A New LC-MS/MS Assay for the Analysis of Sulfated Steroids in Human Serum: Quantification of Cholesterol Sulfate, Pregnenolone Sulfate, 17-Hydroxypregnenolone Sulfate and Androgen Sulfates

Alberto Sánchez-Guijo, Vinzenz Oji, Michaela F. Hartmann, Heiko Traupe and Stefan A. Wudy

Justus-Liebig-University, Centre of Child and Adolescent Medicine, Steroid Research & Mass Spectrometry Unit, Giessen, Germany.
Department of Dermatology, University of Münster, Münster, Germany.

Introduction
Steroids are found in human blood predominantly as sulfated steroids. Conjugation is a physiological mechanism which increases their solubility in biological fluids, facilitating their regulation and excretion. Simultaneous analysis of an extensive number of sulfated steroids in blood is challenging because of their broad range of concentrations. Additionally, chromatographic baseline separation of some sulfated steroids is complicated but mandatory, since some of them are structurally related and provide similar signals in mass spectrometry.

Objectives
- To develop and validate a new LC-MS/MS assay to obtain the most detailed sulfated steroidome in human blood to date.
- Apply this method to study and diagnose conditions related to the metabolism of sulfated steroids, i.e. steroid sulfatase deficiency (recessive X-linked ichthyosis, RXLI).

Methods
- Chromatographic separations were achieved with a phenyl-reversed based fused-core column (Acquity Phenyl-X, 100x2.1 mm, 2.6 μm).
- Electrospray ionization – tandem mass spectrometry (ESI-MS/MS) was used for the identification and quantification of sulfated steroids. The MS/MS product ion for quantification was always the sulfated group HSO₄⁻ (m/z 97).
- Solid phase extraction required 300 μl of serum and was able to isolate both conjugated and unconjugated steroid fractions.
- All compounds were validated at 3 different concentration levels.

Results
- All isobaric/structurally related compounds (i.e. androgen sulfates) were baseline separated.
- DHEAS, CS, AnS, epimers, AndiolS, 16OH-DHEAS and PregS were the sulfated steroids found in higher concentrations in human blood. The validation parameters of all compounds met the standards of the FDA and EMA guidelines for bioanalytical evaluation.
- The sulfates of testosterone (TS), epitestosterone (eTS) and DHT could not be detected in any sample (limits of detection LOD = 0.5 ng/ml and limits of quantification LOQ = 1 ng/ml).
- 17OH-PregS presented important matrix effects which affected its analytical performance, but only at lower concentrations.
- Analytical performance was studied at 3 quality controls (QCs) for each compound. Recoveries ranged between 85.5% and 111.6%. Intra and between day accuracies were all below 20% (relative error). Intra and between day precisions were all below 20% (% coefficient of variation), with the only exception of 17OH-PregS at its lower quality control.
- The concentration of CS in RXLI patients is about 35 times higher than in healthy males and in ichthyosis vulgaris controls.

Conclusions
- We developed and validated a reliable method for the simultaneous quantification of 11 sulfated steroids in human serum, which requires only 300 μl of serum.
- Sample preparation allows for isolation of both unconjugated and sulfated fractions.
- The method has proved its diagnostic value to discriminate between RXLI and other types of skin ichthyoses.

Acknowledgment
This work was supported by the Selbsthilfe Ichthyose e. V., the Medical Faculty (O111409) of the University of Münster, and by the German Research Foundation (DFG) within DFG Research Group 1369 "Sulfated Steroids in Reproduction" to subproject 7 (S.A.W., principal investigator, WU 148/6-2).

Some performance parameters of the LC-MS/MS method

Variation of CS levels in RXLI and controls (logarithmic scale)