Journal of Chromatography A, 1196–1197 (2008) 153–160

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Isolation and quantification by high-performance liquid chromatography–ion-trap mass spectrometry of androgen sulfoconjugates in human urine

Emmanuel Strahm[a], Isabelle Kohler[a], Serge Rudaz[b], Sophie Martel[c], Pierre-Alain Carrupt[c], Jean-Luc Veuthey[b], Martial Saugy[a], Christophe Saudan[a,*]

[a] Laboratoire Suisse d'Analyse du Dopage, Institut Universitaire de Médecine Légale, Chemin des Croisettes 22, 1066 Epalinges, Vaud, Switzerland
[b] Laboratory of Analytical Pharmaceutical Chemistry, School of Pharmaceutical Sciences, EPGL, University of Geneva, University of Lausanne, 30 Quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland
[c] LCT-Pharmacochemistry, School of Pharmaceutical Sciences, EPGL, University of Geneva, University of Lausanne, 30 Quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland

ARTICLE INFO

Article history:
Available online 3 May 2008

Keywords:
Endogenous steroids
Phase II metabolism
Mixed-mode polymeric anion-exchange
Solid-phase extraction
Linear ion-trap mass spectrometry

ABSTRACT

Together with steroid glucuronides, sulfoconjugates may be used as markers of steroid administration as well as endogenous steroid production. A fast and sensitive analytical procedure has been developed for the simultaneous separation, determination and quantification of sulfate and glucuronide derivatives of testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio) and dehydroepiandrosterone (DHEA) in human urine. First, a weak anion-exchange solid-phase extraction support (SPE Oasis WAX) was used for complete and rapid separation of sulfates and glucuronides in two extracts after loading of urine sample (2 mL). Then sulfates were analyzed directly by high-performance liquid chromatography–ion-trap mass spectrometry (LC–MS/MS) with electrospray ionization in negative mode. Chromatographic separation of the targeted sulfoconjugates was achieved using a Waters XBridge C_{18} column (150 mm × 4.6 mm I.D., 5 μm) with gradient elution. Assay validation demonstrated good performance for instance for T sulfate (TS) and E sulfate (ES) in terms of trueness (89–107%), repeatability (3.4–22%) and intermediate precision (5.8–22%) over the range of 2–200 ng/mL (corresponding to 1.5–147 ng/mL as free steroids). Results obtained on biological samples demonstrated the suitability of this analytical strategy for direct measurement of androgen sulfoconjugates and glucuroconjugates in human urine.

1. Introduction

Testosterone metabolism and urinary excretion of androgen steroids are of critical importance for the interpretation of an adverse analytical findings in anti-doping control. The urine profile of endogenous steroids is mainly based on the concentrations equivalent to the glucuronides of testosterone, androsterone, etiocholanolone, epitestosterone and dehydroepiandrosterone (DHEA) together with the testosterone glucuronide (TG)/epitestosterone glucuronide (EG) ratio. According to guidance given by the World Anti-Doping Agency (WADA) in 2004, urine samples should be submitted to isotopic ratio mass spectrometry (IRMS) if levels of the targeted androgen glucuronides are greater than fixed cut-off values or the TG/EG ratio is greater or equal to 4.0 [1].

Steroid concentration in urine is assessed by gas chromatography–mass spectrometry (GC–MS) after deconjugation of the glucuronide moiety by enzymatic hydrolysis (β-glucuronidase) and derivatization (trimethylsilylation) prior to analysis [2,3]. This procedure does not provide complete information about phase II metabolism that mainly encompasses glucuro- and sulfoconjugates. However, analytical methods have been developed to quantify indirectly sulfate concentration by determination of the total amount of steroid conjugates after acid hydrolysis [4,5] or enzymatic hydrolysis [6]. Then, sulfoconjugate concentration is assessed from the difference between total and glucuronide fraction. Nevertheless, it may be pointed out that this method has not received common use in clinical trials and anti-doping controls, due to the need to perform two extractions for each sample and the possible resulting errors in the evaluation of steroid levels.

To avoid chemical transformation steps prior to analysis and thus conserve the information of phase II metabolism, the development of LC–MS/MS methods for identification of intact androgen steroid sulfate in urine specimens have been reported

* Corresponding author. Tel.: +41 21 314 7330; fax: +41 21 314 7095.
E-mail address: Christophe.Saudan@chuv.ch (C. Saudan).

0021-9673/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2008.04.066

in the literatures during the last decade [7–10]. In this study, we propose a method for simultaneous quantification at endogenous levels of the five relevant androgen steroid sulfoconjugates in urine matrix. For that purpose, intact sulfoconjugates were isolated prior to detection and quantification by liquid chromatography–tandem mass spectrometry (LC–MS/MS) with a linear ion-trap quadrupole (LTQ) instrument. To minimize ion suppression during MS analysis [11], enhanced specimens clean-up based on weak anion-exchange solid-phase extraction (SPE Oasis WAX) was developed. The clean-up procedure allowed selective separation of glucuronide and sulfate species contained in urine specimens of humans.

2. Experimental

2.1. Chemicals and reagents

Methanol (99.9%), hydrochloric acid (37%), potassium dihydrogenphosphate, sodium carbonate and sodium hydrogencarbonate were purchased from Merck (Darmstadt, Germany). Formic acid (98%), ammonium hydroxide (28% in water), glacial acetic acid (>99.8%), *n*-pentane (>99.0%), sodium sulfate (>99.0%), sodium acetate (>99%), ammonium iodide (NH_4I) and ethanethiol were obtained from Sigma–Aldrich (Buchs, Switzerland). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was provided by Macherey-Nagel (Düren, Germany). *Escherichia coli* β-glucuronidase (140 U/mg at 37 °C) was purchased by Roche diagnostics (Mannheim, Germany). Ultrapure water was produced by a Milli-Q Gradient A10 water purification system with a Q-Gard 2 and a Quantum EX Ultrapure organex cartridge purchased by Millipore (Billerica, MA, USA). Commercial reference steroids 4-androsten-17β-ol-3-one sulfate sodium salt (testosterone sulfate, TS), 4-androsten-17α-ol-3-one sulfate sodium salt (epitestosterone sulfate, ES), 5β-androstan-3α-ol-17-one sulfate potassium salt (etiocholanolone sulfate, EtioS), 5-androsten-3β-ol-17-one sulfate sodium salt (dehydroepiandrosterone sulfate, DHEAS), 4-androsten-17β-ol-3-one glucosiduronate (testosterone glucuronide, TG) 4-androsten-17α-ol-3-one glucosiduronate (epitestosterone glucuronide, EG), 5β-androstan-3α-ol-17-one glucosiduronate (etiocholanolone glucuronide, EtioG) and 5-androsten-3β-ol-17-one glucosiduronate (dehydroepiandrosterone glucuronide, DHEAG) were purchased from Steraloids (Newport, RI, USA). 5α-androstan-3α-ol-17-one sulfate triethylammonium salt (androsterone sulfate, AS), [16,16,17α-$^2H_3$]androst-4-en-17β-ol-3-one sulfate triethylammonium salt (TS-d_3), [2,2,4,4-$^2H_4$]5α-androstan-3α-ol-17-one sulfate triethylammonium salt (AS-d_4), 5α-androstan-3α-ol-17-one-3-β-D-glucuronide (androsterone glucuronide, AG), [2,2,4,4-$^2H_4$]5α-estran-3α-ol-17-one glucuronide sodium salt (norandrosterone glucuronide-d_4, NAG-d_4) were purchased from Australian Government National Measurement Institute (Pymble, Australia). 17β-hydroxy-17α-methylandrost-4-en-3-one (methyltestosterone, MeT) was purchased from Sigma–Aldrich.

2.2. Instrumentation

The LC–MS system was composed of a Rheos 2000 CPS-LC system pump (Flux Instrument, Basel, Switzerland) and a HTS Pal autosampler (CTC Analytics, Zwingen, Switzerland) coupled to a LTQ Linear ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) using electrospray interface. The column temperature and autosampler tray were set at 30 and 10 °C, respectively. The sample (5 μL) was injected into the system and the chromatographic separation was performed on a XBridge C_{18} column (150 mm × 4.6 mm I.D. 5 μm) (Waters, Milford, MA, USA) at a flow rate of 1 mL/min. Water containing 1% (v/v) acetic acid (A) and acetonitrile (B) were used as mobile phase solvents. The gradient percentage of organic

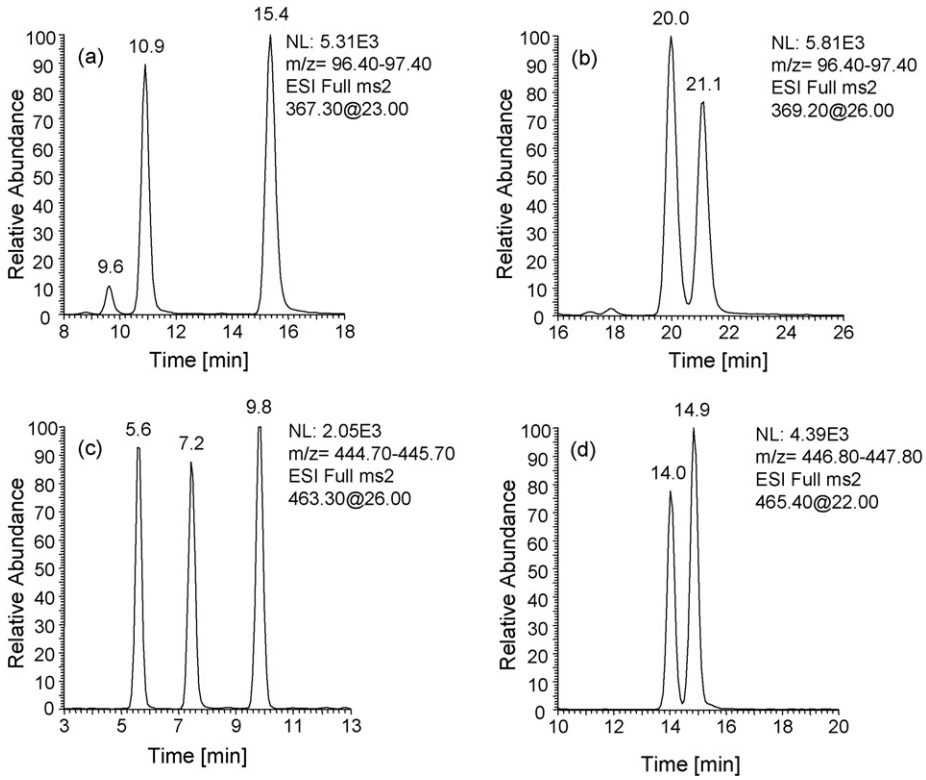

Fig. 1. LC–MS/MS chromatograms of sulfates (0.5 ng on column) TS (t_R = 9.6 min), ES (t_R = 10.9 min), and DHEAS (t_R = 15.4 min) (a); AS (t_R = 20.0 min) and EtioS (t_R = 21.1 min) (b); and glucuronides (2.5 ng on column) TG (t_R = 5.6 min), DHEAG (t_R = 7.2 min) and EG (t_R = 9.8 min) (c) and AG (t_R = 14.0 min) and EtioG (t_R = 14.9 min) (d) in urine specimen.