use of enzyme mixture can cause unwanted conversions of the analytes, *e.g.* the presence of oxidoreductase activity has been reported converting the steroidal 3-ol to a 3-oxo group (Hewitt *et al.*, 2002). It can therefore be argued that a chemical deconjugation should be used for hydrolysis of steroid sulphates (Cawley *et al.*, 2005).

The method used in this study is based on the published method by Poucke and Van Peteghem (Van Poucke *et al.*, 2002) and we have tested use of recombinant *glucuronidase* and solvolysis instead of *Helix pomatia*. The solvolysis was based on the procedure published by Schänzer W. *et al.* (1992) with minor modifications.

Not much has been published on testing of solvolysis conditions for steroid sulphates - Roig *et al.* (2007) reported a recovery of 91% for a C3-conjugated steroid (dehydroepiandrosterone-3-sulphate). For testing the solvolysis, we synthesized sulphate conjugates of selected androgenic steroids. Sulphates of testosterone, boldenone and trenbolone were synthesized and parameters like acidity and incubation time were tested. Steroid sulphate conjugates were synthesised by reaction with excess of triethylamine:sulphurtrioxide in dimethylformamide (Santos *et al.*, 2003) and steroid glucuronides were biosynthesised by incubation with rabbit liver homogenate fortified with uridinediphosphoglucuronic acid (Mulder *et al.*, 1994).

Materials and Methods

Materials and reagents

All reagents were of analytical or HPLC grade and supplied by Merck (Darmstadt, Germany) and Rathburn Chemicals (Walkerburn, Scotland). Water was ultra-purified using a Maxima purification system from USF Elga (Bucks, UK). β -nortestosterone (BNO) and testosterone (TES) were purchased from Sigma-Aldrich, α -trenbolone (ATR), β -boldenone glucuronide, β -nortestosterone glucuronide and α -nortestosterone sulphate, were bought from LGC PromoChem (National Analytical Reference Laboratory, Australia), α -boldenone (ABO), β -boldenon (BNO), β -trenbolone (BTR), α -nortestosterone (ANO), β -boldenone sulphate, testosterone- d_2 (TES- d_2), β -Boldenone- d_3 (BBO- d_3), β -nortestosterone- d_3 (BNO- d_3) and β -trenbolone- d_3 (BTR- d_3) were from RIKILT (Wageningen, The Netherlands). Testosterone sulphate was synthesized from testosterone according to the procedure described later. Stock solutions of each compound were prepared separately in ethanol at concentrations of 1 mg mL⁻¹. Stock solutions of internal standards, however, were prepared by reconstitution of ampoules containing a fixed amount of material with ethanol at a concentration of 0.1 mg mL⁻¹. Stock solutions were stored at -18°C until use for up to 3 year. A final working solution containing all the compounds at 0.1 μ g mL⁻¹, except internal standards, was prepared by diluting stock solutions with ethanol. A final working solution of internal standards containing all four internal standards at 0.1 μ g mL⁻¹ was also prepared by diluting stock solutions with ethanol. Final working solutions were freshly made. Samples were cleaned up on C18 SPE-columns (500 mg, 3 mL, Waters Corp. Milford, MA, USA) and filtered through NH₂ SPE columns (500 mg, 5 mL, Waters Corp.).

Syntheses of steroid sulphates

An amount of 0.1 mg each of testosterone, β -trenbolone and boldenone was dissolved in 1 mL of dry dimethylformamide and each added a spoon (approximately 20 mg) of triethylamine:sulphurtrioxide. The reaction mixtures were left overnight at room temperature. The reaction mixtures were each added 4 mL water and applied on activated and washed Isolute 101 SPE columns (100 mg, Biotage, Sweden). After washing with water the columns were eluted with 1 mL of methanol and 1 mL of ethyl acetate. The eluates were evaporated to dryness.

Biosyntheses of steroid glucuronides.

Three vials containing 0.01 mg each of testosterone, β -trenbolone and boldenone in 10 μ L methanol were each added 1 mL of rabbit liver homogenate (2 mg protein mL⁻¹) and 2 mg uridinediphosphoglucuronic acid (UDPGA) and incubated at 37°C with gentle shaking for 1 h. The incubation mixtures were applied on activated and washed Isolute 101 SPE columns (100 mg, Biotage, Sweden). After washing with water, the columns were eluted with 1 mL 50% methanol. The eluates were evaporated to dryness.

Samples

Urine was collected at slaughterhouses, as part of the national plan for monitoring drug residues in animals and animal products. As soon as the samples were received, they were stored at -18°C until analysis.

Sample preparation

To 1 mL of urine, 1 mL MOPS buffer (50mM, pH 7) and 250 U of glucuronidase (type VII-A, Sigma) were added. After addition of 200 μ L internal standard working solution (20 ng) the sample was incubated at 37°C for 1 h. The sample was applied to the C18 SPE-column (500 mg) pre-activated with 5 mL of methanol and subsequently by 5 mL of water. After washing with 3 mL water, the column was vacuum-dried for 2 min. The analytes were eluted from the column with 3 mL methanol. The extract was dried using a nitrogen evaporator at 40°C and reconstituted with 1 mL of ethylacetate and 40 μ L 4 M H₂SO₄ followed by

incubation at 40°C for 30 min. After addition of 320 µL 1 M tris-buffer the organic phase was evaporated at 40°C using nitrogen. One mL 20% methanol was added and vortexed. The sample was applied to a new C18 SPE-column (500 mg) activated with 5 mL methanol and subsequently by 5 mL water prior to use. After washing with 3 mL water and 3 mL 30% methanol, the column was vacuum-dried for 2 min. The analytes were eluted from the column with 4 mL ethylacetate. The extract was filtered through a NH₂ SPE-column (500 mg) from Waters, activated with 6 mL ethylacetate before use. The extract was dried using a nitrogen evaporator at 40°C. The residue was reconstituted in 400 µL methanol/water/formic acid (25/75/0.1).

LC-MS/MS analysis

HPLC separation was performed on an Agilent 1100 series LC system from Agilent Technologies (Palo Alto, CA, USA) equipped with a high-pressure binary pump, degasser, auto sampler and a column heater. The steroids were separated on an Ascentis express C8 column (2.1mm*100mm, 1.8µm) from Supelco at 30°C using a gradient method (0 min: 90% A; 0.5 min: 75% A; 15 min: 65% A; 20 min: 10% A). Mobile phase A contained 0.1% formic acid in water and mobile phase B contained acetonitrile, and the flow rate was set at 0.25 mL min⁻¹. The sample volume injected was 10 µL.

The mass spectrometer was a Quattro Ultima Pt triple quadrupole instrument (Waters Corp.) with Masslynx v. 4.1 software (Waters Corp.). Ionisation of the analytes was achieved using an electrospray interface in the positive ion mode (ESI⁺), and ionisation source parameters were as follows: capillary voltage (V_{cap}), 3.75 kV; cone voltage (V_{cone}), 35 V; desolvation temperature, 400°C; source temperature, 120°C. Nitrogen was used as the nebulising gas (maximum flow), desolvation gas (flow-rate of 600 L h⁻¹), and as cone gas (flow-rate of 120 L h⁻¹). Argon was used as the collision gas at a pressure of $\sim 2.5 \cdot 10^{-3}$ mbar. Data acquisition was performed in the multiple reaction monitoring (MRM) mode. MRM transitions and collision energies for the analytes are listed in Table 1, and the dwell time was set to 0.1 s for every transition.

Matrix-matched standards were used to quantify the analytes. The fortification levels of the validation curves were: 0.0 (blank sample), 0.5, 1.0, 2.0, 5.0 and 10.0 µg L⁻¹. A fixed amount of internal standard (20 µg L⁻¹) was added to all the samples. Calibration curves were obtained relating relative responses of the analyte (chromatographic peak area of the analyte at different concentration levels in relation to the concentration of the internal standard divided by the peak area of the internal standard) versus nominal concentrations (µg L⁻¹) of the analytes. As a deuterated labelled standard was not available for some steroids a deuterated epimer standard was used. BBO-d₃ was used as an internal standard for ABO, BTR-d₃ was used as an internal standard for ATR and BNO-d₃ was used as an internal standard for ANO.

Table 1. Data acquisition method for analysis by LC-MS/MS.

Name (abbreviation)	MRM transition (m/z) ^a	Collision energy (eV)
Testosterone (TES)	289.1>97.1	18
	(289.1>109.1)	20
α -Boldenone/ β -Boldenone (ABO/BBO)	287.2>121.1	18
	(287.2>135.1)	15
α - Trenbolone/ β -Trenbolone (ATR/BTR)	271.2>199.1	20
	(271.2>253.2)	20
α - Nortestosterone/ β - Nortestosterone (ANO/BNO)	275.2>109.1	22
	(275.2>239.2)	17
Testosterone-d ₂ (TES- d ₂)	291.1>99.1	18
β -Boldenone-d ₃ (BBO-d ₃)	290.2>121.1	15
β -Trenbolone-d ₃ (BTR-d ₃)	274.3>256.0	20
β - Nortestosterone-d ₃ (BNO-d ₃)	278.2>109.1	22

^a Primary MRM transitions used for quantitative purposes are listed first, and secondary MRM transitions for confirmation are in parentheses.

Results and Discussion

Since some steroid conjugates are not commercially available, we synthesized selected steroid sulphates by reaction of the parent compound with trimethylamine:sulphur trioxide and biosynthesized selected steroid glucuronides by incubation of the parent compound with rabbit liver homogenate fortified with UDPGA. These conjugates were used in the optimization of hydrolysis conditions.

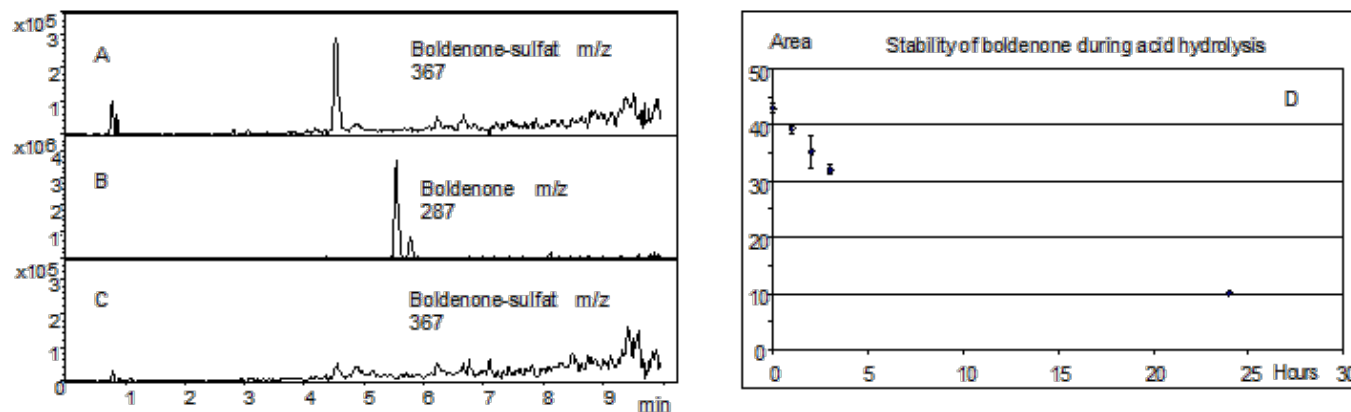


Figure 2. Acid hydrolysis of boldenone sulphate to A: 0 min and B,C: 30 min. Samples were analysed on an Agilent 1100 HPLC connected to an Bruker Daltonics Esquire HCT ion trap mass spectrometer equipped with an ESI ion source operated in positive mode. D: Stability of boldenone during prolonged hydrolysis time.

Solvolysis was investigated by optimising the concentration of sulphuric acid and time of incubation at 40°C. After addition of ethylacetate and 4 M sulphuric acid the decay of boldenone sulphate was monitored by HPLC-MS to various time points. Figure 2 (panel A) shows the extracted ion chromatogram of boldenone sulphate (m/z=367) to time 0. After 30 min of hydrolysis, a boldenone peak appeared (m/z=287) and Figure 2 (panel B and C) shows that boldenone sulphate was hydrolysed after 30 min of incubation. The stability of β -boldenone during the hydrolysis reaction for prolonged hydrolysis time was investigated for up to 24 h. Fig. 2 (panel D) shows that the concentration of β -boldenone decreases with increasing incubation time.

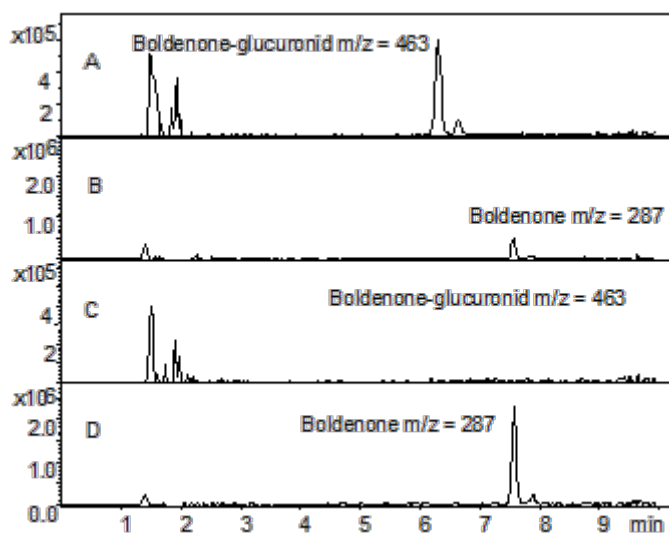


Figure 3. Enzymatic hydrolysis of biosynthesized boldenone glucuronide. A, B: extracted ion chromatograms of boldenone glucuronide and boldenone to time 0. C, D: extracted ion chromatograms obtained after 1 hour of hydrolysis.

After 2 hours of incubation, only 80% β -boldenone was recovered and after 24 hours only 20% was recovered. Concentrations of 1, 2, 3 and 4 M sulphuric acid were tested; 4 M gave the best results (results not shown).

Figure 3 shows the extracted ion chromatograms obtained prior to and after enzymatic hydrolysis with β -glucuronidase from *E. coli* of the biosynthesised boldenone glucuronide. Panels A and B show the chromatograms prior to hydrolysis, the glucuronide with $m/z=463$ elutes at 6 min and a minor peak or unconjugated boldenone elutes to 7.5 min. After hydrolysis for one hour (panel C and D) the boldenone glucuronide peak has disappeared and the boldenone peak has increased in intensity.

For testing the deconjugation procedure on bovine urine samples, we used our originally validated method for steroids in urine where *Helix pomatia* is used for enzymatic hydrolysis of steroid conjugates. The enzymatic step was changed by using β -glucuronidase (type VII-A) for hydrolysing steroid glucuronides and the sulphates were released by hydrolysis under acidic conditions with 4 M sulphuric acid.

An estimate of the concentration of our synthesized testosterone sulphate was calculated by using the deconjugation procedure on a fixed volume of the synthesized standard and the concentration of testosterone in the extract was estimated from the calibration curve from analysis of a standard of unconjugated testosterone.

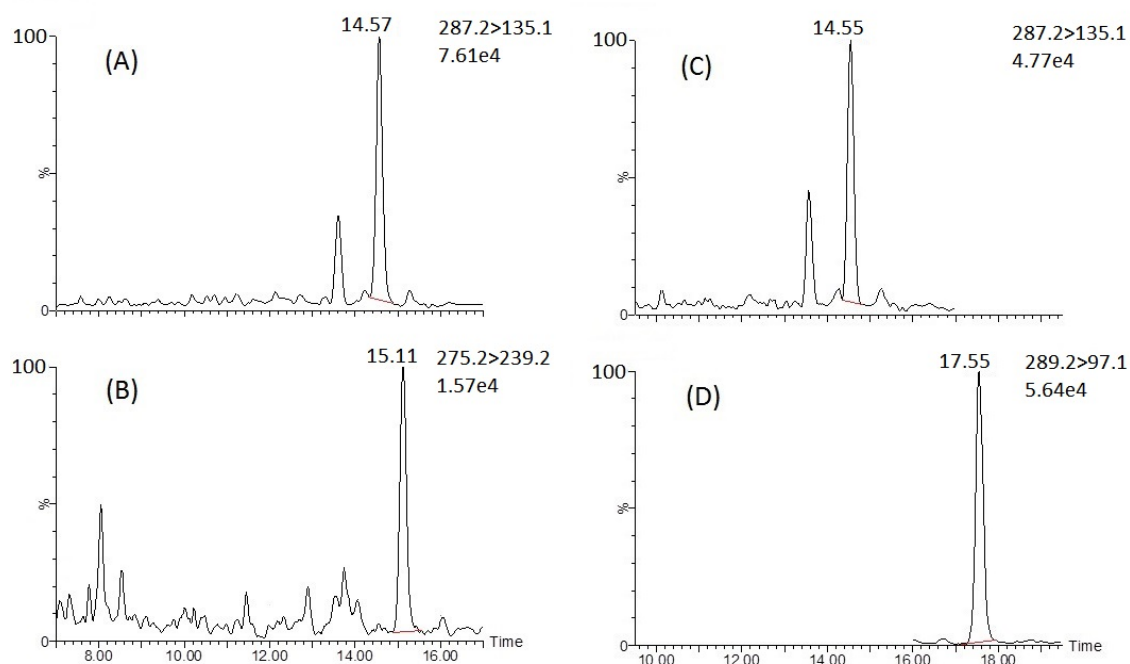


Figure 4. Chromatograms of blank bovine urine sample spiked with $6 \mu\text{g L}^{-1}$ boldenone glucuronide (A), nortestosterone glucuronide (B), boldenone sulphate (C) and $15 \mu\text{g L}^{-1}$ testosterone sulphate (D).

Table 2. Repeatability, intra-laboratory reproducibility and recovery in spiked samples of bovine urine.

Name	Nominal Concentration ($\mu\text{g L}^{-1}$)	Repeatability (%RSD _r)	Intra-laboratory Reproducibility (%RSD _{IR})	N	Recovery (%)
α -Boldenone	1.0-5.0	11	11	24	96
β -Boldenone	1.0-5.0	10	10	24	100
α -Nortestosterone	1.0-5.0	18	18	24	98
β -Nortestosterone	1.0-5.0	18	14	20	92
α -Trenbolone	1.0-5.0	12	14	24	98
β -Trenbolone	1.0-5.0	13	22	24	98
β -Boldenone sulphate	3.0-30.0	13	14	16	57
α -Nortestosterone sulphate	3.0-30.0	14	23	16	52
Testosterone sulphate ^a	7.5-75	7	14	18	50
β -Boldenone glucuronide	6.0	8	12	6	78
β -Nortestosterone glucuronide	6.0	12	12	6	99

^a Concentration of in-house synthesized testosterone-sulphate estimated according to the procedure described in the text.

The method was validated according to the EU legislation (Commission Decision 2002/657/EC, 2002) and analytes were quantified using spiked samples *i.e.* bovine urine spiked with analytes and deuterated internal standards, prior to sample preparation. For calculation of precision and recovery, three concentration levels were selected for the unconjugated steroids (1, 2 and 5 $\mu\text{g L}^{-1}$) and sulphate conjugated steroids (3, 6 and 30 $\mu\text{g L}^{-1}$) and one concentration level for the glucuronide conjugated steroids (6 $\mu\text{g L}^{-1}$) – for testosterone sulphate, though, the concentration levels were 7.5, 15 and 75 $\mu\text{g L}^{-1}$. In Figure 4 the chromatograms are shown for two urine samples spiked with either glucuronides (panel A and B) or sulphates (panel C and D) of steroids.

The residuals and determination coefficient were acceptable ($r^2 > 0.95$) and specificity is acceptable since no interferences are occurring where the analytes are eluting. Decision limit, $CC\alpha$, and detection capability, $CC\beta$, is not significantly different from the originally method and are below 1.0 $\mu\text{g L}^{-1}$. The precision is acceptable for all tested analytes (Table 2). The accuracy, calculated as recovery for the spiked samples, is acceptable for the free steroids, 78 and 99% for the tested glucuronides and 50-57% for the three sulphates tested, taking into account the mass equivalence of steroid from steroid conjugate.

Conclusion

A method has been presented that provides detection of androgenic steroids and phase 2 metabolites of these in bovine urine. The conditions for hydrolysis of glucuronides and especially sulphates have been optimised and validated and procedures for synthesis of glucuronide- and sulphate conjugates of steroids has been presented. Hydrolysis of sulphate conjugates, not hydrolysed by normally used enzyme preparations, is possible with acceptable results.

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