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Doping control analysis of desmopressin in human plasma and urine by HPLC-ESI-MS/MS

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Abstract

In this paper, the qualitative detection of desmopressin in human plasma and urine by high-performance liquid chromatography/electrospray tandem mass spectrometry (LC-ESI-MS/MS) is presented. Sample preparation in urine consisted of solid phase extraction with weak cation exchange cartridges (WCX) after a delipidation step with a 60:40 di-isopropyl ether/*n*-butanol. WCX extraction from plasma was performed after protein precipitation with ammonium sulphate. Lower limits of detection were respectively 50 pg/mL in plasma and 25 pg/mL in urine. The method was tested by analyzing plasma and urine samples obtained after desmopressin administration via intravenous, oral and intranasal routes. In plasma, desmopressin could only be detected after intravenous application. In urine, all administration routes resulted in unambiguous detection.

Introduction

Desmopressin (1-desamino-8-D-arginine-vasopressin, dDAVP) is a synthetic derivative of the endogenous peptide hormone arginine vasopressin (AVP), that plays an important role in homeostasis, by regulating water, glucose, and salts in the blood. Desmopressin, whose structure is depicted in Figure 1, was developed with the aim of having a longer lasting and more potent antidiuretic effect than AVP, which is not suitable for therapeutic use because of unfavorable pharmacokinetic properties [1]. Administration of desmopressin after EPO intake provokes rapid hemodilution, which causes a decrease in hematocrit, an indirect marker of erythropoietins (EPOs) misuse and blood doping [2]. Therefore, desmopressin was added to the World Anti-Doping Agency (WADA) Prohibited List as a masking agent [3].

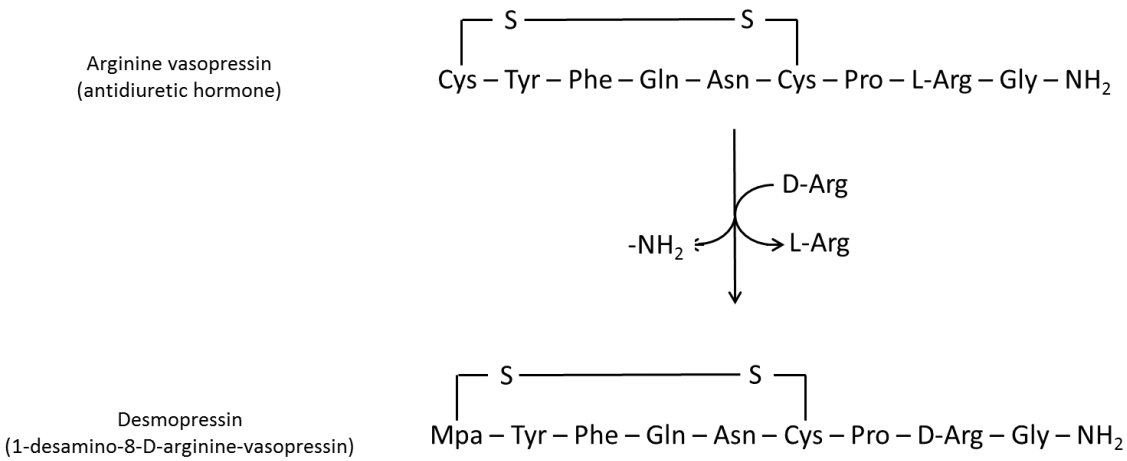

Figure 1: Desmopressin; compared with arginine vasopressin, desmopressin's Cys1 has been deaminated, and the Arg8 is in the dextro form.

 ISBN 978-3-86884-038-4

Recently, the detection of desmopressin in urine after intranasal and oral administration by using a hybrid quadrupole/time-of-flight (Q-TOF) mass spectrometer was described [4]. In the present study, a liquid chromatographic triple quadrupole tandem mass spectrometry method to detect desmopressin in plasma and urine is presented and was applied to the detection of desmopressin after intravenous, oral and intranasal administration.

Experimental

Plasma sample preparation

2 mL of plasma were spiked with 50 µL of [deamino-Cys^{1}, Val^{4},D-Arg^{8}]-vasopressin (50 ng/mL) as internal standard. Then, 2 mL of saturated $(NH_4)_2SO_4$ solution, containing 2% NH_4OH, were added and the samples were centrifuged (4100 *g*, 1 h, 2°C) to precipitate plasma proteins. Then, solid phase extraction (SPE) of the supernatant was performed with WCX (60 mg) cartridges. The column was activated with 2 mL of MeOH and 2 mL of H_2O. After sample loading, the column was washed first with 2 mL of 5% NH_4OH aqueous solution, then with 2 mL of 60:40 H_2O:MeOH mixture. Finally, samples were eluted with 1.25 mL of 80:20 MeOH:(5% HOAc in H_2O). The eluate was subsequently evaporated to dryness and then dissolved in 40 µL of 95:5 H_2O:ACN, 0.1% HOAc, 0.01% TFA prior to LC-MS analysis.

Urine sample preparation

3 mL of urine were spiked with 2 ng of internal standard. After acidifying the samples (pH= 3.5-4.0) with 500 µL of 5% HOAc aqueous solution, they were delipidated by adding 5 mL of di-isopropyl ether:*n*-butanol 60:40 solution, vortexing (30 seconds) and centrifuged (4100 g, 20 minutes, 5°C). The upper layer was discarded and a second delipidation step with di-isopropyl ether:*n*-butanol 60:40 was performed under the same conditions but with a basic pH (9.5-10.0) after addition 500 µL of ammonia buffer. After centrifugation, the upper layer was discarded again. Delipidated urines were then processed by SPE, evaporated and analyzed by LC-MS/MS with the same procedure described for plasma.

LC-MS/MS

A Surveyor MS Pump Plus coupled with a Surveyor Plus autosampler (Thermo Scientific, Bremen, Germany) was used, equipped with a Zorbax 300SB-C18 reverse-phase column (1.0 x 50 mm, 3.5 µm) protected with a C8 guard column. 30 µL were injected using a binary gradient with a constant flow rate of 50 µL/min was used: mobile phase A consisted of H_2O, 0.1% HOAc, 0.01% TFA; mobile phase B consisted of ACN, 0.1% HOAc, 0.01% TFA. Gradient elution was as follows: 95% A for 1.5 minutes, then decreased linear to 0% A in 8.5 minutes, and held at 0% A for 5 minutes, followed by an increase to 95% A in 0.1 minutes and, finally, system riequilibration in 10 minutes.

MRM detection on a TSQ Quantum Discovery Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, Bremen, Germany) was performed in positive mode using product ions at *m/z* 328.0 and *m/z* 120.0 both from the precursor ion at *m/z* 535.5 were used for desmopressin. For the internal standard, the transition *m/z* 542.7 → *m/z* 328.0 was used.

Validation

10 different blank human plasma and urine samples were spiked at different levels (0, 10, 25, 50, 100, 200 pg/mL) to determine limit of detection (LOD), for both matrices. Specificity, extraction recovery and matrix effect were also tested during the validation procedure.

Excretion studies

Six whole blood and urine samples from six patients who received desmopressin were used in the study. Three patients received desmopressin intranasally (~10 mg/dose), two orally (200 mg) and one intravenously (20 mg).

Results and Discussion

LC-MS/MS

Chromatographic separation was achieved by using a C18 column, which allowed satisfactory peak shapes and chromatographic retention for desmopressin and the internal standard. Using this column, a high percentage (90%) of ACN was required to elute desmopressin and the IS.

The full scan mass spectrum of desmopressin exhibited an abundant $[M + 2H]^{2+}$ ion (Figure 2a), whereas the monocharged pseudomolecular ion $[M + H]^{+}$ has approximately half of the intensity. The product ion spectrum of *m/z* 535.5 showed several ions (Figure 2b) with good intensities (*m/z*= 328.0, 120.0, 214.0, 276.1, 430.4 and 526.7).

ISBN 978-3-86884-038-4

Two precursor-product ion transitions were selected for the MRM detection (*m/z*= 328.0, 120.0 from parent ion *m/z*= 535.5), according to the identification criteria for mass spectrometry-based qualitative assay recommended by WADA [5]. For the IS and the carrier peptide only the most abundant transition was selected (respectively *m/z*= 328.0 from precursor ion *m/z*= 542.7, and *m/z*= 328.0 from precursor ion *m/z*= 520.8).

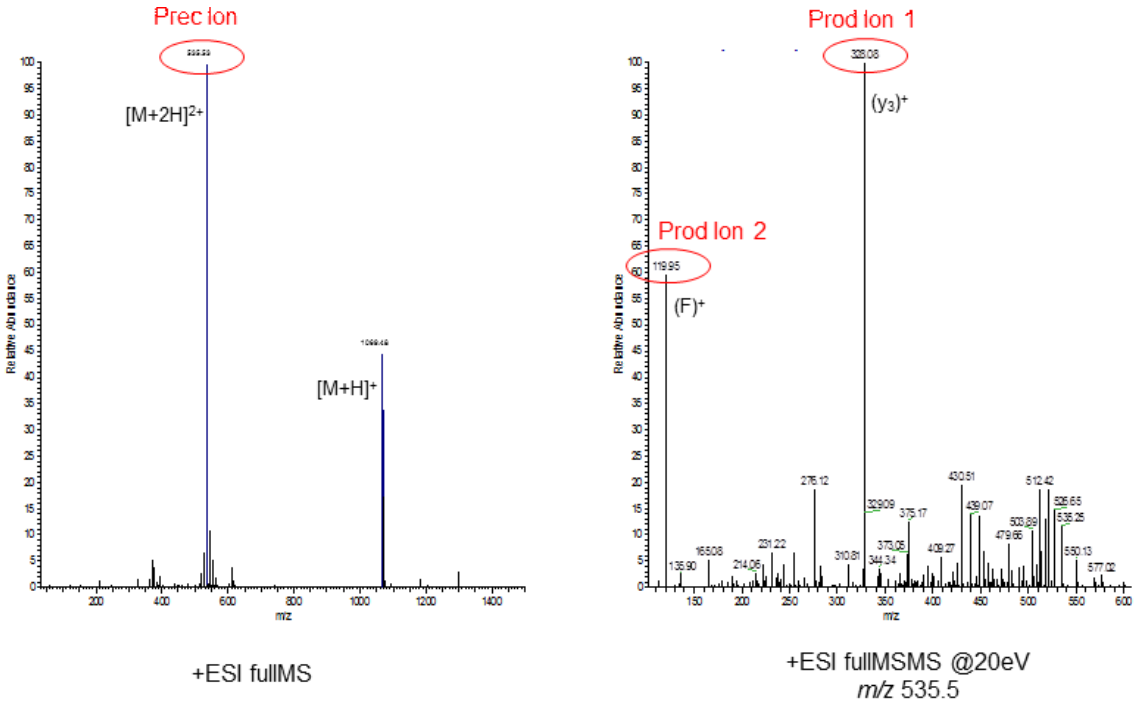

Figure 2: Full-scan MS of desmopressin (a), and product ion scan for the parent ion $[M + 2H]^{2+}$ (b).

Plasma sample preparation

For the optimization of plasma protein precipitation, of acetone, methanol, saturated $(NH_4)_2SO_4$ and ethanol were evaluated. Deproteination of the samples using saturated $(NH_4)_2SO_4$, gave the best results regarding S/N response. SPE was considered to further concentrate and desalt the samples. In particular, WCX were investigated, because desmopressin is a basic peptide with a pKa value of approximately 12. Since the WCX protocol requires a basic pH to load the samples on the SPE column, the $(NH_4)_2SO_4$ saturated solution was basified with NH_4OH (pH 9.2).

Urine sample preparation

Using the WCX cartridges protocol as a stand-alone sample preparation technique; the product ion at *m/z*= 328.0 was clearly detectable at 100 pg/mL. Unfortunately, the second most abundant ion at *m/z*= 120.0 was highly interfered (Figure 3a). To obtain this latter transition with improved sensitivity and specificity, a further purification step was needed in addition to the WCX cartridge. A previous method for plasma delipidation without protein denaturation was adapted [6]. The adapted procedure consists of a liquid-liquid extraction of the lipids with a mixture of di-isopropyl ether:*n*-butanol 60:40 at a volume ratio of 2:1, pH 5.2 and the other extraction was performed at pH 9.5 to remove both acidic and basic interferences. The sample delipidated before SPE (Figure 3b) clearly showed a better signal for both transition compared to the same sample purified only with SPE. The increase in response for the product ion at *m/z*= 328.0 was moderate but still relevant, whereas the quality of the peak chromatogram was dramatically improved for the product ion at *m/z*= 120.0.

Validation

The methods showed an LOD of 50 pg/mL for plasma and 25 pg/mL for urine, with signal to noise ratio greater than 3 for the two diagnostic transitions in all urine samples that were analyzed. Maximum tolerance windows (% of base peak *m/z*= 328.0) were put ±10% (absolute) for *m/z*= 120.0, according to the WADA identification criteria.

ISBN 978-3-86884-038-4

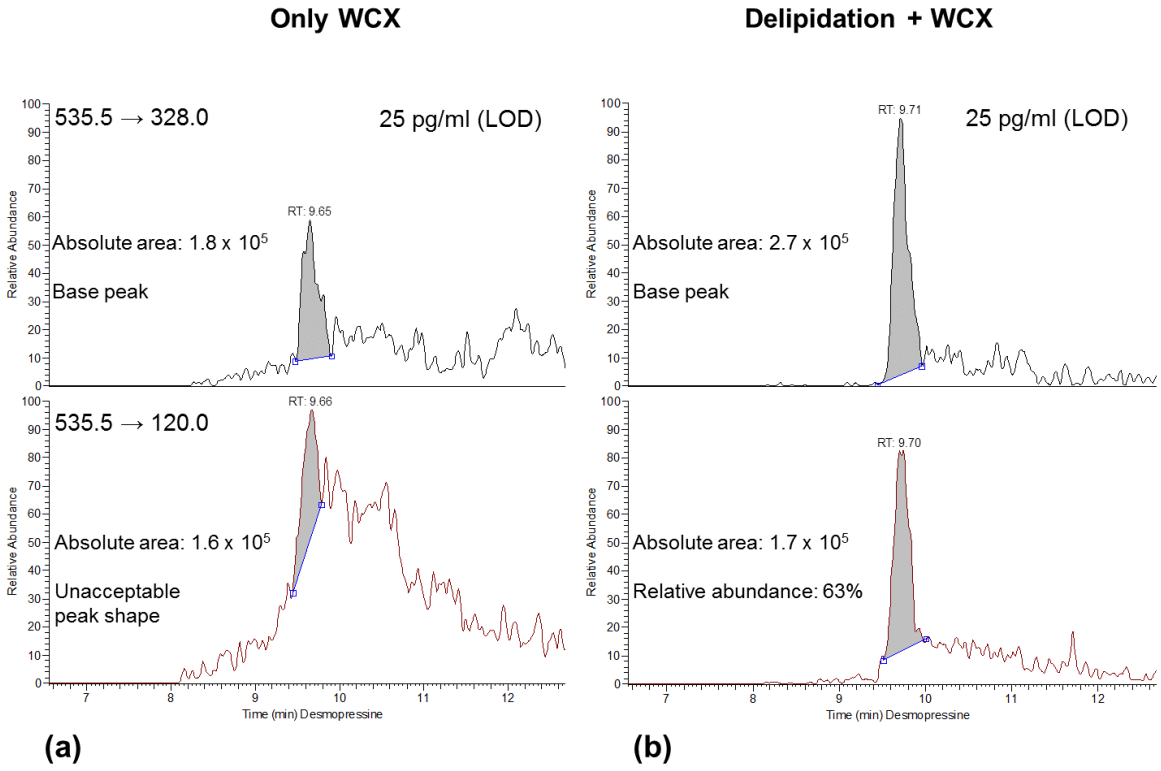

Figure 3: MRM extracted ion chromatograms of the product ions at m/z 328.0 and m/z 120.0 from the parent m/z 535.5 for a urine sample fortified with desmopressin at the limit of detection processed only with WCX cartridges and with the combination of delipidation and WCX.

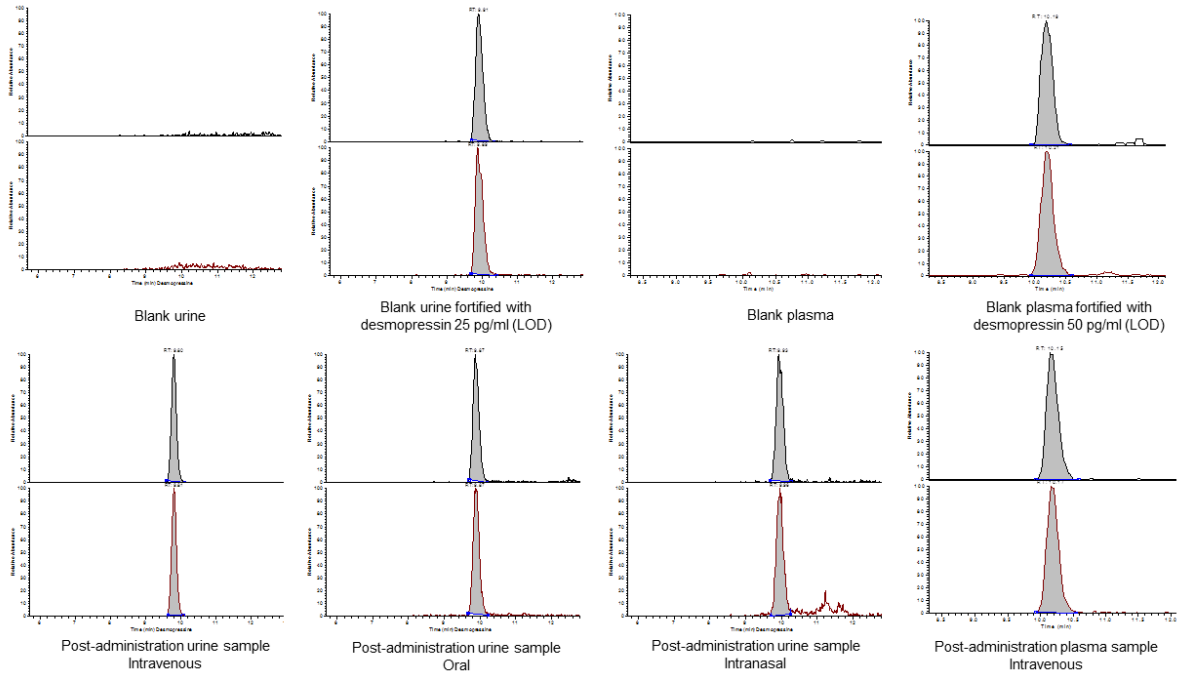

Figure 4: MRM extracted ion chromatograms of the diagnostic product ions at m/z 328.0 and m/z 120.0; desmopressin was unambiguously detected in urine after intravenous, oral and intranasal administration, whereas in plasma was detected only after intravenous administration.

ISBN 978-3-86884-038-4

Extraction recovery and ion suppression
MRM extracted ion chromatogram from a pure reference standard, a sample spiked prior to injection and a sample spiked before extraction were compared. According to the abundances recorded, the average extraction recovery of the preparation was estimated respectively at 40.3% (SD= 13.4) for plasma and 59.3% (SD= 29.4) for urine. *Signal reduction due to ion suppression* in the ESI source was calculated as 5.1% (SD= 14.0) for plasma and 42.7% (SD= 12.9) for urine.
Method application
Plasma and urine samples were collected at the same post-administration time from each patient. Results are summarized in Table 1 and shown in Figure 4 as extracted ion chromatograms of product ions at *m/z* 328.0 and 120.0. Desmopressin was unambiguously detected in all urine samples collected after administration. In plasma, desmopressin was detected only after intravenous administration.
The high elimination rate from blood circulation of desmopressin and the low bioavailability of oral and intranasal formulations were probably the main causes of the non-detection of this compound in plasma after oral and intranasal administration. Urine must therefore be considered the matrix of choice for doping control analysis of desmopressin.

Conclusions

Detection of desmopressin in human plasma and urine by LC-MS/MS has been successfully achieved. In particular, results of the excretion studies demonstrate that urine is the preferred matrix for the detection of desmopressin misuse in sport after oral, intranasal or intravenous administration, whereas desmopressin can realistically be detected in plasma only after intravenous administration.
Additionally, delipidation of urine samples resulted to be a useful tool to improve urine clean-up for the urinary detection of other peptides as well. More details on both methods are available in the full papers [7,8].

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