

HPLC fractionation method for the isolation of endogenous urinary steroids prior to GC/C/IRMS analysis

Swiss Laboratory for Doping Analyses, Epalinges, Switzerland

Carbon isotope analysis by isotope ratio mass spectrometry (IRMS) is becoming a conventional procedure to enable the detection of steroids abuse in sport. The basic principle of this type of procedure relies on the purification steps of the urine. For this purpose, high performance liquid chromatography (HPLC) is a powerful tool. We present herein the first steps in development of a separation method optimized with the help of ultra-high performance liquid chromatography (UPLC) with ultraviolet (UV) detection.

LC method development

Separation method development for 21 steroids was initially performed on an Acquity UPLC System (Waters, Milford, MA, USA). The standards of 5 β -androstan-3 α ,11 β -diol-17-one (11 β -hydroxyetiocholanolone, OHE), 5 α -androstan-3 α ,11 β -diol-17-one (11 β -hydroxyandrosterone, OHA), 1,4-androstadien-17 β -ol-3-one (boldenone, BO), 5 β -androstan-3 α -ol-11,17-dione (11-ketoetiocholanolone, 11KE), 5 α -androstan-3 α -ol-11,17-dione (11-ketoandrosterone, 11KA), 4-androsten-17 β -ol-3-one (testosterone, T), 4-androsten-3,17-dione (androstenedione, Adione), 4-androsten-17 α -ol-3-one (epitestosterone, epiT), 5 β -androst-1-en-17 β -ol-3-one (boldenone metabolite 1, BOM1), 4-androsten-4-ol-3,17-dione (formestane, F), 5-androsten-3 β -ol-17-one (dehydroepiandrosterone, DHEA), 5 β -pregnan-3 α ,17,20 α -triol (PT), 5 β -androstan-3 α ,17 β -diol (5b), 5 α -estran-3 α -ol-17-one (19-norandrosterone, NA), 5 α -androstan-17 β -ol-3-one (dihydrotestosterone, DHT), 5 β -androstan-3 α -ol-17-one (etiocholanolone, E), 5 α -androstan-3 α ,17 β -diol (5a), 5 α -androstan-3 α -ol-17-one (androsterone, A), 5 β -pregnan-3 α ,20 α -diol (PD), 5-pregnen-3 β -ol-20-one (P) and 16,(5 α)-androsten-3 α -ol (16EN) were individually injected (4 to 10 μ g according to the UV response) on an Acquity column (BEH Shield RP₁₈ 50 mm \times 2.1 mm, 1.7 μ m) with a mobile phase of

acetonitrile (ACN) and water (H₂O) increasing from 5% to 95% of ACN in two gradient runs: 6 min (short run) and 18 min (long run). Flow rate was 0.4 ml/min and UV detection from 194 to 300 nm was employed.

To optimize the mobile phase composition, results were transferred into the modeling software OSIRIS (v.4.2, Datalys, Grenoble, France) and a simulated separation was found (Figure 1 and Table 1).

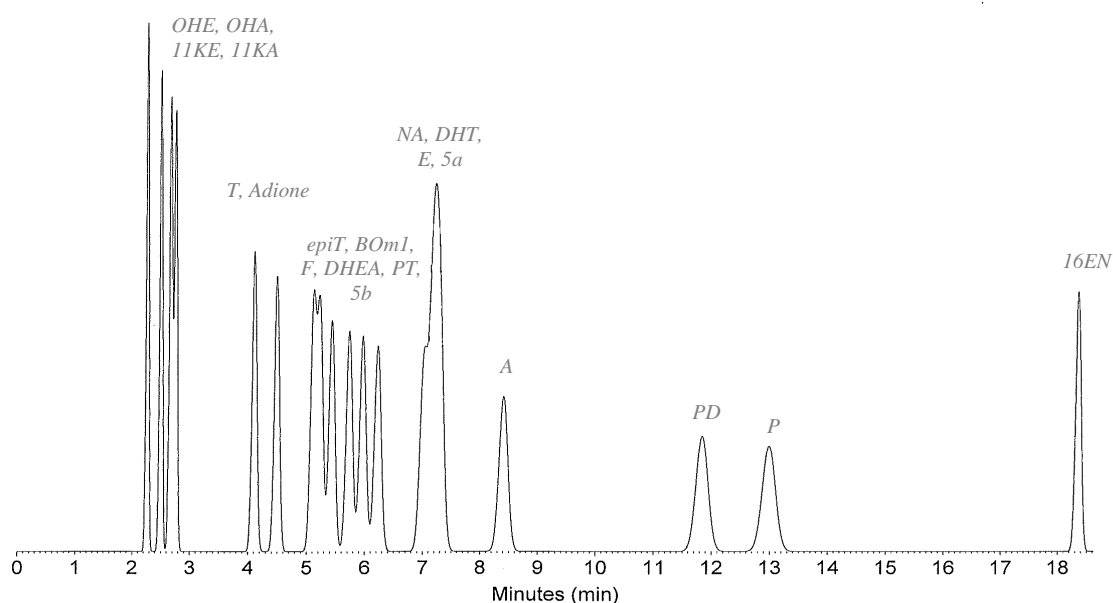


Figure 1. Simulated chromatogram from OSIRIS

The proposed gradient was tested on the UPLC-UV system (data not shown) and the scale up to an HPLC system was performed with the help of an evaluation program for chromatographic performances and methods transfer [1]. The suggested gradient for an XBridge Shield column (RP₁₈ 150 mm x 4.6 mm, 3.5 μm) is shown on Table 1.

Simulation on OSIRIS software			HPLC scale up		
Time [min]	ACN [%]	H ₂ O [%]	Time [min]	ACN [%]	H ₂ O [%]
0.00	30.75	69.25	0.00	30.8	69.2
14.00	34.95	65.05	0.20	30.8	69.2
16.30	61.05	38.95	40.00	33.8	66.2
			50.00	60.0	20.0
			52.00	95.0	5.0

Table 1. Simulated elution gradient from OSIRIS and scaled up elution gradient for HPLC separation

The scale up tests were performed with an Agilent 1100 Series coupled to the 1200 Series fraction collector (Agilent Technologies, Waldbronn, Germany). The before suggested gradient allowed the collection of five fractions containing, in order of appearance, OHE, OHA, BO, 11KE and 11KA (fraction A), T and Adione (fraction B), epiT, BOm1, F, DHEA, PT and 5b (fraction C), NA, DHT, E and 5a (fraction D) and A, PD, P and 16EN (fraction E) (Figure 2). Flow rate was 1.5 ml/min and UV detection of 195 nm was employed.

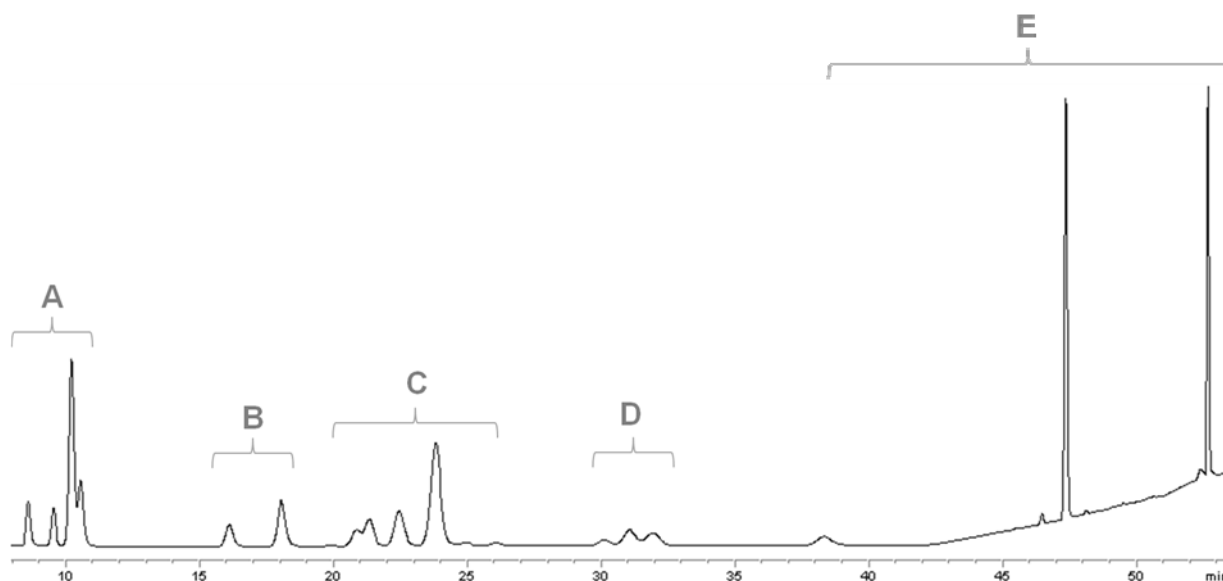


Figure 2. HPLC-UV response (in the same order of appearance on the chromatogram): **Fraction A:** OHE, OHA, BO, 11KE and 11KA; **Fraction B:** T and Adione; **Fraction C:** EpiT, BOm1, F, DHEA, PT and 5b; **Fraction D:** NA, DHT, E, 5a; **Fraction E:** A, PD, P, 16EN.

Preliminary tests on GC/C/IRMS

The collected fractions B, C, D and E were acetylated (with acetic anhydride) and carbon isotope measurements were made on all fractions on a Delta V Plus IRMS system (ThermoFisher Scientific, Bremen, Germany) coupled to an Agilent 7890 GC (Agilent Technologies, Waldbronn, Germany) via a GC-C/TC III interface.

Chromatographic separations, achieved on a DB17-MS column 30 m x 0.25 mm x 0.25 μ m (method previously published [2]), were adequate for most substances except for T and Adione (fraction B), and DHEA and 5b (fraction C). The difference between $^{13}\text{C}/^{12}\text{C}$ ratios of the fractions and unprocessed steroids was on average 0.5‰ showing no significant deviation on the isotopic fractionation.

Furthermore, three different urine samples were extracted (SPE, hydrolysis, LLE [2]), purified with the newly developed HPLC method, derivatized and analyzed both on GC/C/IRMS and GC-MS. Peak shape and purity was satisfactory for most of the steroids. But the high amount of etiocholanolone in urine was a problem for the detection of the steroids in fraction D and recovery for 16EN was quite low.

Conclusion and perspectives

The herein presented method is yet not applicable in routine analysis. Another step is mandatory to ensure complete purification of all compounds especially for T, DHEA, 5b and E. As a solution, a second HPLC purification will be developed with the same method development but, this time, for acetylated steroids. In addition, some optimization has to be done on the entire process to increase, if possible, the 16EN recovery.

Acknowledgments

The authors thank Dr. Davy Guillarme for his scientific assistance.

References

1. Laboratory of Analytical Pharmaceutical Chemistry. HPLC calculator: programme d'évaluation des performances chromatographiques et de transfert de méthode en HPLC. Geneva (2009) <http://www.unige.ch/sciences/pharm/fanal/lcap/telechargement.htm>
2. Saudan C, Emery C, Marclay F, Strahm E, Mangin P, Saugy M. (2009) Validation and performance comparison of two carbon isotope ratio methods to control the misuse of androgens in humans. *J. Chromatogr. B* **877**, 2321-2329.