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Flunisolide – Triamcinolone acetonide. MS differentiation in positive and in negative ionization mode. LC differentiation.

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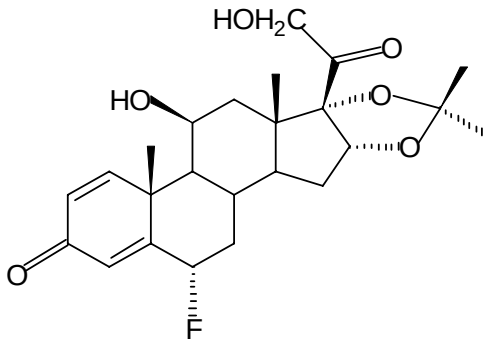
Introduction

Flunisolide and Triamcinolone acetonide belong to the glucocorticosteroid class. All glucocorticosteroids are prohibited in competition and included in the WADA Prohibited List in the section S9 [1].

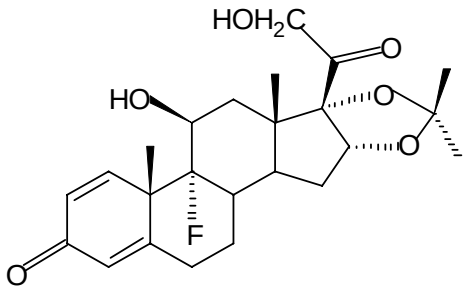
The two glucocorticosteroids have the same molecular mass (434), same molecular formula ($C_{24}H_{31}FO_6$) and similar chemical structures; they differ only by the position of the fluorine atom on the steroid skeleton – 6α for Flunisolide, respectively, 9α for Triamcinolone acetonide.

Therefore, the two prohibited substances may have common ions or MRM transitions and close chromatographic retention times, therefore interfering in each others detection. In the case of the analyze of a doping control sample containing one of these two substances, these interferences may result in a false negative analytical finding or in a misidentification of the substance detected.

This paper presents the results of the tests carried-out by the liquid chromatography coupled with tandem mass spectrometry with triple quadrupole in order to differentiate the two prohibited substances.



Flunisolide $C_{24}H_{31}FO_6$ M=434



Triamcinolone acetonide $C_{24}H_{31}FO_6$ M=434

Figure 1. Chemical structure of Flunisolide and Triamcinolone acetonide

Materials and Methods

The tests have been carried-out on 10µg/ml solutions in methanol of reference materials. The instrumental analysis has been carried-out on two analytic equipments: AGILENT 6410, respectively, VARIAN 1200L. The analytic conditions are presented in table 1.

Table 1. LC/MS^2 analytic parameters

Equipment	**AGILENT 6410**	**VARIAN 1200L**
Column	ZORBAX SB-C18 (50x2.1mm, 5µm)	ChromSep SS OmniSpher 3 C18 (100x2.0mm, 3µm)
Column Thermostat	30^0C	25^0C
Solvent A	5mM NH_4HCOO in water	0.1% acetic acid and 5mM NH_4CH_3COO in water (v/v)
Solvent B	5mM NH_4HCOO in acetonitrile	Methanol
Flow	0.3ml/min	0.25ml/min
Gradient B	30-50% in 1min, 50%-70% in 3min, 5min at 70%, 5min at 30%	30-50% in 1min, 50%-70% in 3min, 1min at 70%, 5min at 30%
Injection volume	2µL	10µL
Ionization	**ESI positive or negative**: Drying gas: 12l N_2/min at 350^0C Nebulysing gas: 50psi N_2 Capillary needle: 4000V	**APCI negative**: Drying gas: 12psi N_2 at 150^0C Nebulysing gas: 58psi air Auxiliary gas: 17psi N_2 at 400^0C Corona current: 5µA
Collision gas	Nitrogen	Argon, 1.5mTorr
MRM transitions selection and collision energy optimization	Analysis in Product Ion Scan mode followed by analyses in MRM mode at various collision energies	Use of the MSMS Breakdown acquisition soft during direct injection of a 10µg/ml solution of reference material

Results and Discussion

MS Differentiation

Both substances (like the majority of the glucocorticosteroids) give good signals both in ESI and in APCI and ionize both in positive mode, forming $[M+H]^+$, and in negative mode, forming adducts with anions from the mobile phase [2,3,4,5].

In figure 2 the Product Ion spectra, at 10V collision energy, in positive mode, are shown for Flunisolide (a) and Triamcinolone acetonide (b) respectively. In table 2, the selected MRM transitions together with the optimized collision energy are given. Table 2 also allows comparison of the (relative) abundances of these selected transitions between both compounds. The ion 417 (loss of H_2O) is specific to Flunisolide, while the ions 415 (loss of HF) and 357 to Triamcinolone acetonide, but even for the common ions, 339 and 321, the relative abundances are too different to satisfy the WADA identification criteria [6] concerning the mass spectrometry if one would try to confirm a sample containing Flunisolide against a reference with Triamcinolone acetonide (or vice versa).

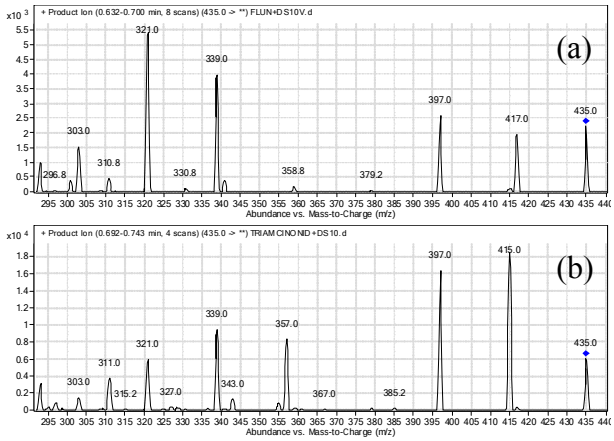


Figure 2. Product Ions Scans in positive ionization mode (Agilent 6410)

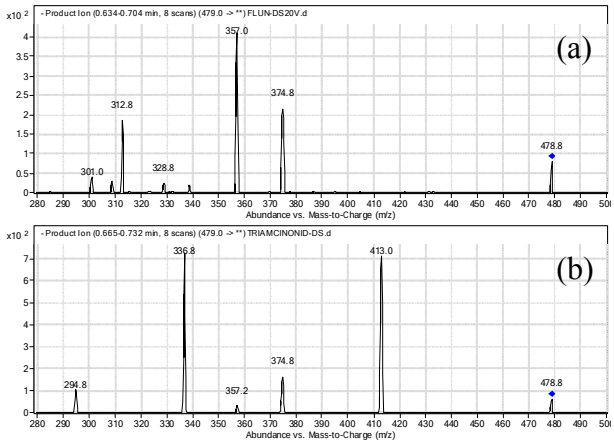
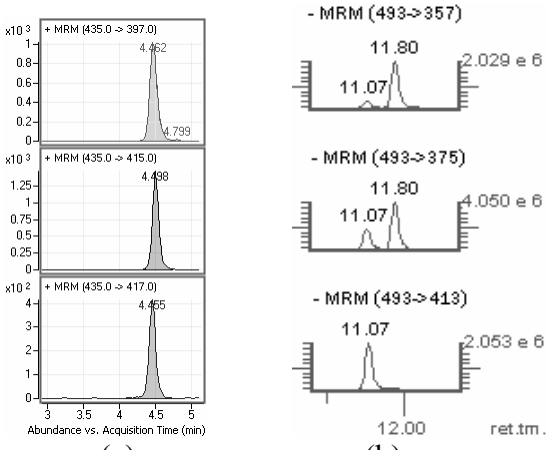


Figure 3. Product Ions Scans in negative ionization mode (Agilent 6410, formate adduct as precursor ion)



(a) (b)

Figure 4. LC/MS^2 analysis of urines spiked with 20ng/ml Flunisolide and Triamcinolone acetonide, prepared and analyzed by the routine screening procedure [5]: (a) on Agilent 6410 in positive mode with water/ acetonitrile, (b) on Varian 1200L in negative mode (acetate adducts) with water/methanol

Table 2. Optimized collision energy MRM's abundances on Agilent 6410 in positive ionization mode

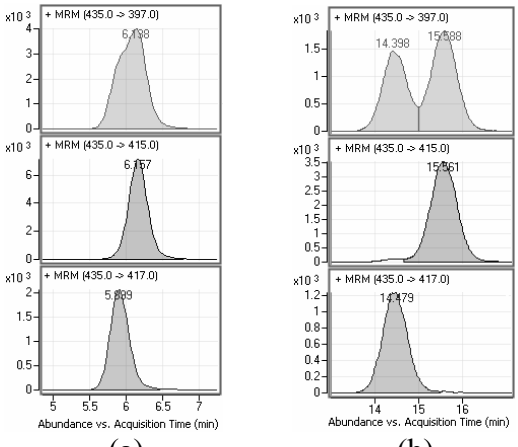
MRM (collision energy)	Flunisolide (Area)		Triamcinolone acetonide (Area)	
(+) 435->321 (10V)	74532	159%	56012	50%
(+) 435->339 (5V)	61752	132%	77323	69%
(+) 435->357 (5V)	684	1%	67586	60%
(+) 435->397 (5V)	46767	100%	112325	100%
(+) 435->415 (5V)	3443	7%	218793	195%
(+) 435->417 (5V)	38770	83%	597	1%

Table 3. Optimized collision energy MRM's abundances on Agilent 6410 in negative ionization mode (formate adduct as precursor ion)

MRM (collision energy)	Flunisolide (Area)		Triamcinolone acetonide (Area)	
(-) 479->313 (30V)	1329	12%	-	-
(-) 479->337 (30V)	396	4%	4825	70%
(-) 479->357 (15V)	6895	63%	1069	16%
(-) 479->375 (15V)	10881	100%	6853	100%
(-) 479->413 (15V)	-	-	8036	117%

Table 4. Optimized collision energy MRM's abundances on Varian 1200L in negative ionization mode (acetate adduct as precursor ion)

MRM (collision energy)	Flunisolide ($h.10^{-3}$)		Triamcinolone acetonide ($h.10^{-3}$)	
(-) 493->185 (28V)	10990	10%	-	-
(-) 493->357 (21V)	50350	44%	8010	19%
(-) 493->375 (14V)	114200	100%	42700	100%
(-) 493->413 (22V)	-	-	50330	118%



(a) (b)

Figure 5. LC/MS^2 analysis of a 5µg/ml Flunisolide and Triamcinolone acetonide solution in methanol analyzed on Agilent 6410 in positive mode with water/acetonitrile on a C18 column eluted: (a) isocratic with 30% solvent B, (b) isocratic with 25% solvent B

In figure 3 (a) and (b) and in table 3 the Product Ion spectra are shown, at 20V collision energy, in negative mode (formate adduct, 479, as precursor ion) and the MRM abundances at optimized collision energy, obtained on Agilent 6410 for the two compounds. The ion 313 is specific to Flunisolide, while 413 and 337 to Triamcinolone acetonide

In table 4 the MRM abundances are shown, at optimized collision energy, obtained in negative mode (acetate adduct, 493, as precursor ion) on Varian 1200L for the two compounds. The ion 185 is specific to Flunisolide, while 413 to Triamcinolone acetonide.

LC Differentiation

In the routine screening analysis chromatographic conditions, the Flunisolide – Triamcinolone acetonide chromatographic separation succeeds when using, on Varian 1200L, water/methanol based mobile phase (figure 4b), but not when using, on Agilent 6410, water/acetonitrile based mobile phase (figure 4a).

In isocratic conditions with 30% solvent acetonitrile (figure 5a), although the peaks are not at all separated, the difference between retention times (0.258min, 4.2%) >4%, which is already sufficient to differentiate: if one would try to confirm a sample containing Flunisolide against a reference with Triamcinolone acetonide (or vice versa), the WADA identification criteria [6] concerning the liquid chromatography (retention time ± 2%) would not be satisfied.

In isocratic conditions with 25% solvent B acetonitrile (figure 5b) the chromatographic separation looks sufficient to avoid major interferences between the two compounds.

References

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