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## **Excretion Study: Betamethasone-17,21-propionate Ointment and Possible Metabolites in Man Analyzed with LC-ESI-MS/MS**

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### **Introduction**

Betamethasone is a glucocorticosteroid enclosed in the WADA/IOC-list for banned substances [1]. It is allowed for out-of-competition use and, with a therapeutic use exemption (TUE), also for selected application forms (i.e. inhalative) for in-competition use [5]. Betamethasone in its various application forms is one of the most frequently used corticosteroids in man. So the aim of this study was to investigate which concentrations of betamethasone and its possible metabolites [2] in human urine are detectable after local therapeutic application of a betamethasone-17,21-dipropionate (BDP) ointment. Assumed metabolites were: 6 $\beta$ -hydroxybetamethasone, betamethasone-17-propionate, betamethasone-21-propionate and betamethasone. Both monopropionates had to be synthesized by partial alkaline hydrolysis.

### **Experimental**

#### *Excretion study*

- (1) *Basics*: The study was performed with *Soderm® Plus* ointment supplied by Dermapharm GmbH, Grünwald, Germany, containing 0.64 mg of BDP per gram. Patient was a 53 years old male with 78 kg of bodyweight. After collection of a blank urine sample, the ointment was administered four times with ~0.5 g (therapeutic dose) lateral at left knee-joint at 0h, 9h, 20h and 32h (all in all 2.2 g consistent with 1.41 mg of BDP). Every urine was sampled. Last specimen was obtained 125h post administration.
- (2) *Preparation and analysis*: Samples were prepared according to established procedures [3] and analyzed with LC-MS/MS (parameters shown in Table 1). New glass tubes were used to avoid contaminations. In addition to the samples of the excretion study, urine specimens spiked with all involved substances (0.2, 0.5, 2 and 5 ng/mL of urine) were analyzed for calibration and quantitation purposes.

#### *Preparation, purification and characterization of betamethasone-monopropionates*

- (1) *Preparation*: Fourty  $\mu$ mol (20 mg) of BDP were dissolved in 2 mL of distilled methanol. Then, a volume of 0.1 mL of a 1M sodium hydrogen carbonate solution as well as 0.5 mL of deionized water were added (pH ~8). The hydrolysis proceeded at room temperature. After 120 min, an optimum

concerning the yield of both monopropionates was reached.

- (2) *Purification*: The reaction mixture was purified on a HP 1090 liquid chromatograph with diode array detector at  $\lambda = 246$  nm using a semi-preparative column (VP 250/10 Nucleosil 100-7 C18, Macherey-Nagel, Düren, Germany). Solvents were: A = deionized water and B = acetonitrile. The gradient started at 5 % B increasing to 50 % B in 3 min and proceeded to 90 % B in 15 min.
- (3) *Characterization*: Owing to identical molecular weights of the monopropionates (448 u), a distinction by means of retention time and fragmentation pattern was required. A difference of retention times was small but visible. Because of missing commercial reference material, the fragmentation of the prepared and purified substances was compared with that of similar, commercially available esters (betamethasone-17-valerate and betamethasone-21-acetate, Fig.1 C and D).

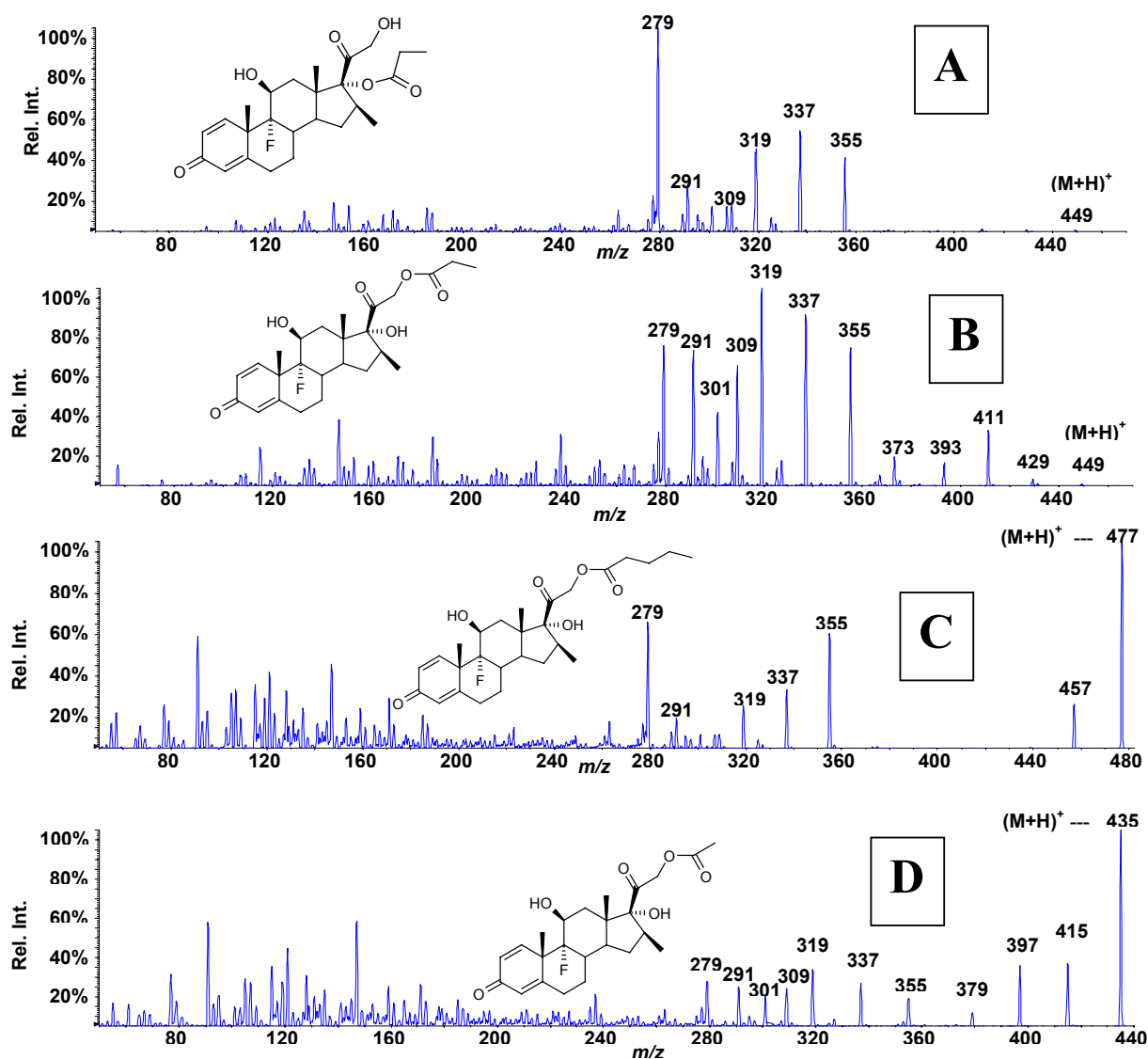


Figure 1: ESI product ion spectra of betamethasone-17-propionate (A) and -21-propionate (B) (mol. wt. = 448 each), obtained at a collision offset voltage of 25V, betamethasone-17-valerate (C) (mol. wt. = 476) and -21-acetate (D) (mol. wt. = 434), obtained at an average collision offset voltage of 45V.

## Results and discussion

The mass spectrometric experiments showed that the 21-esters as well as the 17-esters have specific ESI-fragmentation pathways in the higher  $m/z$  region, which can be used for differentiation (Fig. 1 A and B). Both groups of esters initially lose hydrogen fluoride (-20u, HF) to yield the product ion at  $m/z$  429. The following elimination of water ( $m/z$  429 to 411) is clearly visible in spectra of the 21-esters (Fig. 1 B and 2 right spectrum) but barely in those obtained from the 17-esters (Fig. 1 A and 2 left spectrum). The latter show a gap of fragment ions between  $m/z$  411 and 355, which is part of the degradation of the betamethasone nucleus. Apparently the 17-esters preferentially lose the carboxylic acid ( $m/z$  429 to 355), while the 21-esters eliminate the ester-group in form of (alkyl-)ketene and water. Although the 17-esters show indications for the loss of water as second fragmentation step (followed by the release of alkyl-ketene or ketene), their abundance is marginal (Fig. 3 left spectrum). These assumptions are supported by spectra of other betamethasone-esters (Fig. 1 C and D). Additional MS<sup>3</sup>-experiments (spectra not shown here) indicate that the fragment  $m/z$  393 is not identical with (betamethasone + H)<sup>+</sup> (see also Fig. 4).

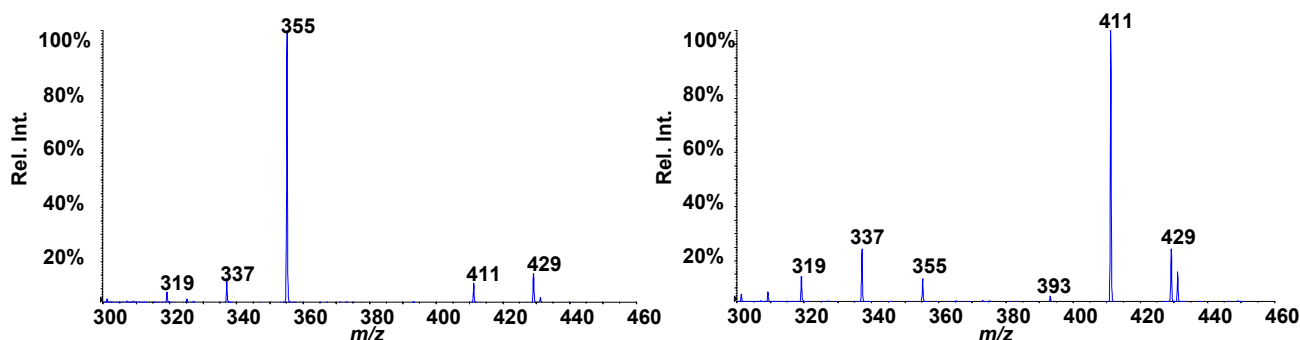


Figure 2 ESI mass spectrum of betamethasone-17-propionate (left side) and betamethasone-21-propionate (mol. wt. = 448 each), obtained in MS<sup>3</sup> mode. Precursors were  $m/z$  449 and 429 (collision offset voltage = 15V, AF2 = 20).

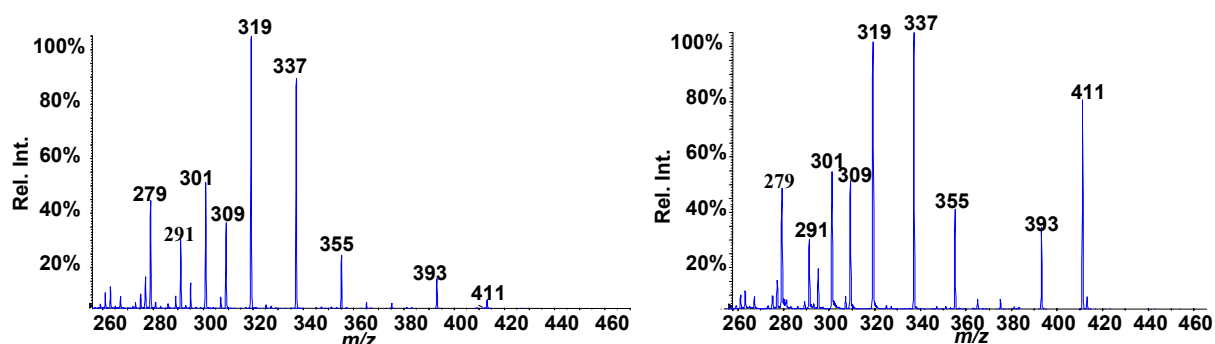
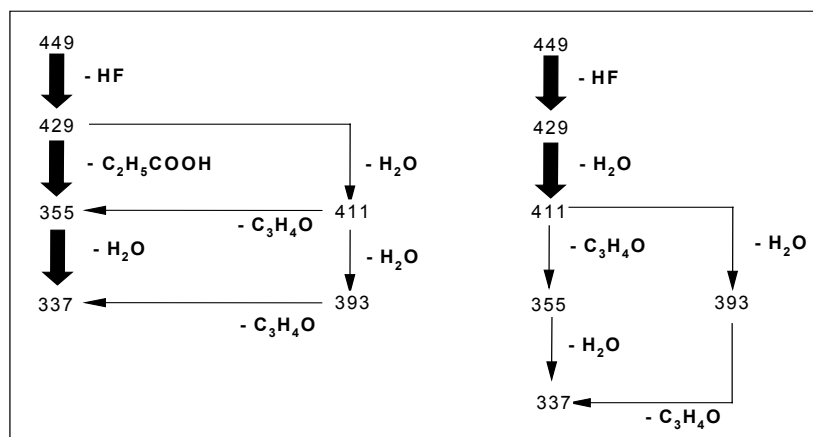


Figure 3 ESI mass spectrum of betamethasone-17-propionate (left side) and betamethasone-21-propionate (mol. wt. = 448 each), obtained in MS<sup>3</sup> mode. Precursors were  $m/z$  449 and 411 (collision offset voltage = 15V, AF2 = 35).

Due to the fact that betamethasone is one of the most common corticosteroids in man, the aim of this study was to investigate the main metabolites of BDP. Preparation of putative metabolites was required to explore their mass spectrometric behavior, as they were not commercially available. The mass spectrometric analysis of various betamethasone-esters showed a difference in fragmentation routes between

17-esters and 21-esters, which may be used to differentiate esters with identical molecular weight. A linear calibration for all investigated substances was obtained, and the LOD was estimated between 0.01 and 0.1 ng/mL. In spite of the very low LOD, no substances were detected in the specimens of the excretion study performed with the BDP ointment (according to the results of other studies [4]). Thus, a positive case in human doping analysis after therapeutic application of BDP ointment seems to be fairly improbable. The results of this study demonstrate the benefit of mass spectrometric use in dope analysis.



**Figure 4** Proposed fragmentation route of betamethasone-17-propionate (left) and -21-propionate (right)

**Table 1** Analytical parameters of LC-MS/MS measurements of excretion specimens

Hewlett Packard HP1100 liquid chromatograph coupled to Applied Biosystems API 4000 QTrap mass spectrometer	
Analytical column:	Agilent Zorbax SB-C18, 150 x 4.6 mm, 3.5 µm particle size
Flow:	0.8 mL/min (splitless)
Solvents:	A: Ammonium acetate buffer (pH = 3.5, 5 mmol ammonium acetate, 1 % glacial acetic acid) B: Acetonitrile
Gradient:	10% Acetonitrile to 90% in 18 min + 1 min isocratic at 90%
Injection volume:	20 µL
Run Time / Post Time:	19 min / 3.5 min
Ion source:	ESI
Interface Temp.:	350°C
Ionisation mode:	Positive, multiple reaction monitoring of protonated molecules (M+H) <sup>+</sup>
Dwell time:	60 ms

## References

- [1] World Anti Doping Agency, The 2005 Prohibited List, Montreal, 2005
- [2] Nakano M., Nishiuchi M., Takeuchi M., Yamada H. *Steroids*, 1981, **37**: 511-25
- [3] Gotzmann, A., Bredehöft, M., Thevis, M., Schänzer, W. Detection of prednisolone, prednisone and 20-dihydroprednisolone in human and equine urine by means of LC/MS/MS. In: Schänzer, W., Geyer, H., Gotzmann, A., Mareck, U. (eds) *Recent Advances in Doping Analysis (11)*. Sport und Buch Strauß, Köln 2003: 33-41
- [4] Deventer, K., Delbeke, F.T. Excretion studies with corticosteroids. In: Schänzer, W., Geyer, H., Gotzmann, A., Mareck, U. (eds) *Recent Advances in Doping Analysis (12)*. Sport und Buch Strauß, Köln 2004: 37-4
- [5] World Anti Doping Agency, „International Standard for Therapeutic Use Exemptions, Montreal 2005