Journal of Steroid Biochemistry & Molecular Biology 149 (2015) 1–10

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

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

Validated LC–MS/MS simultaneous assay of five sex steroid/neurosteroid-related sulfates in human serum

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ARTICLE INFO

Article history:
Received 8 October 2014
Received in revised form 8 December 2014
Accepted 11 January 2015
Available online 13 January 2015

Keywords:
Dehydroepiandrosterone sulfate
Estrone sulfate
Androsterone sulfate
Pregnenolone sulfate
Allopregnanolone sulfate
Neurosteroid sulfates
LC–MS/MS

ABSTRACT

Conventionally, the concentration of steroidal sulfates was estimated by indirect or immuno-based assays before the use of liquid-chromatography tandem mass spectrometry (LC–MS/MS). In the present study, a validated LC–MS/MS method is described for the simultaneous quantification of dehydroepiandrosterone sulfate (DHEA-S), estrone sulfate (E_1-S), androsterone sulfate (ADT-S), pregnenolone sulfate (Preg-S) and allopregnanolone sulfate (Allopreg-S). E_1-S binding to serum proteins was observed, especially for the high concentration quality control serum samples, leading to −10 to −15% bias using a polymer-based SPE. This protein binding can be efficiently eliminated using a Waters Oasis™ WAX following the same extraction procedure. Most likely, the E_1-S binding elimination on Oasis™ WAX can be attributed to its different sorbent structure, where the benzeno group of E_1-S can interact with the benzene of the backbone of Oasis™ WAX. With this improvement, the method has been fully validated according to the FDA guidelines. The low quantification limits (LLOQs) are 40 ng/mL, 40 pg/mL, 5 ng/mL, 1.5 ng/mL and 0.25 ng/mL for DHEA-S, E_1-S, ADT-S, Preg-S and Allopreg-S, respectively. A good linearity is obtained with $R > 0.99$ for all compounds within the appropriate calibration range. Accuracies of all levels of QCs are within the range of 10% for DHEA-S, E_1-S, ADT-S and Preg-S while for Allopreg-S, the accuracy is within the 15% range. The interday coefficient variance is 5.5–9.5% for the low limits of quantification of all five compounds while values of 1.3–9.9% are found for higher levels of QCs of all five compounds. Recovery of the five compounds in stripped serum is equivalent to that in unstripped serum. The average recovery difference is less than 5% between stripped and unstripped serum for each compound. All results of other test parameters such as matrix, hemolysis and lipemic effects as well as stabilities meet the acceptance criteria of EndoCeutics SOPs and FDA guidelines.

1. Introduction

Sulfonation plays an important role in steroid metabolism [1–4]. Following sulfonation, steroids and their metabolites become more polar with increased water solubility and are more easily released into the blood, where they can be eliminated by the liver and kidneys [5,6]. On the other hand, steroid sulfatase can form some active steroids from circulating sulfate metabolites [4].

The circulating level of steroid sulfates is typically much higher than that of the free/unconjugated corresponding compounds, thus making their measurement technically less challenging. Before the availability of high performance liquid chromatography mass spectrometry (LC–MS or LC–MS/MS), indirect methods were typically used where the sulfates were hydrolysed before analysis by immunoassay or further derivatized and analysed by gas chromatography coupled to mass spectrometry (GC–MS and GC–MS/MS) [7–9]. The indirect detection and quantification of the sulfated compounds may underestimate some conjugates due to limited hydrolysis. Moreover, sample preparation in the indirect assay is laborious and time-consuming with the additional risk of introducing more errors or interferences.

Introduction of the polar sulfate group onto steroids and their metabolites facilitates the detection of sulfated compounds by mass spectrometry with negative mode as they are ionized in all biological systems [6]. Accordingly, liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI–MS/MS) is well-suited for the quantification of steroid sulfates in serum, plasma, blood, tissue and urine. In most cases, the sample

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http://dx.doi.org/10.1016/j.jsbmb.2015.01.006
0960-0760/

preparation procedures for LC–MS/MS assays are much simpler than that for GC–MS/MS, including the use of solid phase and protein precipitation extractions. When the sensitivity is not an issue, the direct and simple protein precipitation can even be a choice for sample preparation. However, solid phase extraction (SPE) is the most popular strategy for steroid sulfates as they are highly polar and acidic compounds with pKa at about 0. So far, dehydroepiandrosterone sulfate (DHEA-S), estrone sulfate (E_1-S), androsterone sulfate (ADT-S) and pregnenolone sulfate (Preg-S) have been reported to be quantitated using the LC–MS/MS method [1,2,10–14].

Accurate measurement of steroid sulfates should be particularly useful for studies of total metabolism of the sex steroids. To the authors' knowledge, it is the first report of a LC–MS/MS method for the quantitation of allopregnanolone sulphate (Allopreg-S) in human serum. In 2005, Mitamura et al. reported a LC–MS/MS method for the simultaneous quantitation of DHEA-S, ADT-S and epiandrosterone sulphate (Epi-ADT-S) without E_1-S and Preg-S [13] while, more recently, Galuska et al. reported a LC–MS/MS method for a panel of six steroid sulphates with a LLOQ of 400 pg/mL for E_1-S [10]. In the present work, we describe a sensitive, specific, accurate and fully validated liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the simultaneous direct quantification of DHEA-S, E_1-S, ADT-S, Preg-S and Allopregnanolone sulfate (Allopreg-S), where LLOQs of E_1-S and Allopreg-S are 40 pg/mL and 0.25 ng/mL respectively. Polymer weak anion exchange solid phase extraction (SPE) plates from Waters (Waters Canada Limited, Missisauga, Canada) and Phenomenex (USA) were used for the extraction. The importance of a choice of SPE for sample workup has been discussed in detail to avoid protein binding for the accurate measurement of E_1-S. To achieve better separation and sensitivity, a UPLC column Eclipse plus RRHD C18 (1.8 μm 50 × 2.1 mm) (Agilent technologies, Mississauga, Canada) was used on the UPLC-API 5000 system (AB Sciex, Concord, Canada). Validation of the assay was performed in agreement with EndoCeutics standard operating procedures (SOPs) and the FDA guidelines [15].

2. Chemicals and methods

2.1. Chemicals

Sulfate standards and internal standards (IS) are commercially available: DHEA-S (99.0%), ADT-S (98.35%), Preg-S (98.46%), and Allopreg-S (99.33%) were all purchased from Steraloids Inc., (Newport, USA). The internal standard (IS) of E_1-S-d_4 (99.0%) and E_1-S (98.5%) were from ResearchPlus (Barnegat, USA) CDN isotopes (Pointe-Claire, Canada), respectively. Preg-S-$^{13}C_3$ was from Isosciences (King of Prussia, USA), while DHEA-S-d_5 (99.2%) and ADT-S-d_4 (97.9%) were from Cerilliant (Texas, USA). (3β)-Allopregnanolone Sulfate (Epi-Allopreg-S) was purchased from TRC (Toronto, Canada).

28∼30% ammonium hydroxide aqueous solution was from EMD (Mississauga, Canada) while ammonium acetate (99.9%) was obtained from Sigma. On the other hand, reagent ethanol and high purity methanol (LC–MS grade) were from Fisher Scientific (Ottawa, Canada). Stripped human serum MSG 3000 was purchased from Golden West Biologicals (Temecula, USA). Postmenopausal human serum was obtained from Bioreclamation LLC (Westbury, NY, USA). Ultrapure water was obtained in house using the Millipore system. Polymer weak anion exchange SPE Oasis™ WAX was from Waters (Waters Canada Limited, Missisauga, Canada) while Strata-X-AW was from Phenomenex (Torrance, USA).

2.2. Sample preparation

Stock solutions, working solutions (spiking solutions) and internal standards of steroid sulfates were prepared in reagent grade ethanol. All stock solutions were prepared at 50 μg/mL and diluted in the working solution at the required concentation. The concentration ratio of the working (spiking) solutions to the final concentation of spiked calibration curves and Quality Controls (QC)s is 50. The calibration curve of each compound includes eight calibrants, i.e., STDB, STDC, STDD, STDE, STDF, STDG, STDH and STDI. The calibration ranges are 40–4000 ng/mL for DHEA-S, 40–4000 pg/mL for E_1-S, 5–800 ng/mL for ADT-S, 1.5–150 ng/mL for Preg-S and 0.25–25 ng/mL for Allopreg-S, respectively. The calibration curves were prepared in stripped serum while QC samples were prepared in both stripped and unstripped serum (all unstripped sera correspond to unstripped postmenopausal human serum). Four levels of QCs were prepared as low limit of quantitation (LLOQ), Low QC (LQC), Medium QC (MQC) and High QC (HQC) in stripped serum and Endo QC, Endo Low QC (Endo LQC), Endo Mediun QC (Endo MQC) and Endo High QC (Endo HQC) in unstripped serum. The Endo QC was made from pooled unstripped serum which contains the five analytes at approximately 3 times the LLOQ concentration in stripped serum. The Endo LQC, Endo MQC and Endo HQC were prepared by spiking Endo QC with the appropriate QC working solution. During validation, 6 replicates for each QC were processed in every validation assay.

Matrix as well as hemolysis and lipemic effects were investigated at the level of the Endo LQC and Endo HQC. Six individual lots of postmenopausal human sera were used for the matrix effect and one individual lot of postmenopausal human serum was spiked with 2% of the postmenopausal whole blood for the hemolysis effect. The lipemic postmenopausal human serum was the commercial product. The Low QCs and High QCs were prepared in the same manner as Endo LQC and Endo HQC while 3 replicates for each QC were processed to evaluate these effects.

To evaluate stabilities of sulfate compounds in the matrix, six replicates of Endo QC, Endo LQC and Endo HQC were processed. For the short term stability test in biological matrix, samples were thawed and kept at room temperature for the appropriate time

Table 1
MS parameters of MRM for steroid sulfates and their internal standards.

Compounds	Q1 mass (*m*/*z*)	Q3 mass (*m*/*z*)	DP (V)	CE (V)	CXP (V)	Dwell time (ms)	FP (V)
E_1-S	349.2	269.2	−130	−43	−25	100	−10
E_1-S-d4	353.2	273.2	−130	−43	−25	50	−10
DHEA-S	369.3	97.1	−130	−70	−25	50	−10
DHEA-S-d_5	372.3	98.1	−130	−70	−25	50	−10
ADT-S	369.2	97.1	−130	−120	−25	75	−10
ADT-d4	373.2	98.1	−130	−120	−25	75	−10
PREGS	395.2	97.1	−180	−55	−12	100	−10
PREGS-^{13}C	399.2	97.1	−180	−55	−12	75	−10
Allopreg-S	397.3	97.1	−180	−55	−12	150	−10

DP: declutering potential; CE: collision energy; CXP: collision cell exit potential; FP: focusing potential.