Rapid analysis of steroid metabolites using field asymmetric waveform ion mobility spectrometry combined with liquid chromatography and mass spectrometry

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Overview

- A rapid, sensitive method is reported for the determination of isobaric steroid sulfate and glucuronide metabolites utilizing field asymmetric waveform ion mobility spectrometry (FAIMS) in combination with liquid chromatography (LC) and mass spectrometry (MS)
- Three optimised FAIMS conditions, used in conjunction with hydrophilic interaction chromatography (HILIC) and time-of-flight (TOF) MS, allow seven targeted steroid metabolites to be separated
- An 8 minute LC-FAIMS-MS analysis quantitative method with LODs in the range 1-6 ng/mL and LOQs in the range 3-20 ng/mL

Introduction

- A targeted LC-FAIMS-MS approach[1] has been applied to the analysis of isobaric steroids, relevant in doping control analysis
- Seven steroid metabolites were targeted, consisting of two isobaric pairs of steroid glucuronides and an isobaric trio of steroid sulfates:

MW 464	Testosterone glucuronide (**TG**) and epitestosterone glucuronide (**ETG**)
MW 466	Etiocholanolone glucuronide (**ECG**) and androsterone glucuronide (**ADG**)
MW 368	Dehydroepiandrosterone sulfate (**DHEAS**), testosterone sulfate (**TS**) and epitestosterone sulfate (**ETS**)

- Due to the isobaric pairs the current analysis methods require lengthy LC or GC chromatographic run times for complete separation of these steroid metabolites.
- Here the combination of miniaturised FAIMS with LC–MS allows the rapid determination of isobaric steroid metabolites in human urine with an 8 min chromatographic run time

Methods

- A prototype FAIMS device (ultraFAIMS, Owlstone Ltd.) located in the modified source region of a TOF-MS (Agilent 6230 TOF) has been combined with liquid chromatography (Agilent 1200 HPLC)
- A rapid LC-FAIMS-MS method has been developed for the analysis of steroids metabolites in human urine (Figure 1)
- Initial FAIMS scanning experiments conducted using FAIMS-MS with the dispersion field (DF) values ranging from 180-300 Td and compensation field (CF) values ranging from -2-5 Td (Figure 2)

LC
- Agilent Poroshell 120 HILIC column: (4.6 x 50 mm, 2.7 µm)
- MPA: H_2O + 10 mM NH_4Ac + 0.1% acetic acid; MPB: ACN:H_2O 98:2 + 10 mM NH_4Ac + 0.1% acetic acid
- LC method: 0-0.5 min 100% B, 0.5-2.5 min 92% B, 2.5-3.5 min 82% B, 6-8 min 100% B

FAIMS
- FAIMS conditions for LC-FAIMS-MS chosen from FAIMS-MS scanning analysis
- Sodium acetate infused post LC column, for the formation of the doubly sodiated [M+Na-H]$^+$ ions of the steroid metabolites
- FAIMS conditions for LC-FAIMS-MS analysis: DF 260 Td, CF 1.35 Td, CF 2.05 Td, CF 2.70 Td

MS
- MS operated in positive ion mode, with a *m/z* range of 80 – 1500
- MS scan rate at 2 scan/s for LC-FAIMS-MS analysis and a fragmentor voltage of 200 V
- Data processing software used: Agilent MassHunter B.05.00, Microsoft Excel 2010, Origin 2015 Academic Version: 92E, and MATLAB R2014a

Figure 1: Summary of LC-FAIMS-MS method conditions

- Steroid standards were provided by the Drug Control Centre (King's College London, UK)
- Urine sample pre-treatment by solid phase extraction using reversed phase C18 cartridges and reconstituted in starting LC mobile phase

Results

- FAIMS-MS analysis of the steroid metabolites (Figure 2), *via* direct infusion into the electrospray ionization source, yielded an optimal FAIMS separation by monitoring the doubly sodiated [M+2Na-H]$^+$ ion of each steroid at DF 260 Td (Figure 2 inset)

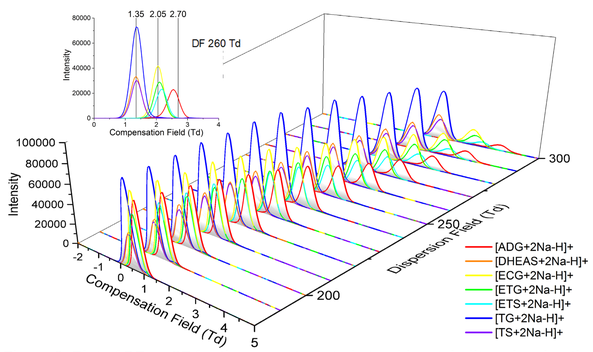

Figure 2: Plot of DF vs CF for the seven targeted steroid metabolites showing the separation of the isobaric steroid ions as the electric field strength increases. Inset: CF spectrum at a DF of 260 Td

- Note that using FAIMS-MS (Figure 2) ETS is separated from TS and DHEAS, but TS and DHEAS are not separated by FAIMS

Table 1: Summary of steroid separation using LC-FAIMS-MS at DF 260 Td

MS	LC	FAIMS
DHEAS/TS/ETS *m/z* 413.1369	DHEAS t_R 1.93 min	DHEAS CF 1.35 Td
	TS/ETS t_R 2.20/2.22 min	TS CF 1.35 Td
		ETS CF 2.05 Td
TG/ETG *m/z* 509.2122	TG/ETG t_R 7.23/7.09 min	TG CF 1.35 Td
		ETG CF 2.05 Td
ADG/ECG *m/z* 511.2278	ADG/ECG t_R 6.99/7.07 min	ADG CF 2.70 Td
		ECG CF 2.05 Td

- **A combination of chromatographic (t_R), FAIMS (CF) and mass (m/z) separation allows all steroid sulfates and glucuronides to be separated and quantified (Table 1, Figure 3)**

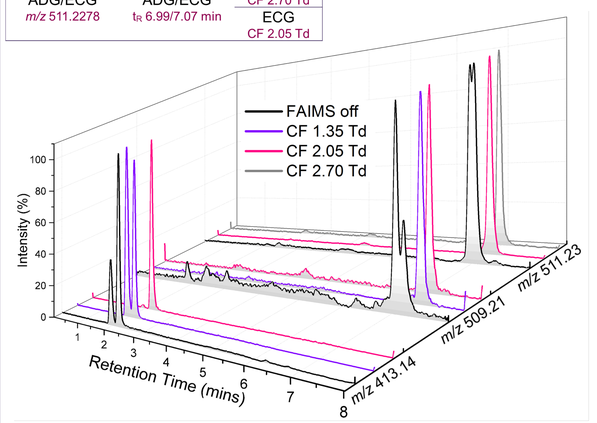

Figure 3: Mass selected chromatograms for the seven steroids spiked (1 µg/mL) into urine with LC-MS (FAIMS off) chromatograms shown in black and LC-FAIMS-MS chromatograms (at DF 260 Td) with CF values color coded (see legend) selected to resolve the steroid metabolite isobars

- Using FAIMS in combination with LC-MS allows the rapid separation of the isobaric steroid metabolites so they do not all need to be chromatographically separated (Figure 3)
- LC-FAIMS-MS is also an effective tool for separating target responses and background ions from the matrix using FAIMS CF selection (Figure 4)

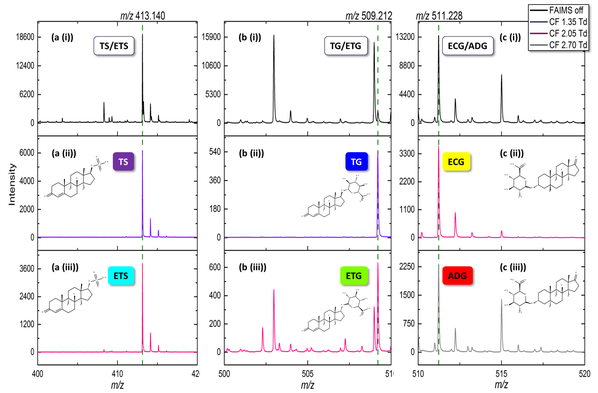

Figure 4: Comparison of extracted mass spectra for the unresolved steroid isobars in LC-MS (a, b and c (i)) and the resolved steroid isobars using LC-FAIMS-MS at DF 260 Td (a, b and c, (ii) and (iii)) with CF values color coded (see legend)

- LC-FAIMS-MS quantitative response for each steroid metabolite is shown in Figure 5 and Table 2
- Linearity is observed in the range 31-1000 ng/mL for all steroid metabolites with LODs in the range 1-6 ng/mL and LOQs in the range 3-20 ng/mL

Steroid	R^2 Linear	LOD (ng/mL)	LOQ (ng/mL)
ADG	0.9900	6.1	20.3
DHEAS	0.9975	1.1	3.8
ECG	0.9927	2.8	9.3
ETG	0.9927	3.7	12.3
ETS	0.9919	1.1	3.7
TG	09926	1.0	3.4
TS	0.9955	1.3	4.2

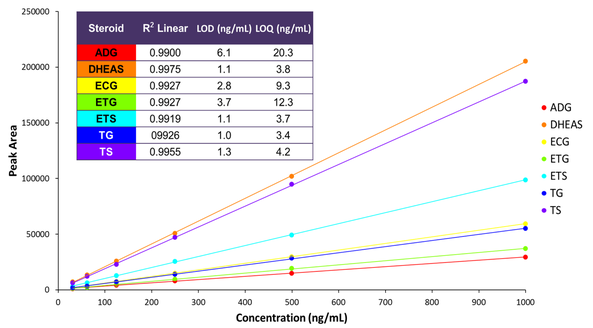

Figure 5: Calibration curves for each of the steroids using LC-FAIMS-MS using the three CF values of 1.35, 2.05 and 2.70 Td at DF 260 Td, with LODs and LOQs in inset table

- Discrimination (Table 2) between isobaric steroid metabolites analysed individually and as a mixture is ≥ 95% (with DHEAS as a measure of the error of the system)
- Reproducibility using LC-FAIMS-MS was equal to or improved compared to LC-MS alone, with no internal standard present (Table 2)

Table 2: Discrimination between steroid metabolites and reproducibility (% RSD) analysed by LC-FAIMS-MS and LC-MS

Steroid	Percent Discrimination (%)	%RSD 6 x n=1 spiked urine extracts	
		LC-MS	LC-FAIMS-MS
DHEAS	97.0	5.6	4.3
TS	99.5	4.9	5.1
ETS	96.2		2.8
ADG	95.1	12.8	5.8
ECG	97.2		3.6
ETG	96.0	12.2	4.9
TG	95.1	8.7	9.1

- Steroid metabolites ADG and ECG detected in unspiked urine by LC-MS and LC-FAIMS-MS are compared in Figure 6
- Using LC-FAIMS-MS the two isobars can be individually identified
- S:N ratios increased by >200 % for the individual components in comparison to the unresolved peak for the pair

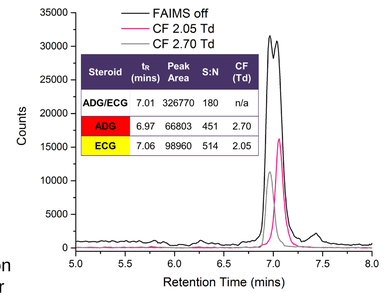

Figure 6: LC-FAIMS-MS (at DF 260 Td) of ECG/ADG in unspiked urine, compared with LC-MS, with CF values color coded (see legend)

Conclusions

- FAIMS-MS analysis of seven steroid metabolites shows that all of steroid isobars can be separated using FAIMS apart from DHEAS/TS
- Three dimensional analysis using mass extracted ion chromatograms at selected FAIMS CF values (FAIMS) aids the identification and quantification of seven steroid metabolites relevant to anti-doping analysis in sports
- Combining FAIMS with chromatography and MS allows for the analysis time to be kept short: 8 mins per run
- Steroid glucuronides and sulfates are detected in unspiked human urine using the LC-FAIMS-MS method developed
- Further work includes the combination of FAIMS with tandem MS for increased sensitivity to improve LODs and LOQs in the analysis of human urine for anti-doping analysis

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