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A simple method for the small scale synthesis and solid-phase extraction purification of steroid sulfates

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ABSTRACT

Steroid sulfates are a major class of steroid metabolite that are of growing importance in fields such as anti-doping analysis, the detection of residues in agricultural produce or medicine. Despite this, many steroid sulfate reference materials may have limited or no availability hampering the development of analytical methods. We report simple protocols for the rapid synthesis and purification of steroid sulfates that are suitable for adoption by analytical laboratories. Central to this approach is the use of solid-phase extraction (SPE) for purification, a technique routinely used for sample preparation in analytical laboratories around the world. The sulfate conjugates of sixteen steroid compounds encompassing a wide range of steroid substitution patterns and configurations are prepared, including the previously unreported sulfate conjugates of the designer steroids furazadrol (17β-hydroxyandrostan[2,3-d]isoxazole), isofurazadrol (17β-hydroxyandrostan[3,2-c]isoxazole) and trenazone (17β-hydroxyestra-4,9-dien-3-one). Structural characterization data, together with NMR and mass spectra are reported for all steroid sulfates, often for the first time. The scope of this approach for small scale synthesis is highlighted by the sulfation of 1 μg of testosterone (17β-hydroxyandrost-4-en-3-one) as monitored by liquid chromatography–mass spectrometry (LCMS).

1. Introduction

Steroid sulfates are a major class of phase II steroid metabolite that are of growing importance in fields such as anti-doping analysis [1], the detection of residues in agricultural produce [2] and medicine [3]. This is in part driven by improvements in liquid chromatography mass spectrometry (LCMS) technology that empower the direct detection of phase II conjugates [4,5] but also arises due to the important information unveiled by a thorough analysis of phase II metabolism [6,7]. In the field of anti-doping science the analysis of human sulfate metabolites can afford greater retrospectivity for the detection of steroidal agents [8–10] and also serve as markers to distinguish between steroids of exogenous and endogenous origin [11–13]. Although there are a range of reliable approaches to analyse for phase II metabolites in both humans and animals, the range of available steroidal sulfate reference materials is incomplete and the ability to rapidly make and manipulate steroid sulfates as standards or reference materials has limitations [1]. This may mean that significant steroid sulfate markers cannot be quantified or even identified [11,13].

A range of methods have been developed to access steroid sulfates including the reaction of the parent steroid with sulfate salts and acetic anhydride [14], chlorosulfonic acid [15], amine complexes of sulfur trioxide [16–19], sulfuric acid and carbodiimides [20], sulfamic acid [21], or more recently by novel sulfuryl imidazolium salts [22,23]. These reactions however, whilst effective in affording the desired sulfate compounds, generally require significant chemical expertise and may also require harsh or hazardous conditions [15,19,20], specialised reagents [22,23], or complicated purification methods. These factors make small scale synthesis of steroid sulfates for analytical purposes a somewhat challenging undertaking. Simple synthetic access to steroid sulfates would facilitate the identification of metabolites and assist in the development of methods targeting these analytes. In this paper we report a general method for the small scale synthesis and purification of steroid sulfate compounds for anti-doping research and other analytical applications that is suitable for adoption by analytical laboratories. The method takes advantage of a rapid purification by solid-phase extraction (SPE), a technique familiar to analytical laboratories but with untapped potential in chemical synthesis.

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2. Experimental

2.1. Materials

Chemicals and solvents including sulfur trioxide pyridine complex (SO_3·py), sulfur trioxide triethylamine complex (SO_3·NEt_3) and 1,4-dioxane, were purchased from Sigma–Aldrich (Castle Hill, Australia) and were used as supplied unless otherwise stated. Androsterone (3α-hydroxy-5α-androst-17-one), epiandrosterone (3β-hydroxy-5α-androst-17-one), etiocholanolone (3α-hydroxy-5β-androst-17-one), methandriol (17α-methylandrost-5-ene-3β,17β-diol) and testosterone (17β-hydroxyandrost-4-en-3-one) were obtained from Steraloids (Newport RI, USA). Estra-4,9-dien-3,17-one was obtained from AK Scientific (Union City CA, USA). Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one) was obtained from BDH (Poole, UK). Lithocholic acid (3α-Hydroxy-5β-cholan-24-oic acid) was obtained from L. Light & Co. (Colnbrook, UK). Epitestosterone (17α-hydroxyandrost-4-en-3-one) was synthesised from testosterone using literature methods [24]. MilliQ water was used in all aqueous solutions and in the liquid chromatography mobile phase. Liquid chromatography (gradient) grade methanol was obtained from Merck (Kilsyth, Australia) and was used for preparing the liquid chromatography mobile phase and steroid standard solutions. *N*,*N*-Dimethylformamide (DMF) and aqueous ammonia solution were obtained from Chem-Supply (Gillman, Australia). Formic acid was obtained from Ajax Chemicals (Auburn, Australia). Ethyl formate and methanol were distilled separately from calcium hydride under a nitrogen atmosphere before use. Chlorosulfonic acid [CAUTION!] was distilled under a nitrogen atmosphere before use. Tetrahydrofuran was distilled from sodium wire before use. Solid-phase extraction (SPE) was performed using Waters (Rydalmere, Australia) Oasis weak anion exchange (WAX) 6 cc cartridges (186004647).

2.2. Instruments

Melting points were determined using a SRS Optimelt MPA 100 melting point apparatus and are uncorrected. Optical rotational were determined using a Perkin–Elmer 241MC polarimeter (sodium D line, 298 K) in the indicated solvents. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using either Varian 400 MHz, Bruker Ascend 400 MHz, Bruker Avance 400 MHz or Bruker Avance 600 MHz spectrometers at 298 K using deuterated methanol solvent unless otherwise specified. Data is reported in parts per million (ppm), referenced to residual protons or ^{13}C in deuterated methanol solvent (CD_3OD: ^{1}H 3.31 ppm, ^{13}C 49.00 ppm) unless otherwise specified, with multiplicity assigned as follows: br = broad, s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet. Coupling constants *J* are reported in Hertz. Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) were performed using positive electron ionisation (+EI) on a Micromass VG Autospec mass spectrometer or negative electrospray ionization (−ESI) on a Micromass ZMD ESI-Quad, or a Waters LCT Premier XE mass spectrometer. Reactions were monitored by analytical thin layer chromatography (TLC) using Merck Silica gel 60 TLC plates (7:2:1 ethyl acetate:methanol:water, unless otherwise specified) and were visualised by staining with a solution of potassium permanganate [$KMnO_4$ (3 g), K_2CO_3 (20 g), NaOH (0.25 g), H_2O (305 mL)], with heating as required. Liquid chromatography mass spectrometry (LCMS) was performed using an Agilent Technologies Infinity 1260 LC system equipped with an Agilent 1290 HTS LC Injector and an Agilent 6120 Quadrupole detector. Injections (10 μL) were resolved with an Agilent Zorbax SP-C18 UPLC column (2.1 mm × 50 mm, 1.8 μm, 600 bar) with an isocratic mobile phase consisting of 38% aqueous ammonium acetate (26.3 mM): 62% methanol. The column and sample modules were set to 30 °C and 4 °C respectively.

2.3. Chemical synthesis

2.3.1. General method for the small scale steroid sulfation reaction with purification by SPE

A solution of SO_3·py (10.0 mg, 62.8 mmol) in DMF (100 μL) was added to a solution of steroid (1.0 mg) in 1,4-dioxane (100 μL) and the resulting solution was then stirred in a capped vial at room temperature for 4 h. The reaction was then quenched with water (1.5 mL) and subjected to purification by SPE. An Oasis WAX SPE cartridge (6 cc) was pre-conditioned with methanol (5 mL) followed by water (15 mL). The reaction mixture (1.7 mL) was then loaded onto the cartridge and eluted under a positive pressure of nitrogen at a flow rate of approximately 2 mL min^{-1} with the following solutions: formic acid in water (2% v/v, 15 mL), water (15 mL), methanol (15 mL) and saturated aqueous ammonia solution in methanol (5% v/v, 15 mL). The methanolic ammonia fraction was concentrated *in vacuo* to yield the desired steroid sulfate as the corresponding ammonium salt.

2.3.2. General method for the small scale steroid sulfation reaction with conversion determined by ^{1}H NMR analysis

A steroid sulfation reaction was performed as per Section 2.3.1 above. A modified SPE protocol eluting with only formic acid in water (2% v/v, 15 mL), water (15 mL) and saturated aqueous ammonia solution in methanol (5% v/v, 15 mL), followed by concentration of the methanolic ammonia fraction yielded a mixture containing both the starting steroid and the corresponding steroid sulfate as the ammonium salt. A ^{1}H NMR spectrum was obtained and integration of a suitable signal (typically C3-H or C17-H) of both steroid and steroid sulfate provided a ratio of the two compounds which was used to determine the percent conversion of the sulfation reaction. The mixture was then subjected to a second SPE purification using the conditions outlined in Section 2.3.1 to yield pure steroid sulfate as the corresponding ammonium salt.

2.3.3. General method for the steroid sulfation reaction with purification by recrystallisation

Sulfation was performed by minor modification of known literature procedures [17]. A solution of steroid (100 mg) in pyridine (1 mL) was added drop-wise to solid SO_3·NEt_3 or SO_3·py (1.1–1.6 equiv). The resulting solution was stirred at room temperature for 20 h unless otherwise specified. The reaction was then added to diethyl ether (20 mL) and the resulting precipitate was collected by filtration. The crude steroid sulfate salt was recrystallised from refluxing dichloromethane/diethyl ether, washed with small portions of cold diethyl ether and finally dried *in vacuo* to yield the desired steroid sulfate as its corresponding triethylammonium or pyridinium salt.

2.3.4. Testosterone 17-sulfate, ammonium salt **1a** [17]

A solution of testosterone (1.0 mg, 3.47 μmol) in 1,4-dioxane (100 μL) was treated with a solution of SO_3·py (10.0 mg, 62.8 μmol, 18.1 equiv) in DMF (100 μL) and purified by SPE as per Section 2.3.1 to yield the *title compound* **1a** as a white solid. Performing the sulfation reaction as per Section 2.3.2 showed full conversion. R_f 0.34; δ_H (400 MHz): 5.71 (s, 1H, C4-H), 4.34 (t, *J* 8.6 Hz, 1H, C17-H), 2.53-0.95 (m, 19H), 1.24 (s, 3H, C18-H_3), 0.87 (s, 3H, C19-H_3); δ_C (100 MHz): 202.4 (C3), 175.2 (C5), 124.1 (C4), 87.9 (C17), 55.4, 55.1, 51.3, 43.8, 40.0, 37.7, 36.8, 34.7, 33.9, 32.8, 29.1, 24.3, 21.6, 17.7 (C18), 12.0 (C19); LRMS (−ESI): *m/z* 367 (100%, $[C_{19}H_{27}O_5S]^-$); HRMS (−ESI): found 367.1579, $[C_{19}H_{27}O_5S]^-$ requires 367.1579. Copies of the 400 MHz ^{1}H NMR, 100 MHz ^{13}C