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## **Glucocorticosteroids in Routine Doping Analysis: Metabolism and Detection of Fluticasone-17-propionate**

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### ***Abstract***

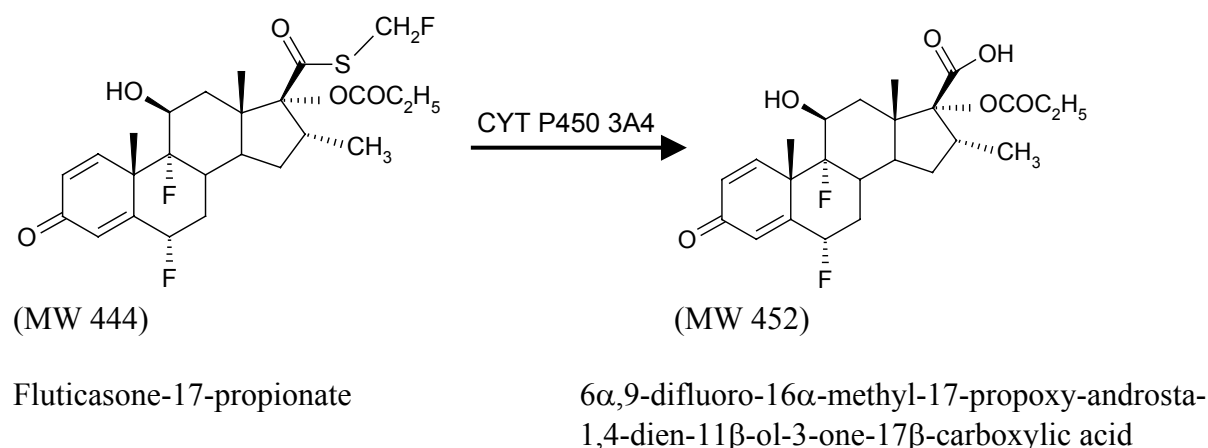
Since January 1st 2004, the administration of glucocorticosteroids by non-systemic-routes (inhalative, intraarticular, topic, etc.) requires a therapeutic use exemption, abbreviated process (TUE2), defined by the ‘International Standard for Therapeutic Use Exemptions’ [1, 2].

First experiences after one year of implementation of sample preparation for glucocorticosteroids into screening procedure 4, detection of anabolic androgenic substances (combined fraction), followed by LC-MS/MS analysis is presented. In addition, the metabolism of fluticasone-17-propionate was studied. Fluticasone-17-propionate is available for inhalation and as nasal drops. The only existing metabolite is fluticasone-17-propionate-carboxylic acid (6 $\alpha$ ,9-difluoro-16 $\alpha$ -methyl-17-propoxy-androsta-1,4-dien-11 $\beta$ -ol-3-one-17 $\beta$ -carboxylic acid), which is formed in humans through the cytochrome P450 3A4 pathway. The metabolite was synthesized in our laboratory by alkaline hydrolysis of the thio-ester-group of fluticasone-17-propionate and implemented into the existing screening procedure 5 – detection of diuretics and acidic compounds in human urine by LC-MS/MS, negative ionization mode, after solid phase extraction (Serdolit® PAD I). The extraction yield was estimated with 98%, the limit of detection with 3 ng/ml.

### ***Introduction***

After implementation of a screening procedure for glucocorticosteroids for more than one year in routine doping analysis, the number of screened glucocorticosteroids and their respective metabolites has increased constantly [3,4]. The glucocorticosteroids budesonide, its main metabolite 16 $\alpha$ -hydroxyprednisolone, 16-methyleneprednisolone, desonide, flunisolide,

flucortolone and fluticasone-17-propionate have been added to the existing screening procedure. On the pharmaceutical market, fluticasone-17-propionate is available only for inhalation and as nasal drops. No formulations for systemic administration have been developed. On several doping control forms the administration of fluticasone-17-propionate was indicated and it became obvious that fluticasone-17-propionate is widely used in sports that are performed at extremely low temperatures, e.g. ice skating. However, in no case the parent compound itself was detected in the routine screening procedure for glucocorticosteroids. Possible reasons are the very low locally administered dose and the metabolic pathway of fluticasone-17-propionate in the human body. The only described metabolite, 6 $\alpha$ ,9-difluoro-16 $\alpha$ -methyl-17-propoxy-androsta-1,4-dien-11 $\beta$ -ol-3-one-17 $\beta$ -carboxylic acid, a fluticasone-17-propionate carboxy metabolite [FPCM] is formed in humans through the cytochrome P450 3A4 pathway (Fig. 1). FPCM is excreted in urine [5]. This metabolite is not available on the market, but is readily synthesized as reference compound as shown below.



**FIGURE 1: Metabolic pathway of fluticasone-17-propionate in human.**

The detection of FPCM had to be implemented into a screening procedure different from the commonly employed screening 4, *comprehensive sample preparation for anabolic steroids, glucocorticosteroids, beta-receptor blocking agents, selected anabolic androgenic steroids and buprenorphine in human urine* [3]. Due to its chemical structure, an extraction at pH 9.6 (as performed in the existing screening procedure for glucocorticosteroids), will result in an extremely low recovery. Therefore, it was implemented into the existing screening procedure

for diuretics and acidic compounds where a solid phase extraction with Serdolit® PAD I is performed [6].

## ***Experimental***

### ***Preparation, purification and characterization of fluticasone-17-propionate metabolites***

One hundred µl of an ampoule of the formulation *Flutide Nasetten* (GlaxoSmithKline, München, D), containing 0.4 mg of fluticasone-17-propionate in 0.4 ml aqueous solution were transferred into a glass tube. A total of 400 µl of distilled methanol was added as well as 500 µl of an aqueous solution containing 10 mol/l potassium hydroxide, resulting pH: 14. After heating for 30 min at 60°C and cooling to room temperature the mixture was neutralized by slowly adding hydrochloric acid (1 mol/l) and pH control.

The reaction mixture was analyzed using an HP 1090 liquid chromatograph with diode array detector (Hewlett Packard, Waldbronn, D) at a wavelength of 246 nm. The column used was a Nucleosil 100-5, C18, 125 x 5 mm (Macherey-Nagel, Düren, D), and solvents were: A = 5 mmol ammonium acetate, 1 % glacial acetic acid, pH 3.5 and B = acetonitrile. After holding for 3 min at 20% B, gradient started increasing to 50 % in 12 min and proceeded to 90 % B in 1 min, holding for 3 min.

Two fractions were collected under visual control from 10.7 – 12.3 min and from 13.7 – 15.3 min into separate glass tubes for further analytical investigations by LC-MS/MS. The analysis of both aliquots and a standard solution of fluticasone-17-propionate, 1 mg/ml in methanol were performed on a Hewlett Packard HP1100 liquid chromatograph coupled to a PE Sciex API 2000 triple quadrupole mass spectrometer. All instrumental parameters are shown in Table 1.

### ***Validation***

Preliminary investigations of specificity, limit of detection, recovery, and robustness have been performed for the detection of FPCM in human urine after solid phase extraction with Serdolit® PAD I (research grade, Serva, Heidelberg D). Sample preparation was conducted as described elsewhere [6].

**TABLE 1: Analytical parameters, negative, multiple reaction monitoring of product ion scans experiments.**

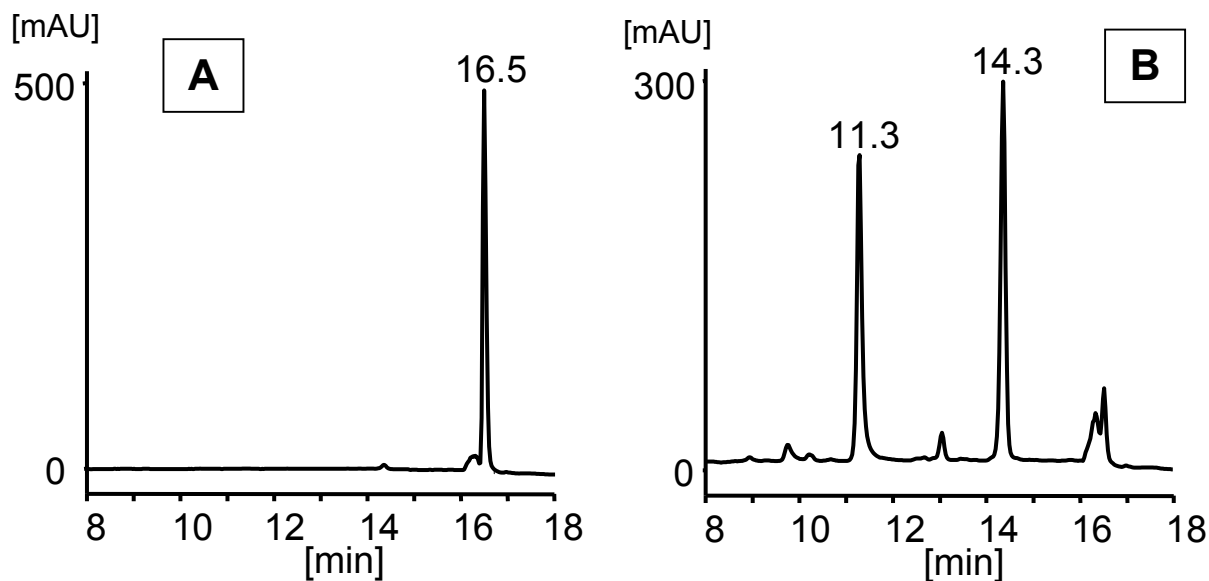
Flow:	0.8 ml/min (post column split 1:10)
Solvents:	A: Ammonium acetate buffer (pH = 3.5, 5 mmol ammonium acetate, 1% glacial acetic acid) B: Acetonitrile
Column:	Nucleodur RP-18, 70x4 mm i.d., 5 µm particle size, Macherey&Nagel (Düren, D)
Gradient:	0% Acetonitrile to 100% in 9 min
Injection volume:	10 µl
Run Time / Post Time:	9.5 min / 3.5 min
Ion source:	APCI
Interface Temperature:	400°C
Ionisation mode:	Negative, multiple reaction monitoring or product ion scans of deprotonated molecules (M-H) <sup>-</sup>

**Results and discussion**

After the reaction mixture was injected into the LC/UV system, 2 clearly separated peaks, retention times 11.3 and 14.3 min, were observed. Compared to parent compound fluticasone-17-propionate, retention time 16.5 min, the products are more polar (*Fig. 2*).

LC-MS/MS analysis, positive ionisation mode of both fractions revealed the presence of the carboxy metabolite in the fraction collected from 13.7 – 15.3 min. The product ion scan of the protonated molecule at  $m/z$  453 gave rise to characteristic product ions at  $m/z$  415 (- HF - H<sub>2</sub>O) and  $m/z$  359 (-C<sub>3</sub>H<sub>4</sub>O), see *Fig. 4*.

Due to poor recovery of FPCM with liquid-liquid extraction at pH 9.6, screening for this metabolite is not possible with the routine procedure for glucocorticosteroids. The extraction yield of FPCM in native human urine after solid phase extraction with Serdolit PAD I appeared to be better and was estimated with 98%. Therefore, the inclusion of FPCM for the detection of misuses of fluticasone-17-propionate into the screening procedure for diuretics and acidic compounds is recommended [6].



**FIGURE 2: HPLC-UV chromatogram at a wavelength of 246 nm of**  
**A) fluticasone-17-propionate, 20  $\mu$ l of a methanolic solution containing**  
**100  $\mu$ g/ml and**  
**B) reaction mixture (for details and HPLC conditions see text).**

The analysis of these compounds is routinely done by liquid chromatography coupled to a API 2000 triple quadrupole mass spectrometer with negative, multiple reaction monitoring. The product ion scan of the deprotonated molecule at  $m/z$  451 ( $M-H$ )<sup>-</sup> of FPCM generated significant fragment ions at  $m/z$  395, 377, 329 and 77 (Fig. 5). The most predominant fragment ion at  $m/z$  73 was selected for screening purposes. For confirmation, the fragmentation pattern provided further suitable ion transitions at  $m/z$  457/73, 451/329 and 451/395. Satisfactory results for the investigations of robustness and specificity were obtained; the limit of detection was estimated with 3 ng/ml via signal to noise ratio 3:1. This method was found to be adequate for the detection of misuse of fluticasone-17-propionate and has been applied for screening and confirmation expeditiously for all in-competition samples in our laboratory.

Shortly after implementation, a routine doping control sample was found to be suspicious for FPCM after screening. The presence of FPCM could be confirmed according to the requirements of WADA's technical document *Identification criteria for qualitative assays incorporating chromatography and mass spectrometry* [7], (results see Table 2).

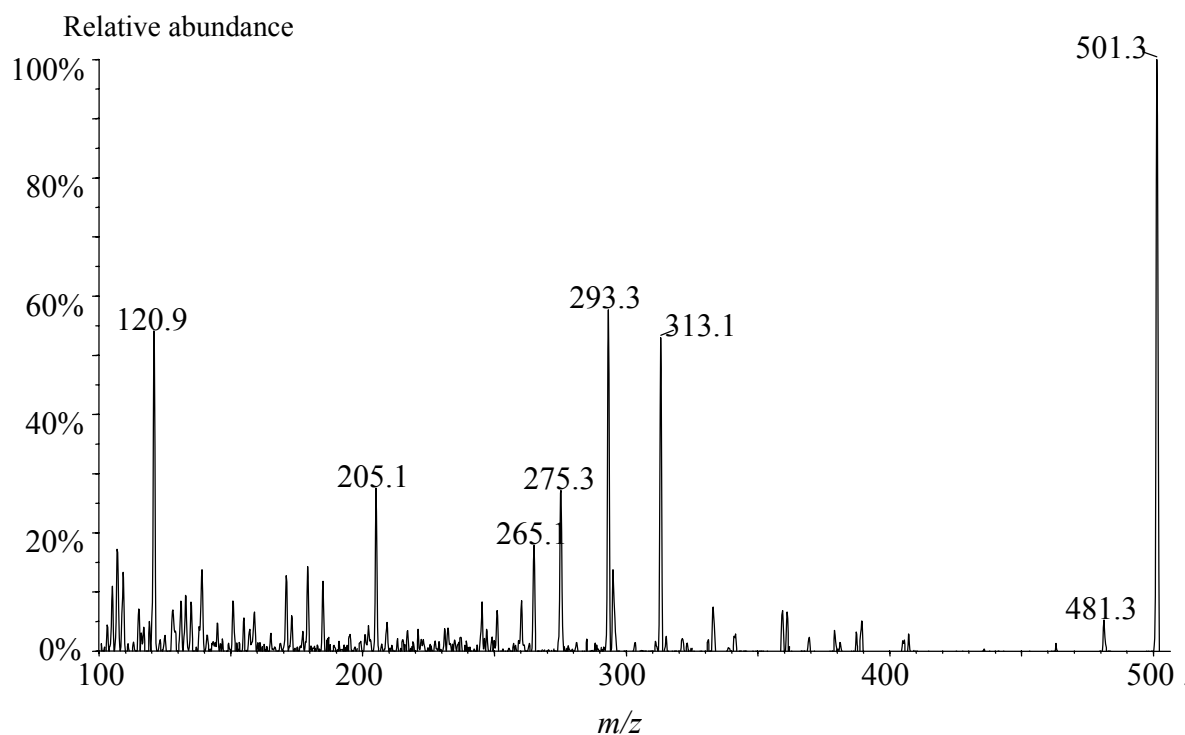
**TABLE 2: Identification of 6 $\alpha$ ,9-difluoro-16 $\alpha$ -methyl-17-propoxy-androsta-1,4-dien-11 $\beta$ -ol-3-one-17 $\beta$ -carboxylic acid in routine sample 1098**

Reference standard FPCM (10 ng/ml)					Sample 1098		
R.T. (min)	m/z	Height (cts)	Relative Abundance (%)	Acceptance Range	Relative Abundance (%)	Height (cts)	R.T. (min)
5,55	451-73	64938	100		100	44613	5,54
	451-329	7992	12,3	2,3-22,3	12,0	5373	
	451-395	7326	11,3	1,3-21,3	11,5	5141	

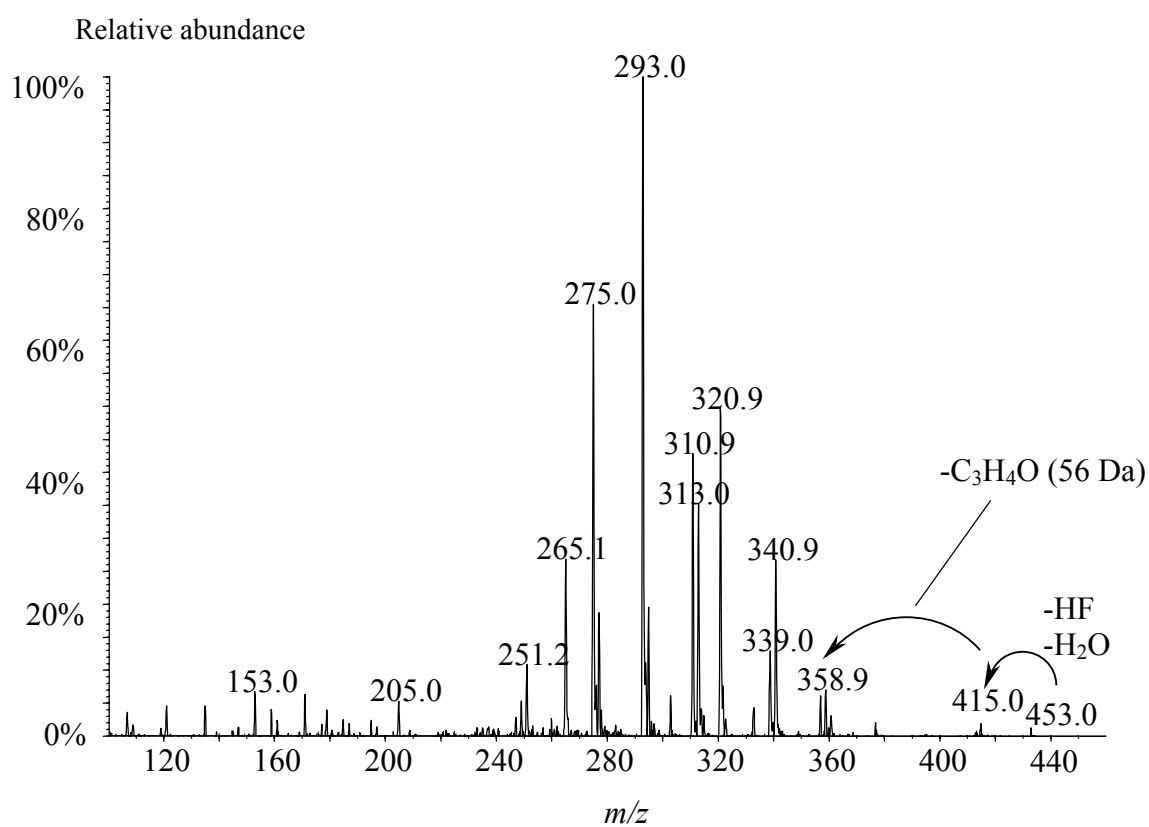
The corresponding chromatograms of sample 1098, blank urine and reference compound FPCM at a concentration of 10 ng/ml are shown in *Fig. 6*. The concentration of FPCM in sample 1098 was estimated roughly to be 7 ng/ml.

### References

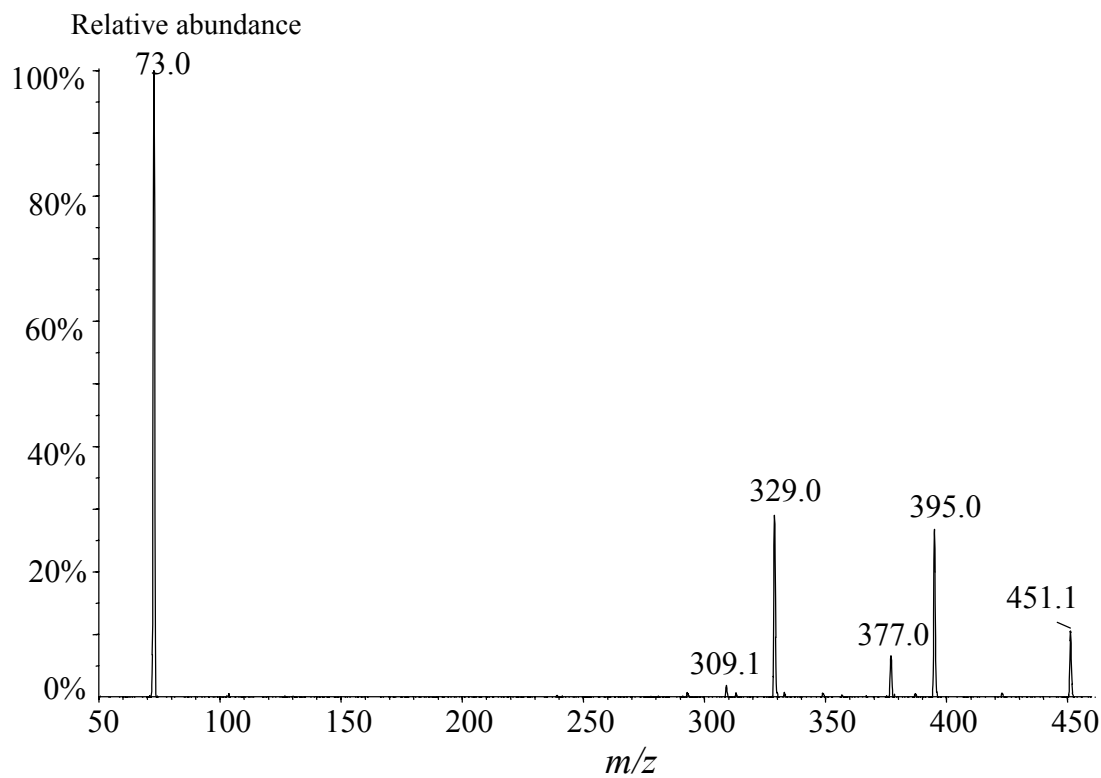
- [1] World Anti-Doping Agency. The 2005 Prohibited List. International Standard, Montreal 2005 [http://www.wada-ama.org/rtecontent/document/list\\_2005.pdf](http://www.wada-ama.org/rtecontent/document/list_2005.pdf) (access date 10.02.2005)
- [2] World Anti-Doping Agency. International Standard for Therapeutic Use Exemptions, Montreal 2005 [http://www.wada-ama.org/rtecontent/document/international\\_standard.pdf](http://www.wada-ama.org/rtecontent/document/international_standard.pdf) (access date 10.02.2005)
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- [7] World Anti-Doping Agency. Identification criteria for qualitative assays incorporating chromatography and mass spectrometry, TD2003IDCR, Montreal 2004 [http://www.wada-ama.org/rtecontent/document/criteria\\_1\\_2.pdf](http://www.wada-ama.org/rtecontent/document/criteria_1_2.pdf) (access date 10.02.2005)



**FIGURE 3:** Product ion scan of the protonated molecule  $m/z$  501 ( $M+H$ )<sup>+</sup> of fluticasone-17-propionate



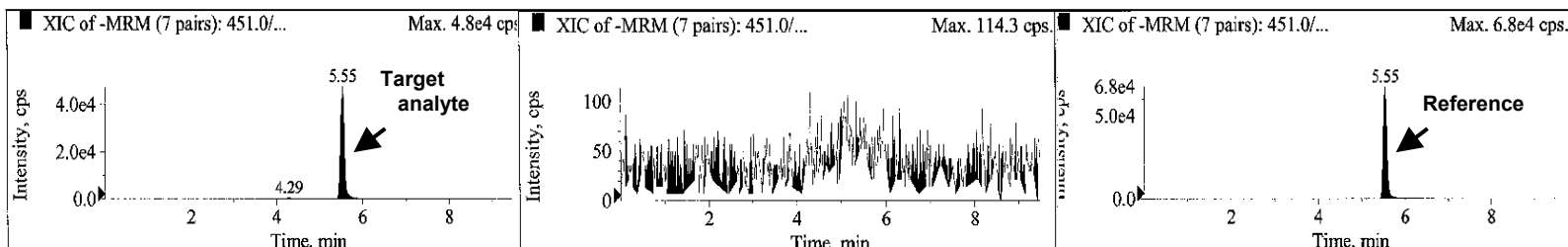
**FIGURE 4:** Product ion scan of the protonated molecule  $m/z$  453 ( $M+H$ )<sup>+</sup> of 6 $\alpha$ ,9-difluoro-16 $\alpha$ -methyl-17-propoxy-androsta-1,4-dien-11 $\beta$ -ol-3-one-17 $\beta$ -carboxylic acid



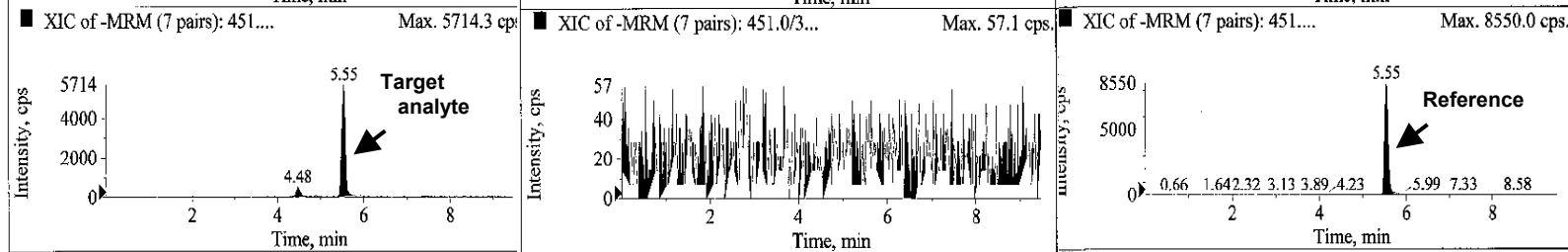
**FIGURE 5:** Product ion scan of the deprotonated molecule  $m/z$  451 ( $M-H$ )<sup>-</sup> of 6 $\alpha$ , 9-difluoro-16 $\alpha$ -methyl-17-propoxy-androsta-1,4-dien-11 $\beta$ -ol-3-one-17 $\beta$ -carboxylic acid



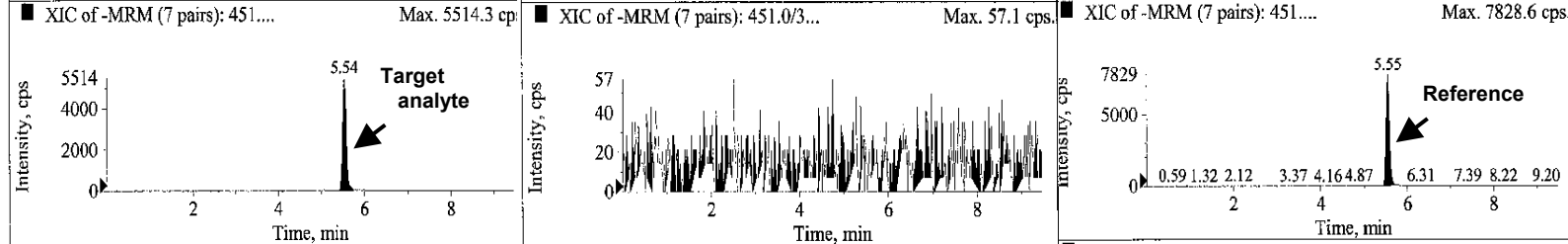
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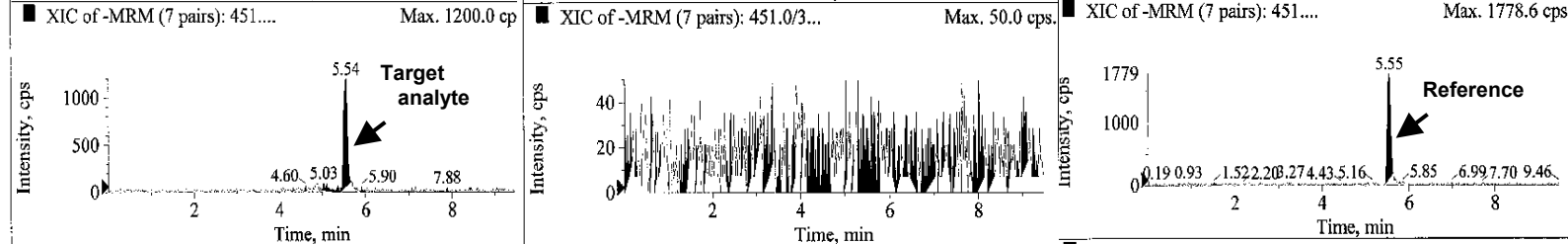
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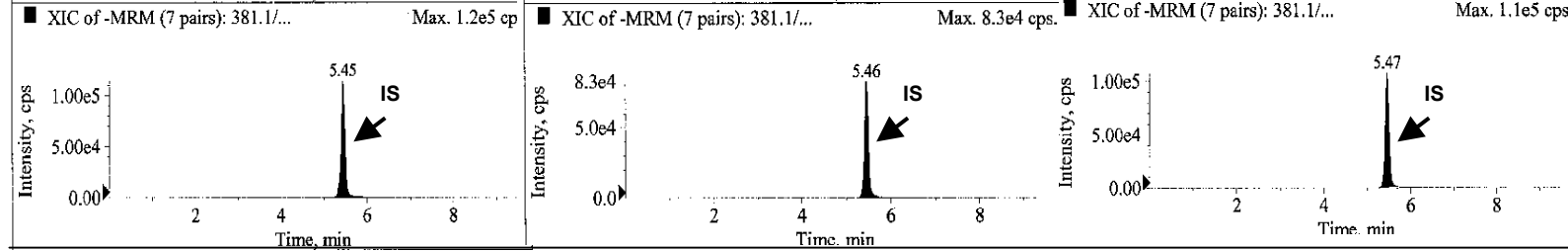
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**FIGURE 6:** A) Doping control sample with mefruside as internal standard [IS],  
 B) Blank urine sample with mefruside as IS,  
 C) Blank urine spiked with FPCM (10 ng/ml) and mefruside as IS.