#C04

Rapid separation of steroid and secosteroid metabolites by ultraFAIMS-MS for high-throughput clinical analysis

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1. Introduction

Mass spectrometry of steroids and secosteroids has many diverse applications in the clinical environment.

- Many steroids have isomeric forms, which may differ in functionality, and can impede steroid quantitation; a method of distinguishing the target steroid from the isomer form is required (Figure 1).

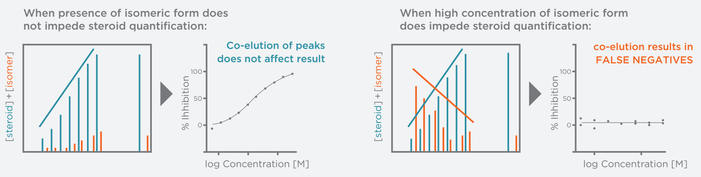


Figure 1. Quantifying steroids can be difficult in presence of isomers

- When no unique multiple reaction monitoring (MRM) transition can distinguish between isomers, liquid chromatography (LC) separation is used. This is not ideal because:
- Long LC run times and/or special columns are required to prevent co-elution.
- Means method can be to expensive for routine applications in clinical settings.
- A faster method for clinical analysis of steroid is highly desirable.

In-source separation techniques such as field asymmetric ion mobility spectrometry (FAIMS) can alleviate these problems, offering the ability to separate isomeric compounds on timescales compatible with on-line and high throughput 96-well plate solid phase extraction (SPE).

- FAIMS separates ions according to field-induced changes in their collision cross section (Figure 2).
- Highly orthogonal to *m/z* separation in a mass analyser.

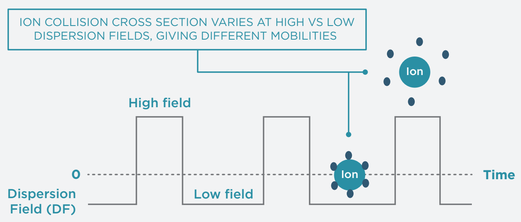


Figure 2. FAIMS separates ions according to field-induced changes in their collision cross section

- Can be set to selectively transmit ion of interest prior to mass spectral detection (Figure 3).

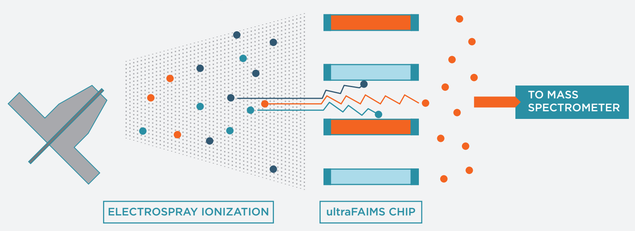


Figure 3. Selective FAIMS transmission of ion of interest

FAIMS can replace LC completely, by removing salts via SPE and separating isomers prior to mass spectrometry (Figure 4).

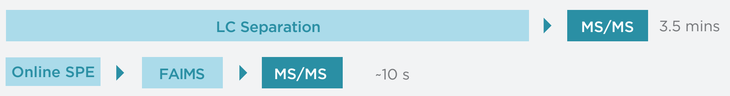


Figure 4. Comparison of timings of chromatographic and high throughput analysis techniques

2. Applications

Testosterone is used to measure the effect of drug-drug interactions on metabolic pathways.

- Formation of 6β-hydroxytestosterone (6β-HT) is monitored to quantify the functional activity of the enzyme CYP3A4.
- Some candidate drugs inhibit 6β-HT formation while promoting 2β-hydroxytestosterone (2β-HT) formation (Figure 5).

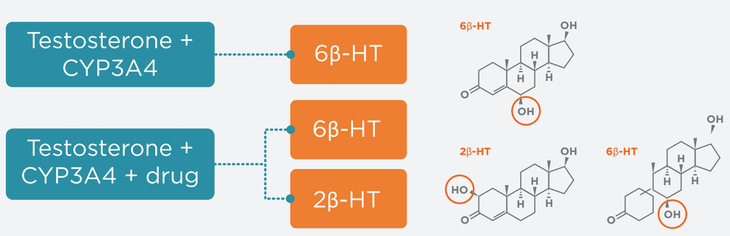


Figure 5. Drug-drug interactions, their effect on metabolite pathways and structures of 6β-HT and 2β-HT

Vitamin D_3 is metabolized in the liver to form 25-hydroxyvitamin D_3 (25-OH D_3).

- 25-OH D_3 levels are routinely measured for diagnostic assessment of vitamin D related diseases
- Biologically inactive 3-epi analogs of 25-OH D_3, 3-epi-25-hydroxyvitamin D_3 (3-epi-25-OH-D_3), have been reported
- These differ in stereochemical orientation of one hydroxy group (Figure 6).

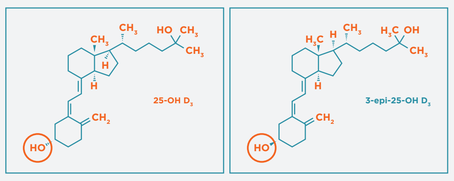


Figure 6. Structures of 25-OH D_3 and 3-epi-25-OH D_3

Here we describe the application of FAIMS-MS to the analysis of hydroxyvitamin D_3 epimers and isomeric testosterone metabolites, eliminating the lengthy liquid chromatography (LC) step for the high-throughput clinical laboratory.

3. Methods

Experiments were carried out with an ultraFAIMS-A1 system (Owlstone Medical Ltd., Cambridge, UK) installed on an Agilent 6460 triple quadrupole MS and 6230 TOF-MS (both Agilent, Santa Clara, US) fitted with a JetStream electrospray ionisation (ESI) source operated in positive ion mode (Figure 7).

ultraFAIMS is a miniaturised FAIMS device in which the electrodes are formed from a micro-manufactured chip.

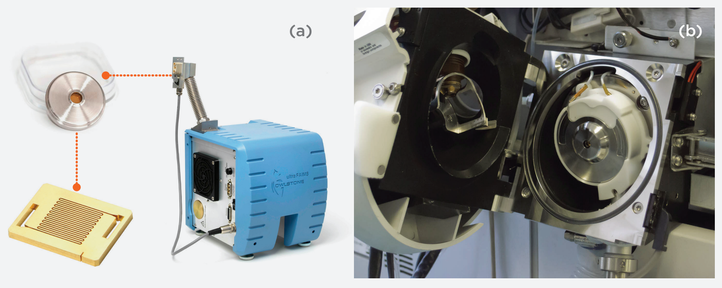


Figure 7. (a) ultraFAIMS A1 device and (b) installed on Agilent 6230 ToF-MS

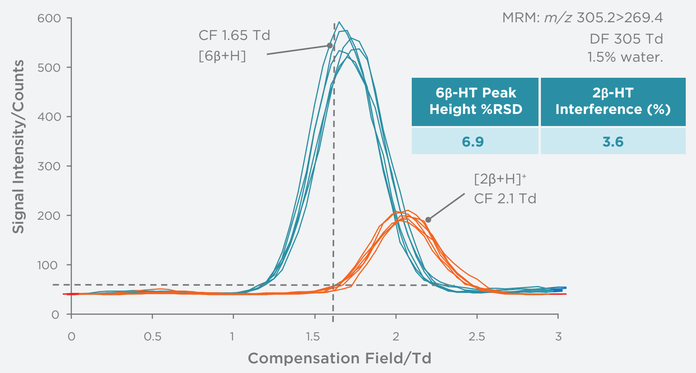
The device consists of a set of parallel gaps in a metal substrate that forms the electrodes. The key dimensions are:

- 100 µm electrode gap and 700 µm channel length.

This small scale enables very fast separations due to short ion residence times and low-voltage electronics.

4a. Results - 2β-HT and 6β-HT

Optimum conditions for FAIMS separation of 2β-HT and 6β-HT were investigated. The addition of water vapour at 1.5% (v/v) enabled separation of 2β-HT and 6β-HT with a drying gas temperature 150°C and 305 Td DF (Figure 8).



6β-HT Peak Height %RSD	2β-HT Interference (%)
6.9	3.6

Figure 8. Optimised separations 2β-HT and 6β-HT with 1.5% (v/v) water vapour

The isomer separations obtained using the optimised conditions were applied to determine selectivity for the target steroid in the presence of the isomeric form.

Calibration curves were constructed to determine the level of quantitation for the ESI-FAIMS-QQQ method. Calibrations were compared from loop injections of 6β-HT, with and without the 2β-HT isomer contribution, with and without FAIMS selection (Figure 9).

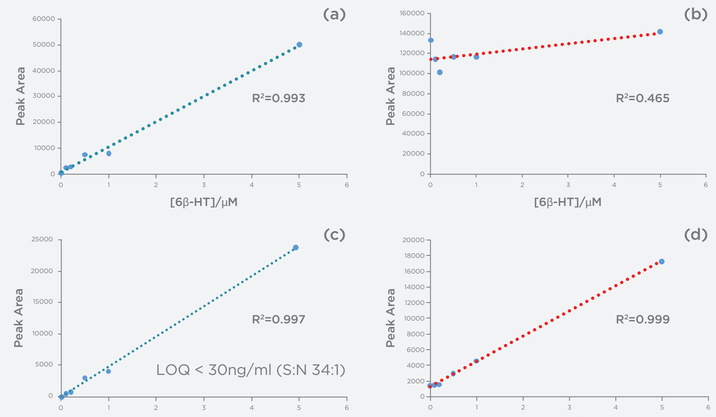


Figure 9. (a) Loop injections of 6β-HT without FAIMS separation, (b) Loop injections of 6β-HT, and 2β-HT without FAIMS separation, (c) Loop injections of 6 β-HT with FAIMS set to transmit 6β-HT (d) Loop injections of 6β-HT and 2β-HT with FAIMS set to transmit 6β-HT

- R^2 values for 6β-HT increased from 0.465 to 0.999 with the addition of FAIMS selection, due to the removal of the 2β-HT isomer contribution.
- As well as removing interference from the isomer, FAIMS also improved S/N of the 6β-HT response.
- The LOQ of 6β-HT was determined to be <0.1 µM (34:1 S/N) showing the sensitivity of the technique was suitable for detecting biologically relevant concentrations using high-throughput online SPE method.

4b. Results - 25-OH D_3 and 3-epi-25-OH D_3

Optimal separation of 25-OH D_3 and 3-epi-25-OH D_3 was obtained using rubidium adducts (Figure 10).

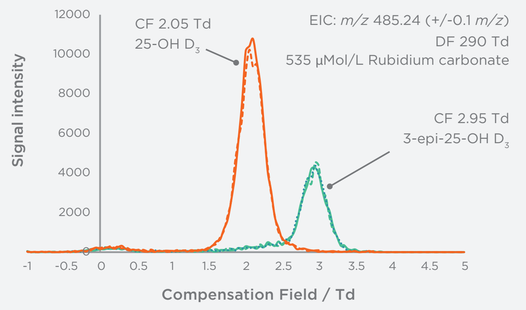


Figure 10. Optimised separations 25-OH D_3 and 3-epi-25-OH D_3 Rb^+ adducts

Table 1: Selectivity performance

	25-OH D_3 (CF = 2.05 Td)	3-epi-25-OH D_3 (CF = 2.95 Td)
Average signal	10283	4393
SD	110.03	125.48
% RSD	1.07	2.86
Interference %	2.38	2.19
Variance (+/- %)	0.32	0.77

Assessment of the interference of the 3-epi-25-OH D_3 isomer was carried out with equimolar solutions of 25-OH D_3 and 3-epi-25-OH D_3, with samples directly infused.

- Interference from 3-epi-25-OH D_3 was ~2% when FAIMS conditions were set to transmit 25-OH D_3,
- Also ~2% from 25-OH D_3 when set to transmit 3-epi-25-OH D_3.

Linear calibration curves were generated for 10:1 ratio mixtures of 25-OH D_3 and 3-epi-25-OH D_3, over a 25-OH D_3 concentration range of 50 nMol L^{-1} to 5 µMol L^{-1} (Figure 11).

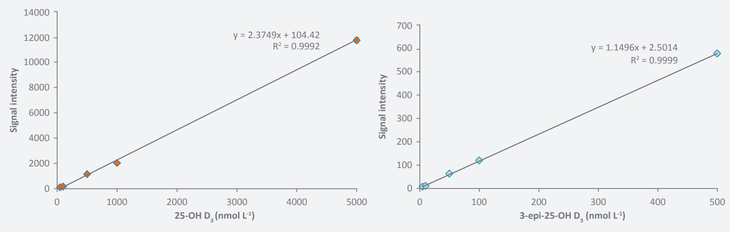


Figure 11. 25-OH D_3 and 3-epi-25-OH D_3 Rb^+ adducts

A 50 nMol L^{-1} 25-OH D_3 sample was analysed to test the calibration (Table 2).

Table 2: Actual and calculated 25-OH D_3 concentration from calibration curve

True concentration (n Mol L^{-1})	Calculated concentration (n Mol L^{-1})	Error
50	53	+ 6%

Increased levels of 3-epi-25-OH D_3 decreased the 25-OH D_3 response, leading to inaccuracies in quantification.

- Addressed using scaling factor determined from the ratio of 25-OH D_3 to 3-epi-25-OH D_3 response to back-calculate the 25-OH D_3 concentration (Figure 12).

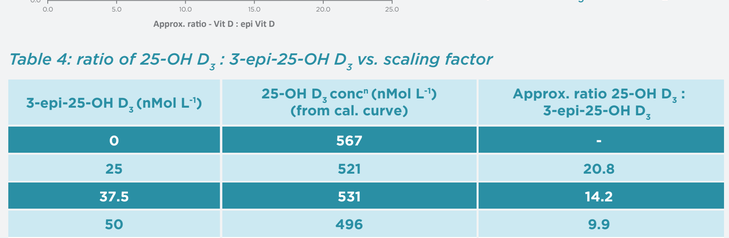


Figure 12. Response of 25-OH D_3 in the presence of increased 3-epi 25-OH D_3 concentration

Table 3: Calculated 25-OH D_3 response based on suppressed ion current and scaling factor

3-epi-25-OH D_3 (nMol L^{-1})	25-OH D_3 ion signal	25-OH D_3 conc^n according to calibration curve (nMol L^{-1})	Difference from true value (nMol L^{-1})
25	1132	521	21
37.5	1157	531	31
50	1072	496	-4
100	967	451	-49
250	919	431	-69

The approximate ratio of 25-OH D_3 to 3-epi 25-OH D_3 was calculated based on the initial measurements and plotted. (Figure 13 and Table 4). This allowed for the selection of the appropriate scaling factor for accurate 25-OH D_3 concentration for unknown samples.

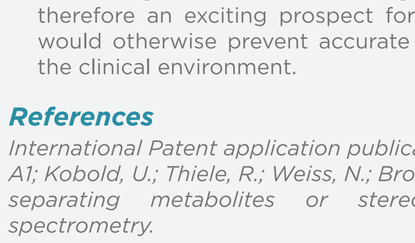


Figure 13. Plot of ratio of 25-OH D_3 : 3-epi-25-OH D_3 vs. scaling factor

Table 4: ratio of 25-OH D_3 : 3-epi-25-OH D_3 vs. scaling factor

3-epi-25-OH D_3 (nMol L^{-1})	25-OH D_3 conc^n (nMol L^{-1}) (from cal. curve)	Approx. ratio 25-OH D_3 : 3-epi-25-OH D_3
0	567	-
25	521	20.8
37.5	531	14.2
50	496	9.9
100	451	4.5
250	431	1.7

5. Conclusions

- Combining ultraFAIMS with mass spectrometry enabled quantification of 6β-HT and 25-OH D_3 by removing biological, isomeric interferences, replacing a lengthy LC separation step with sample to sample analysis times of < 1 minute.
- Combining ultraFAIMS-MS with high-throughput SPE methods is therefore an exciting prospect for when isomeric interference would otherwise prevent accurate determination of steroids in the clinical environment.

References

International Patent application publication number WO 2015/185487 A1; Kobold, U.; Thiele, R.; Weiss, N.; Brown, L.J.; Method and device for separating metabolites or stereoisomers by ion mobility spectrometry.

Acknowledgements

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