K. Deventer[1], S. Esposito[1], D. De Boer[2], O.J. Pozo[3], G. Tsjoen[4], F.T. Delbeke[1], P. Van Eenoo[1]

# Is a simple detection approach possible for desmopressin?

1) Doping Control Laboratory (DoCoLab), Ghent University-UGent, Technologiepark 30, B-9052 Zwijnaarde, Belgium

2) Department of Clinical Chemistry, University Hospital Maastricht, Maastricht, The Netherlands

3) Institut Municipal d'Investigacio Medica (IMIM), Unitat de Farmacología, Barcelona, Spain

4) Department of Endocrinology–Andrology, Center for Sexology and Gender problems, Universitair Ziekenhuis Gent, De Pintelaan 185 9000 Gent Belgium

## Introduction

Desmopressin is a small cyclicpeptide with a molecular weight of 1068 Da and is a synthetic analogue for arginine-vasopressin (AVP), the hormone that regulates urine production (Table 1). Due to the increased retention of water hemodilution can occur. This hemodilution can hamper the detection of blood doping which is generally monitored by blood-concentration dependent parameters like hematocrit and the absolute number and permillage of reticulocytes [1].

**Table 1**: vasopressin-analogues

| Compound | Exact mass (Da) | Structure |
|---|---|---|
| Arginine-vasopressin (AVP) | 1083 | Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH$_2$ |
| Desmopressin | 1068 | Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-D-Arg-Gly-NH$_2$ |
| Terlipressin | 1226 | Gly-Gly-Gly-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH$_2$ |
| Lypressin ([Lys8]-vasopressin) | 1056 | Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH$_2$ |
| [deamino-Cys1, Val4, D-Arg8]-Vasopressin | 1039 | Mpa-Tyr-Phe-Val-Asn-Cys-Pro-D-Arg-Gly-NH$_2$ |
| Oxytocin | 1006 | Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH$_2$ |

Therefore, WADA has placed this compound on the prohibited list since January 2011 [2]. Several analytical methods to detect this compound in plasma using immunoassays have been described [3-5]. In the field of doping analysis, mass spectrometric techniques are preferred for the detection of doping agents, since this technique provides both good selectivity and specificity. To the best our knowledge no LC-MS/MS method for its detection in plasma has been described so far. Therefore the aim of this work was to develop and validate a plasma detection method for desmopressin. To evaluate the performance of the method plasma samples from patients who received desmopressin acetate for the treatment of Diabetes Insipidus were analysed.

## Experimental

### Chemicals and reagents

All the chemicals and solvents used for sample preparation and chromatography were of analytical grade or HPLC grade. Desmopressin was a kind gift from Ferring-Pharmaceuticals (Malmö, Sweden). AVP and [deamino-Cys1, Val4, D-Arg8]-vasopressin were purchased from Sigma Aldrich (Saint Louis, USA). Oxytocin, lypressin ([Lys8]-vasopressin), and terlipressin were purchased from Selleck Chemicals LLC (Houston, USA). Desmopressin and analogues were all available as acetate salts. Stock and working solution were all prepared using 2% acetic acid (HOAc) aqueous solutions. Acetonitrile (ACN) and water ($H_2O$) were purchased from BioSolve (Lexington, USA); methanol (MeOH) was purchased from Fisher Scientific (Aalst, Belgium); 25% ammonia ($NH_4OH$) aqueous solution and glacial HOAc were purchased from Merck (Darmstadt, Germany); trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich.

Ammonium sulfate (($NH_4)_2SO_4$) saturated solution was prepared by dissolving 800 g of ($NH_4)_2SO_4$ (Merck, Darmstadt, Germany) in 1000 mL of $H_2O$ at room temperature. $C_{18}$-solid phase extraction cartridges were purchased from Varian (type Bond-Elut, 500 mg, 3 mL).

Only low binding 1.5 mL vials (Eppendorf, Hamburg, Germany) and low retention pipette tips (Sorenson Biosciences, Salt Lake City, USA) were utilized to store stock solutions (100 µg/mL) and prepare working solutions (10 ng/mL). AVP was always added to desmopressin working solutions at a concentration of 2.5 ng/mL, in order to prevent desmopressin adsorption to solid surfaces.

**Pharmacokinetic studies**

Six desmopressin containing EDTA/heparin/citrate plasma samples were obtained from the Department of Internal Medicine (Endocrinology and Hematology) of the Ghent University hospital, with the approval of the Ethical Committee of the Ghent University (reference: B67020108809).

Four patients received desmopressin acetate via intranasal route and two orally.

**Sample treatment**

Two mL of plasma, was fortified with 50 µL of the carrier-solution (Lypressin, 1µg/mL) followed by the addition of 200 µL of saturated $(NH_4)_2SO_4$-solution. After vortexing (30 s) and centrifugation at 4100 ×g, the supernatant was transferred to a $C_{18}$-cartridge which was conditioned with 1 mL of MeOH and 1 mL of $H_2O$. After loading the sample on the column two washing steps were applied. The first consisted of 2 mL of aqueous 1% HOAc solution and the second one consisted of 2 mL of MeOH/ $H_2O$ (1:1; v/v). Elution was performed with 1.2 mL of ACN/$H_2O$ ( 1:1; v/v). Samples were then evaporated overnight at 45 °C using an Eppendorf®-concentrator. Samples were redissolved in 40 µL $H_20$/ACN (95:5; v/v, containing 0.1% HOAc/ 0.01% TFA) containing the internal standard ([deamino-Cys1, Val4, D-Arg8]-Vasopressin, 1 ng/mL).

**Apparatus**

HPLC separation was achieved with a Surveyor MS Pump Plus coupled with a Surveyor Plus autosampler (Thermo Scientific, Bremen, Germany) using a Zorbax 300SB-$C_{18}$ reverse-phase column (1.0 x 50 mm, 3.5 µm) protected with a $C_8$ guard column. For each sample, 30 µL were injected. A binary gradient 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 min, then decreased linear to 0% A in 8.5 min, and held at 0% A for 5 min, followed by an increase to 95% A in 0.1 min. Then the system was equilibrated for 10 min before next injection (total run time: 25 min). A constant flow rate of 50 µL/min was maintained.

SRM-method development for detection of desmopressin in human plasma was performed on a TSQ Quantum Discovery Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with an ESI source operating in positive mode. The ESI–MS operating variables used in this study were as follows: capillary voltage, 3.5 kV; source

temperature, 350 °C; sheat gas pressure, 30 psi; auxiliary gas pressure, 10 psi; tube lens offset, 84 V.

**Validation**

In accordance with Eurachem validation guidelines [36], 6 human plasma samples, were spiked at different levels (100, 250, 500, 1000 pg/mL) to determine the LOD.

The detection limit was defined as the lowest level at which a compound could be identified in all 6 plasma samples with 2 diagnostic ions present with a signal-to-noise (S/N) ratio greater than 3 and a retention time difference of less than 0.2 min to the reference.

Selectivity was tested by analysing desmopressin-analogues at a concentration of 1 ng/mL.

The analogues included the endogenous hormones AVP and oxytocin, and the synthetic derivatives [deamino-Cys1, Val4, D-Arg8]-vasopressin, lypressin ([Lys8]-vasopressin), and terlipressin.

Specificity was tested during the validation procedure. The 6 blank plasma samples used for determining detection capability were extracted and analysed as described above.

To evaluate the extraction recovery, the 6 plasma samples, used for the validation, were spiked with desmopressin at 50 pg/ml and processed together with non-spiked plasma samples. The extracts of the non-spiked plasma samples were spiked after evaporation and before HPLC injection. After analysis the obtained peak areas of the two sets of samples were compared.

## Results

### Mass spectrometry

Direct infusion experiments of desmopressin yielded a very abundant double charged ion at $[M+2H]^{+2}$ (Figure 1).
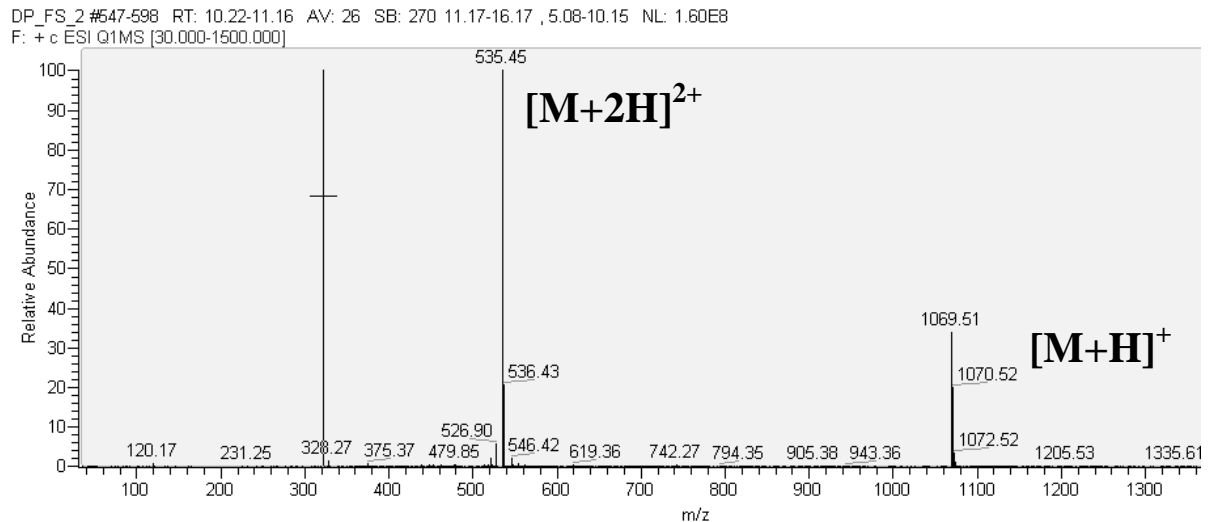


**Figure 1:** Full scan MS spectrum of desmopressin

This high intensity is directly related to the high ionization efficiency due to the presence of an arginine residue (Arg8), containing a very basic guanidinium group (pKa ≈ 12). The single charged ion $[M+H]^{+}$ only achieved a relative abundance of 30% (Figure 1). The collision-induced dissociation of desmopressin base peak (m/z 535.5) led to the formation of several fragments. Fragment ions at m/z 328.0 and m/z 120.0 were selected as diagnostic (Figure 2).
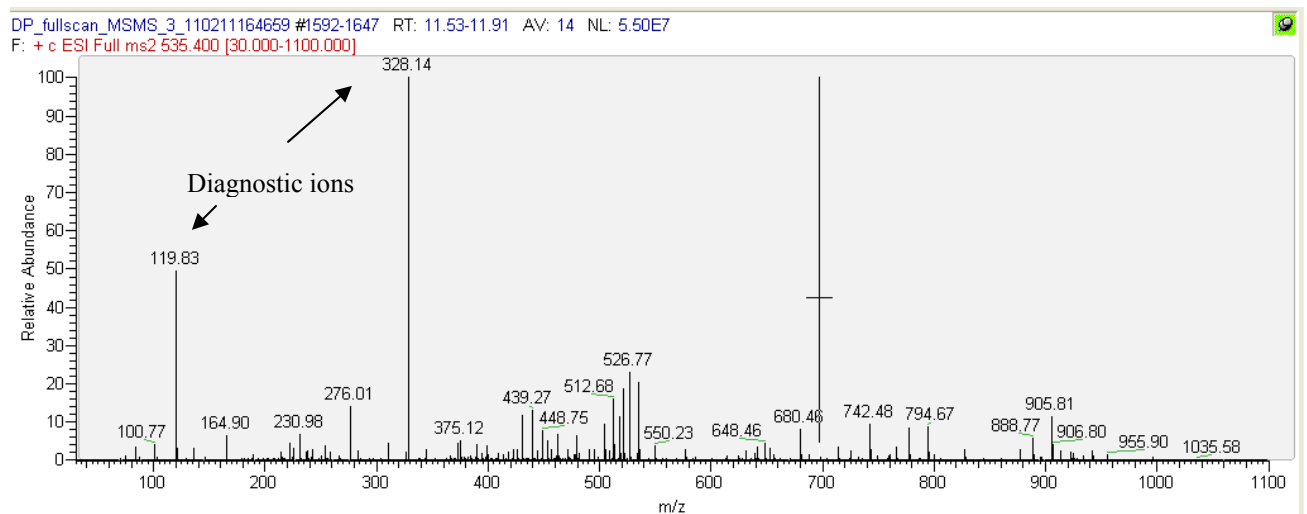


**Figure 2:** Full scan MS/MS spectrum of $[M+2H]^{2+}$ of desmopressin at a CE of 30 eV

Several other product ions, also present in the spectrum, were also included in the SRM method, but they were highly interfered or not sensitive enough to be considered for detection. The other vasopressin-related peptides presented similar ESI spectra. Also, the ion $[M+2H]^{2+}$ appeared as base peak in the full scan MS spectra of each peptide. Additionally, the peptides presented several common fragments, but different precursor ions and retention times were observed.

**Stability**

In doping analysis, stability of doping agents is of utmost importance, because the B-analysis is generally performed several weeks later than the analysis of the A-sample. The stability of desmopressin in aqueous solutions has been investigated by Schmitz et al. [6]. They concluded that desmopressin degrade significant faster at high pH. Therefore, stability of desmopressin was investigated by exposing an acidified solution and a basified solution of desmopressin (50 ng/mL) at a temperature of 56 °C for 6 h. The results of this experiment are presented in figure 3. In the basified solution no desmopressin could be detected, in the acidified solution desmopressin was still present, confirming the experiments of Schmitz and co-workers.
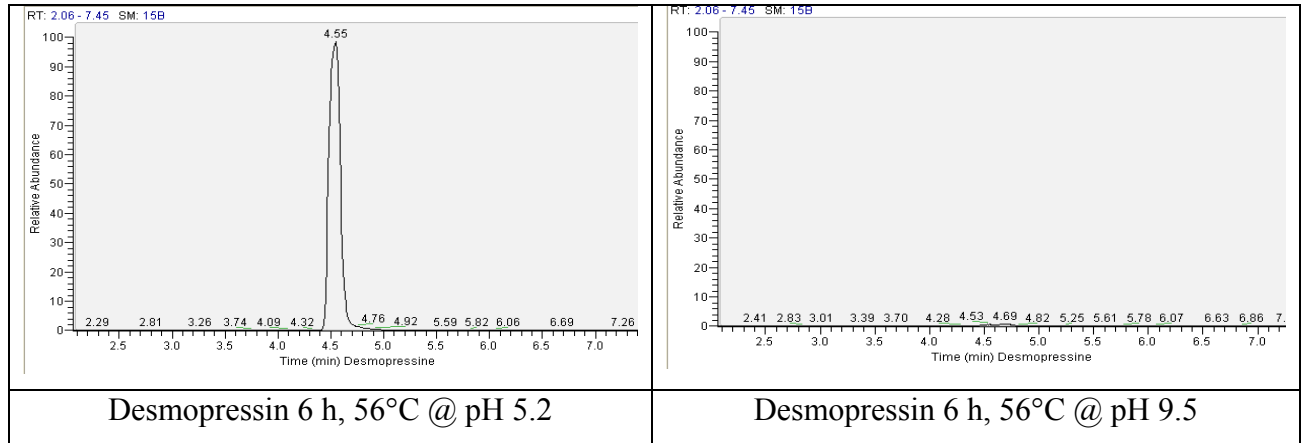


| Desmopressin 6 h, 56°C @ pH 5.2 | Desmopressin 6 h, 56°C @ pH 9.5 |

**Figure 3**: Stability experiment with aqueous solutions of desmopressin (1ng/mL)

To avoid degradation in the plasma samples, acetic acid was added before commencing sample preparation. Since peptides can show unwanted adsorption to the pipette tips and recipients [7], lypressin was added as carrier peptide. Long term stability of acidified working solutions, subject to >20 freeze-thaw cycles, was verified by comparing after one month, peak areas with freshly prepared solutions. No significant difference between the solutions was observed, proving the good stability of desmopressin.

**Sample preparation**

As already mentioned in the introduction, several methods for the detection of desmopressin in plasma by immunoassays have been described. Agersǿ et al. [3] uses a bulk protein precipitation step with acetone. Since the high efficiency of such a precipitation-step, this technique was further investigated. Both acetone and ACN precipitated samples were very dirty compared to plasma precipitated by the addition of saturated $(NH_4)_2SO_4$-solution. Hence, the latter was further preferred. To desalt the precipitated samples a protocol using $C_{18}$-SPE cartridges was adapted from Tausch et al. [8].

**Validation**

Desmopressin could be detected in all 6 plasma samples at a concentration of 250 pg/mL. A representative chromatogram is presented in figure 3.
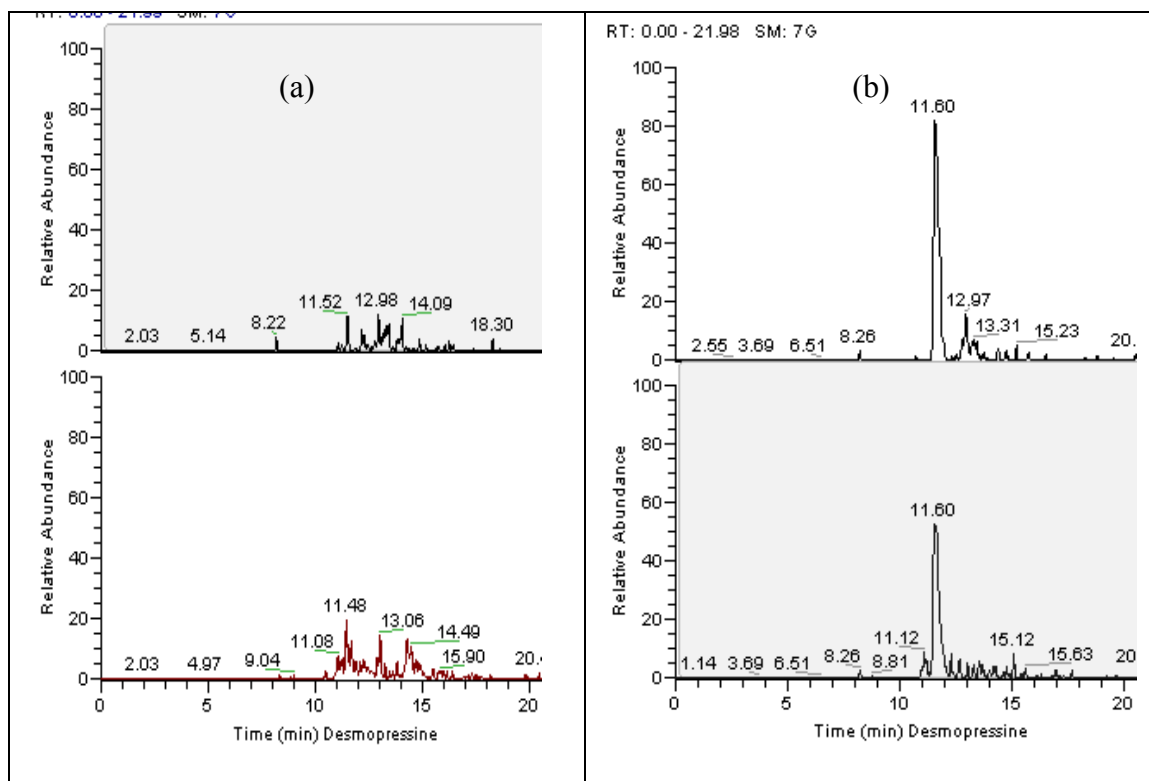


**Figure 3:** (a) blank plasma, (b) plasma spiked with desmopressin at 250 pg/mL, upper extracted ion chromatogram (XIC) is for ion at m/z 328, lower XIC is for transition at m/z 120.

Taking into account the WADA criteria for mass spectrometric identification [2] only at 500 pg/mL, the ion ratios were within the accepted ranges. Regarding chromatographic criteria, retention times were only stable within 0.4 min retention time window. Selectivity and specificity of the method were satisfactory, no endogenous interference and interference from similar substances were observed. Evaluation of the extraction showed only a 10% recovery.

**Application to real samples**

Analysis of real administration samples allow to evaluate the applicability of a detection method in the field of doping analysis. Analysis of the 6 administration samples did only yield in one sample a peak for desmopressin for the two transitions monitored (Figure 4). However, the WADA criteria for identification were not fulfilled. In the other 5 samples no desmopressin was detected.
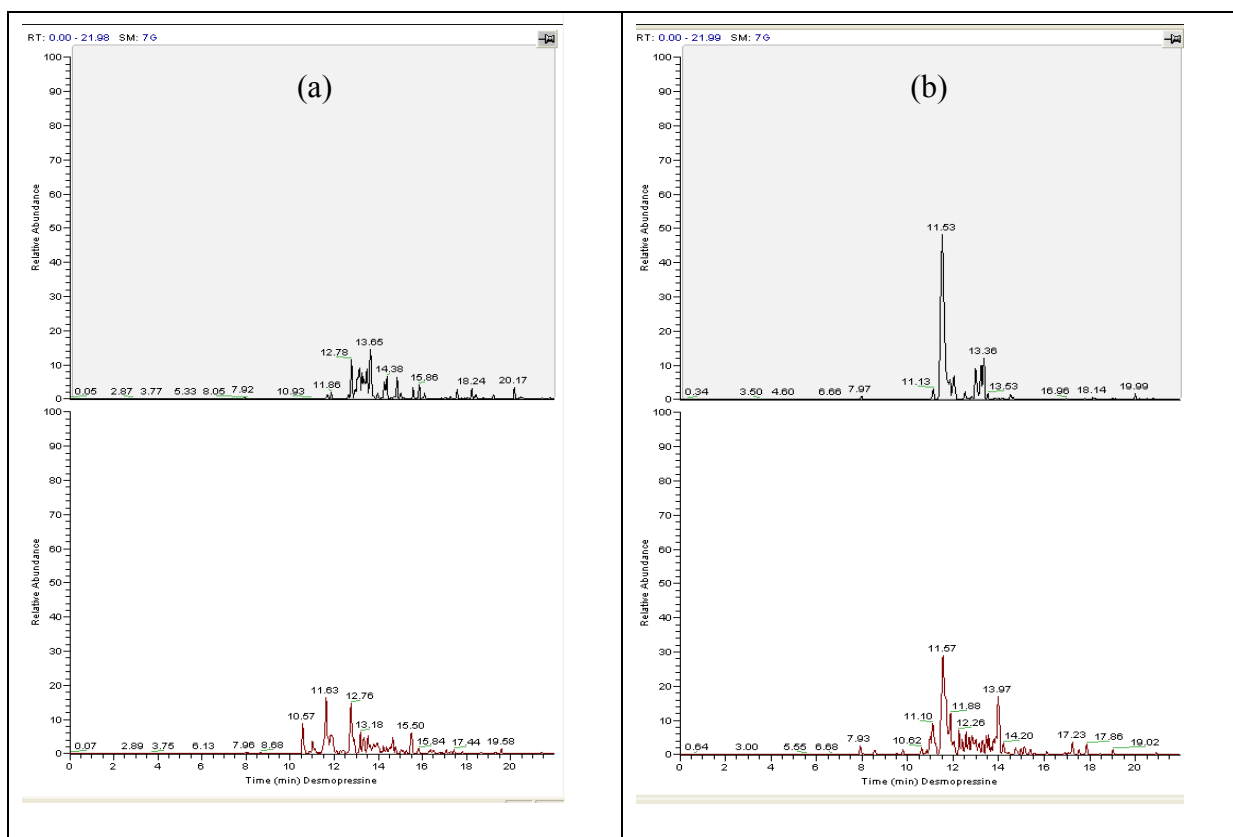


**Figure 4:** (a) blank plasma sample; (b) plasma sample obtained from a patient 3 h after oral administration of 200 μg of desmopressin acetate.

## Conclusions

Due to the presence of the easy ionisable arginine moiety in desmopressin, ESI-efficiency of this compound is very high. Desmopressin also shows to be a stable compound in acidified solutions. Application of the protein-precipitation step shows to be very useful to remove bulk plasma proteins. Taking into account the detection limit obtained, the method should be further improved a factor 10-20 to reach concentration ranges observed after pharmacokinetic studies with oral and intra-nasal preparations. Improvement of the identification criteria regarding chromatography and mass spectrometry for the unambiguous identification of desmopressin should be performed as well. Nevertheless, the method illustrates the potential of LC-MS/MS for the detection of desmopressin.

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