Qualitative detection of desmopressin in plasma by liquid chromatography-tandem mass spectrometry.

Short title: LC-MS DETECTION OF DESMOPRESSIN IN PLASMA

Keywords: desmopressin, LC-MS, doping, plasma

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Abstract

This work describes a liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) method for detection of desmopressin in human plasma in the low femtomolar range.

Desmopressin is a synthetic analogue of the antidiuretic hormone arginine vasopressin and it might be used by athletes as masking agent in the frame of blood passport controls. Therefore, it was recently added by the World Anti-Doping Agency (WADA) to the list of prohibited substances in sport as a masking agent.

Mass spectrometry characterization of desmopressin was performed with a high-resolution Orbitrap-based mass spectrometer. Detection of the peptide in the biological matrix was achieved using a triple quadrupole instrument with an electrospray ionization (ESI) interface after protein precipitation, weak cation solid phase extraction and HPLC separation with an octadecyl reverse phase column. Identification of desmopressin was performed using three product ions, m/z 328.0, m/z 120.0, and m/z 214.0, from the parent ion, m/z 535.5.

The extraction efficiency of the method at the limit of detection was estimated at 40% (n=10), the ion suppression at 5% (n=10), and the limit of detection was 50 pg/ml (S/N> 3).

Selectivity of the method was verified against several endogenous and synthetic desmopressin related peptides. The performance and the applicability of the method were tested by analysis of clinical samples after administration of desmopressin via intravenous, oral, and intranasal routes. Only after intravenous administration, desmopressin could be successfully detected.

Introduction

Desmopressin, 1-desamino-8-D-arginine-vasopressin (dDAVP), is a synthetic analogue of arginine [Arg^8]-vasopressin (AVP), a cyclic peptide hormone with antidiuretic properties, synthesized in the hypothalamic cells and released from neurohypophysis. Desmopressin (monoisotopic mass: 1068.4269 Da) is obtained by deamination at the N-terminal 1 position of Avp and replacement of 8-L-arginine with its D-isomer (Figure 1) [1-3].

Compared with AVP, desmopressin has a longer lasting and more potent antidiuretic effect and is devoid of vasopressor effects [2, 4, 5]. Desmopressin is therapeutically used for the treatment of diabetes insipidus [4; 5], primary nocturnal enuresis (bed wetting) [6-9] type I von Willebrand disease, and hemophilia A [11-14].

Desmopressin can induce hemodilution decreasing hematocrit levels and hemoglobin concentrations. These values are important hematological parameters used to detect blood doping in sports [15-16].

Therefore, desmopressin has recently been added by the World Anti-Doping Agency (WADA) to the 2011 Prohibited List as a masking agent [17].

Previous methods for the detection of desmopressin in biological fluids were performed only for clinical purposes, by immunoassays. These methods provided sensitive and cost effective analyses [8, 18-22], but with some important limitations, in particular concerning specificity [23].

Liquid chromatography-mass spectrometry (LC-MS) shows good sensitivity together with a high degree of selectivity and specificity to allow for unequivocal confirmation of the

presence of a substance based on its molecular weight. Hence LC-MS methods have become the method of choice for the analysis of peptide [24].

Until now, only one study using LC-MS to identify desmopressin has been published [25]. This method was applied for the analysis of skin samples and has a limit of detection (LOD) of 10 ng/ml ($\approx$ 10 picomoles). Obviously, this matrix is not applicable in the field of sport drug testing. Hence, the present work had the aim to develop and validate a LC-ESI-MS/MS method for detection of desmopressin in plasma fit for anti-doping purposes.

Materials and methods

Chemicals and reagents

All the chemicals and solvents used for sample pretreatment and chromatography were analytical grade or HPLC grade. Desmopressin was a kind gift from Ferring Pharmaceuticals (*Malmö*, Sweden). AVP and [deamino-Cys[1], Val[4], D-Arg[8]]-vasopressin were purchased from Sigma Aldrich (Saint Louis, USA). Oxytocin, [Lys[8]]-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.

Saturated ammonium sulfate ((NH_4)$_2SO_4$) solution was prepared by dissolving 800 g of (NH_4)$_2SO_4$ (Merck, Darmstadt, Germany) in 1000 ml of H_2O at room temperature. The resulting solution was gently poured into a second glass bottle in order to get rid of the undissolved crystals of (NH_4)$_2SO_4$, then 20 ml of 25% NH_4OH were added to 1 l of the (NH_4)$_2SO_4$ solution.

Only low binding microcentrifugal vials, type Eppendorf (Eppendorf, Hamburg, Germany) and low retention pipette tips (Sorenson Biosciences, Salt Lake City, USA) were used to prepare stock solutions (100 µg/ml) and 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.

Plasma samples

Method development and validation were performed using left-over plasma samples obtained from Red Cross Blood Transfusion Center (Gent, Belgium, approval number 20100609). Desmopressin containing 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).

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. A further purification of the supernatant was performed by solid phase extraction with Oasis$^®$ WCX (60 mg) cartridges, purchased from Waters (Milford, USA). The column was first activated with 2 ml of MeOH and subsequently rinsed with 2 ml of H_2O. After the sample was loaded, 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 a solution consisting of 80:20 MeOH:(5% HOAc in H_2O). The eluate was subsequently evaporated to dryness with a centrifugal evaporator (45°C, 240 g, 6-8 h), and then dissolved in 40 µl of 95:5 H_2O:ACN, 0.1% HOAc, 0.01% TFA prior to LC-MS analysis.

Liquid chromatography

HPLC separation was achieved with a Surveyor MS Pump Plus coupled with a Surveyor Plus autosampler (Thermo Scientific, Bremen, Germany) using a Zorbax 300SB-C18 reverse-phase column (1.0 x 50 mm, 3.5 µm) protected with a Zorbax 300SB-C8 guard column, both

from Agilent Technologies (Santa Clara, USA). The use of the C8 guard column was adopted from a previous work from Thevis [26].

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 minutes, then decreased linearly 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. Then the system was equilibrated for 10 minutes before next injection (total run time: 25 minutes). A constant flow rate of 50 µl/min was maintained.

Mass spectrometry

MS/MS 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, 84V.

Mass spectrometry characterization of desmopressin and related peptides was performed on an Exactive benchtop Orbitrap-based mass spectrometer (Thermo Scientific, Bremen, Germany) operating in positive HCD scan at 50 eV. The sheath gas was set to 60 (arbitrary units), the aux gas set to 30 (arbitrary units) and the capillary temperature set to 350°C. The capillary voltage and spray voltage were set to 30 V and 3 kV, respectively. The instrument was operated in full scan mode from m/z 60–1200 at 100,000 resolving power. The data acquisition rate was 1 Hz.

Disulfide bridge reduction

In order to get additional information on the MS behavior of desmopressin, reduction of the disulfide bridge (Mpa1-Cys6) was performed by adding 200 µl of dithiothreitol in 0.2 mM ammonium acetate buffer at pH=5.5 (both from Sigma-Aldrich) to readily prepared solutions of the peptide and incubating the resulting solution for 30 minutes at 56 °C.

Validation

In accordance with Eurachem validation guidelines [27], 10 human plasma samples, were spiked at different levels (5, 10, 25, 50, 100 pg/ml) to determine the LOD.

The detection limit was defined as the lowest level at which a compound could be identified in all 10 plasma samples with the 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 plasma samples spiked with desmopressin and its analogues at a concentration of 100 pg/ml.

The analogues included the endogenous hormones AVP and oxytocin, and the synthetic derivatives [deamino-Cys[1], Val[4], D-Arg[8]]-vasopressin, [Lys[8]]-vasopressin, and terlipressin.

Specificity was tested during the validation procedure in order to check the absence of endogenous interferences. The 10 blank plasma samples used for determining analytical performances of the method were extracted and analyzed as described above.

Extraction efficiency and matrix effect

To evaluate the extraction efficiency, the 10 negative 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 for the product ion at *m/z* 328.0 of the two sets of samples were compared.

Matrix effect was evaluated by comparing the peak areas (*m/z* 328.0) of the plasma samples spiked after extraction with a reference solution of desmopressin at 50 pg/ml, corresponding to a 100% recovery (0% matrix effect).

Excretion studies

7 plasma samples from patients who received desmopressin were used in the study. Four patients received desmopressin via intranasal ($\approx$ 10 µg/dose), two orally (200 µg) and one intravenously (20 µg). Blood samples were immediately centrifuged and plasma was separated. When not directly analyzed, plasma samples were stored at <-20°C awaiting analysis.

Results and discussion

Mass spectrometry

Direct infusion of desmopressin and analogues was performed in order to determine the best mass spectrometry conditions for the design of the MRM method. A full scan MS analysis was first performed in order to identify the most abundant precursor ions and subsequently the collision energy was optimized for the most diagnostic fragments.

Desmopressin showed excellent ionization, due to the presence of an arginine residue (Arg_8), containing the guanidinium group with a high proton affinity. The double charged ion $[M+2H]^{+2}$ was observed as base peak for desmopressin (Figure 2) but also for the analogues. The single charged ion $[M+H]^{+}$ only achieved a relative abundance of 20%. 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, *m/z* 120.0 and *m/z* 214.0 were selected as diagnostic. Several other product ions, also present in the spectrum, were also recorded during mass spectrometry characterisation, but they were not specific or not sensitive enough using a criteria of S/N >3 to be considered for reliable 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, therefore method selectivity was guaranteed. Results from infusion experiments for MS/MS optimization of the investigated compounds are summarized on Table 1.

The three diagnostic ions selected for MRM method were further investigated by high-resolution mass spectrometry. Figure 3 shows the fragmentation patterns of the native (a) and the reduced (b) peptide generated by HCD at 50 eV at high resolution. All the relevant fragments are single-charged and some of them present a neutral loss of NH_3. Moreover, it is possible to notice that in the native molecule all the significant high-mass fragments (y_2-NH_3, y_3-NH_3, y_3, b_6) contain the Arg_8. The b_6-NH_3 fragment, which presents an intramolecular disulfide bridge between C1 and C6 and is adjacent to a proline residue, represents the only exception [28]. Reduction of the disulfide bridge provided additional information on the MS characterization of desmopressin, yielding to the identification of the complete y_3-y_7 sequence.

The contemporary presence in fragment ion y_3 of Arg_8 and Pro_6 at the N-terminal, which usually causes enhanced fragmentation attributed to the greater basicity of prolin substituted nitrogen group [29], can explain the elevated abundance of this fragment in the MRM mode.

Liquid chromatography

Chromatographic separation was achieved with a C18 column, which allowed satisfactory peak shapes and chromatographic retention for desmopressin, the internal standard and the carrier peptide. A high percentage (90%) of ACN was required to elute desmopressin, which presented an average retention time (tR) of 10.07 and the IS (tR=10.75 min). The carrier peptide Avp that contains the terminal amino moiety eluted earlier (tR=3.63 min). Additionally, other desmopressin related peptide, including the endogenous hormone oxytocin and the synthetic analogues [Lys^8]-vasopressin, and terlipressin were analyzed to further investigate method selectivity. Results, also summarized in Table 1, demonstrated that the chromatography is sufficient to separate desmopressin from these peptides. In fact, oxytocin elute at 9.88 min, [Lys^8]-vasopressin at 3.50 min, and terlipressin at 3.76 min.

Sample preparation

Since no LC-MS based method for detection of desmopressin in plasma has been described previously, the sample preparation procedures available from the literature were all related to the function of immunoassays. These approaches are based mainly on previous work of Lundin et al [21, 22]. Desmopressin is a small, highly basic peptide, resulting in good solubility, without a significant tertiary structure that can undergo denaturation during protein precipitation. Therefore Lundin et al. [21] successfully used protein precipitation with ice-cold acetone as sample clean-up. To optimize this protein precipitation step, the application of acetone, methanol, saturated $(NH_4)_2SO_4$ and ethanol [30] were evaluated during method development. Deproteination of the samples using saturated $(NH_4)_2SO_4$, gave the best results regarding S/N response. Additionally, omitting the use of an organic solvent to precipitate bulk proteins avoids the requirement to evaporate the deproteinated sample before application to the SPE in the next step. Finally, the use of the protein precipitation allowed a 5-fold increase of the MS response compared with samples that were processed only with SPE. To further decrease sample preparation time, 15 and 30 min centrifugation instead of 60 min was considered. However, due to the lower centrifugation time, deproteination was less complete. Using centrifugation devices with higher speed can further shorten centrifugation times.

SPE was considered to further concentrate and desalt the samples. In particular, cation exchange columns were investigated, because desmopressin is a basic peptide with a pKa value of approximately 12. Mixed-mode ion-exchange (MCX) columns were initially investigated. However, desmopressin could not be recovered from this type of column during the elution step since this type of column is only applicable for weak basic compound.

Weak-cation ion exchange WCX)- columns proved to be particularly suitable for the clean up of strong basic compounds allowing, compared to octadecyl (C18) and hydrophilic-lipophilic

balance (HLB) sorbents, to eliminate efficiently acid and slightly basic matrix components. These resulted in a higher grade of purification.

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). No differences in desmopressin responses were registered for protein-precipitation compared with the non-basified $(NH_4)_2SO_4$ saturated solution. The presence of 20% water in the elution solvent, resulting in a longer evaporation time, guaranteed the best results in terms of sensitivity.

Choice of internal standard and carrier peptide

[deamino-Cys[1], Val[4], D-Arg[8]]-vasopressin was chosen as internal standard since it presents a very similar chemical structure, pKa, and retention times ($\Delta tR \approx 0.7$ min) to desmopressin, and therefore a similar behavior during the entire analytical process. Since the degree of adsorption of a peptide or protein is not always predictable, several precautions were adopted to prevent this phenomenon, including the use of low binding plastic and the use of a carrier peptide. AVP was considered as a suitable carrier peptide for working solutions since it presents a very different retention time ($\Delta tR \approx 6.4$ min) from the target analyte, which means no interferences with its detection, and, additionally, a low cost for the standard reference. We registered only a slight decreasment (approximately 10´%) in MS response when diluted working solutions of 10 ng/ml of desmopressin, not containing AVP as carrier peptide, were analyzed, whereas no significant differences were recorded when AVP was added to plasma sample (500 ng/ml) at the beginning of the sample preparation. Hence, desmopressin adsorption did not occur or was negligible during sample preparation.

Additionally, stability of desmopressin working solution stored at 4°C was tested by preparing a new solution (10 pg/ml) every month and comparing it with the previous by LC-

MS analysis. After 4 months, no significant difference was noticed in peak areas of the various solutions. Moreover, desmopressin working solution was demonstrated to be stable after incubation at 56°C for 6 hours.

Method validation

The method showed an LOD of 50 pg/ml, with a signal to noise ratio (S/N) greater than 3 for the three transitions in all plasma samples that were analyzed. Maximum tolerance windows (% of base peak *m/z* 328.0) were set at ±10% (absolute) for m/z 120.0 and ±5% (absolute) for m/z 214.0, according to the WADA identification criteria for LC-MS qualitative assays [31] that recommend the use of minimum two transitions for MRM-based methods. Nevertheless, using only one transition is also allowed.. According to the abundance recorded using the product ion at *m/z* 328.0, the average extraction efficiency (n=10) of the preparation was estimated at 40% (±4%). Signal reduction due to ion suppression in the ESI source (n=10) was limited to 5% (±14%).

The method also exhibited good selectivity since the related peptides presented different retention time, as shown in Table 1. Mass overlapping for mass spectrometry detection was avoided, thereby ensuring analytical selectivity. AVP, [Lys[8]]-vasopressin, and terlipressin, whose N-terminal is not deaminated, elute earlier (3.5-3.80), whereas desmopressin, [deamino-Cys[1], Val[4], D-Arg[8]]-vasopressin (both deaminated) and oxytocin (which presents the hydrophobic amino acid isoleucine) require a higher percentage of organic solvent.

The method can be used for the analysis of an elevated number of samples per batch and the whole analysis can be performed in a single day, since the only time consuming step (evaporation of the SPE eluate) requires only few hours.

Method application

The suitability of our method was evaluated by the analysis of 7 plasma samples from patients who received desmopressin. Four patients received desmopressin intranasally, two orally and one intravenously. Administered dose and collection times are summarized in Table 2. Detection of desmopressin after oral administration could not be achieved, even taking into account only the most abundant transition (*m/z* 535 -> 328, LOD= 10 pg/ml). Indeed, orally administered desmopressin has a very low bioavailability. Osterberg et al [19] reported maximum plasma levels < 50 pg/ml (t= 90 min) after oral administration of a therapeutic dose of 240 µg.Similarly, desmopressin was not detectable after nasal administration. The lower dose (≈ 10 µg) and the generally lower bioavailability of this type of formulation could explain the non-detection. After intravenous administration of a 20 µg dose, desmopressin could be detected in an unambiguous way (Figure 4b).

It can be concluded that desmopressin detection in plasma, related to anti-doping analysis is not realistic after oral or intranasal administration. In particular since oral administration has shown to effect hematocrit levels [12]. Intravenous application seems unlikely due to its invasive nature. Hence, it will be necessary to consider other detection strategies to increase the possibility to detect the misuse of this peptide.by LC-MS. According to Agerso et al. [20], an important fraction of desmopressin is eliminated from the body via renal clearance. Hence urinary detection of desmopressin will be further investigated.

Conclusions

For the first time a sensitive LC-ESI-MS/MS method for the detection of desmopressin in human plasma for doping purposes was described. Validation of the method showed an LOD of 50 pg/ml when three transitions were considered, and 10 pg/ml when only the most abundant transition (*m/z* 328) was monitored. The method exhibited also good selectivity and specificity. Application of the method to administration samples showed that desmopressin was detectable when administered intravenously, but not after oral and intranasal administration. The results of the analysis of the administration samples are in agreement with the literature describing low bioavailability and short plasmatic half-lives after oral and intranasal application, hampering its detection.

Acknowledgements

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Laboratories/WADA_TD2010IDCRv1.0_Identification%20Criteria%20for%20Qualitativ

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Figure captions

Figure 1

Compared to arginine vasopressin, desmopressin's Cys_1 has been deaminated, and the Arg_8 is in the dextro rather than the levo form.

Figure 2

High-resolution full scan MS spectra of desmopressin (a), and isotopic pattern of its base peak $[M + 2H]^{(2+)}$ (b).

Figure 3

HCD spectra of desmopressin acquired at 50eV (a). The fragmentation scheme shows that all the most abundant high MW fragments contain the arginine residue, except b_6, that is stabilized by the disulfide bridge. Reduction of the disulfide bridge (b) yield to the identification of the sequence y_3-y_7.

Figure 4

Extracted ion chromatograms for the product ions m/z 328.0, m/z 120.0, m/z 214.0 (precursor ion m/z 535.5) respectively in a blank plasma (a), after fortification with desmopressin at LOD concentration (50 pg/ml) (b), before (c) and after administration of intravenous desmopressin (d). Samples were all prepared and analyzed according to the described assay.

Table captions

Table 1

Amino acid sequence, retention times and MRM conditions for ESI-MS/MS analysis of desmopressin and related peptides. Desmopressin and its analogues present several common product ions, but different the retention time and parent ion. The double charged ion resulted to be the most abundant for all the investigated peptides.

Table 2

Summary of blood specimens from excretion study, collected after administration of desmopressin via different routes.
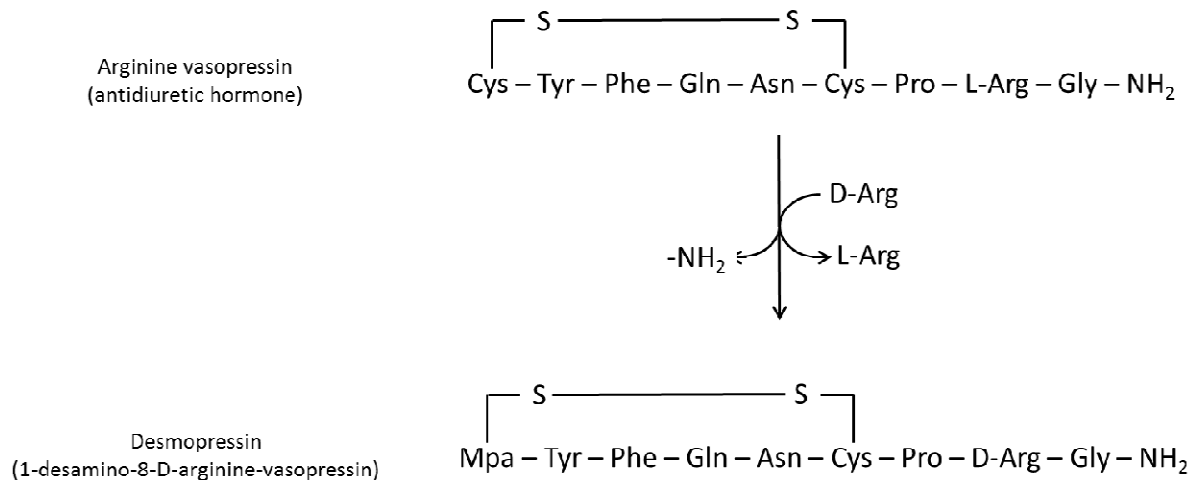
Figure 1

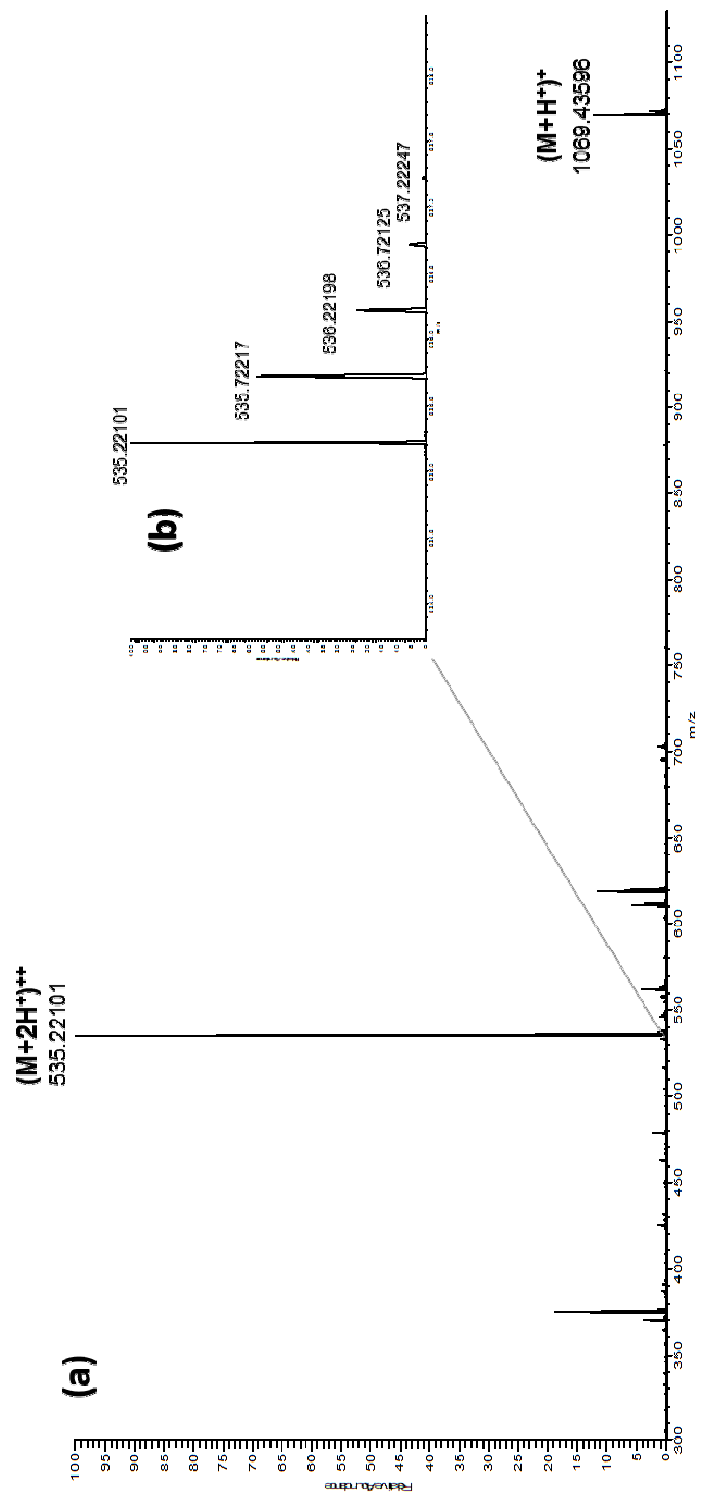Arginine vasopressin
(antidiuretic hormone)

```
      ┌─ S ──────────── S ─┐
Cys – Tyr – Phe – Gln – Asn – Cys – Pro – L-Arg – Gly – NH₂
```

$$\text{D-Arg}$$

$$-\text{NH}_2 \quad \text{L-Arg}$$

Desmopressin
(1-desamino-8-D-arginine-vasopressin)

```
      ┌─ S ──────────── S ─┐
Mpa – Tyr – Phe – Gln – Asn – Cys – Pro – D-Arg – Gly – NH₂
```
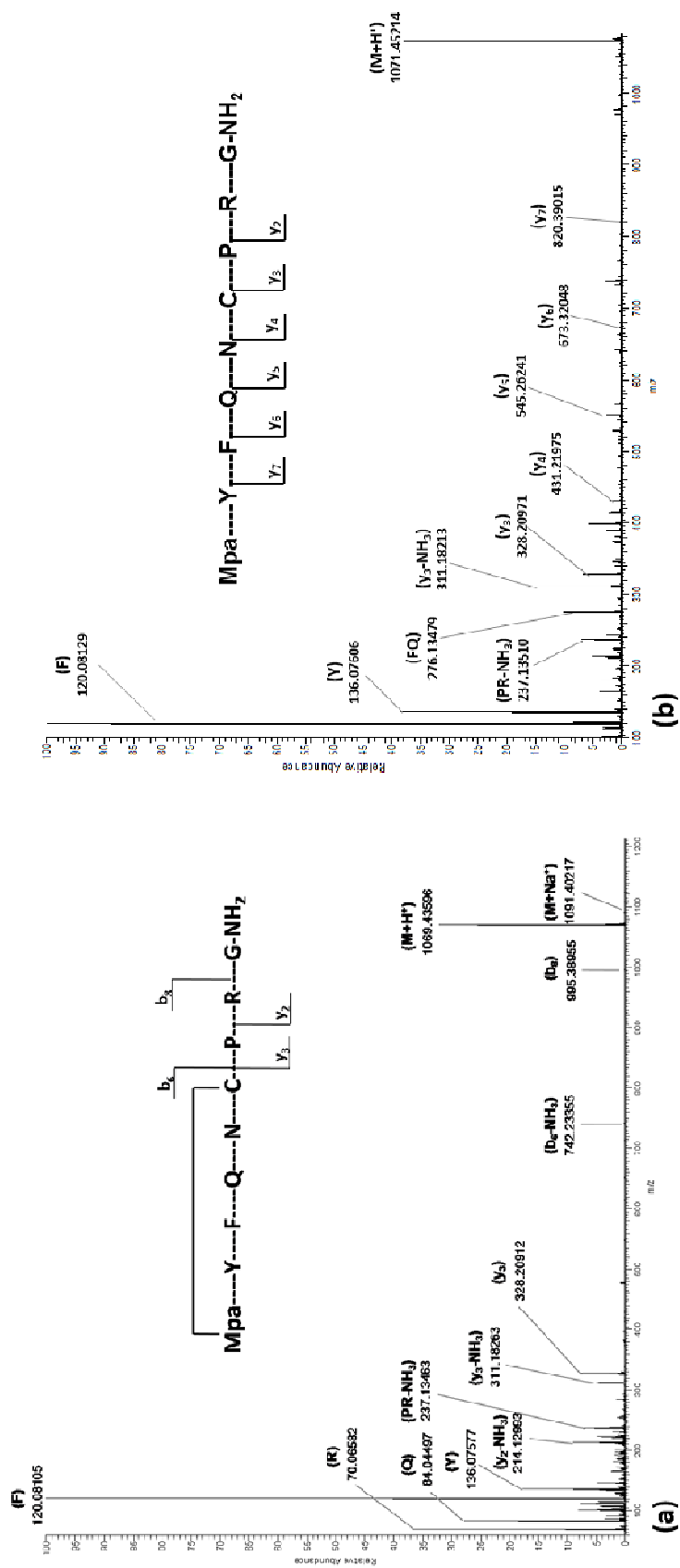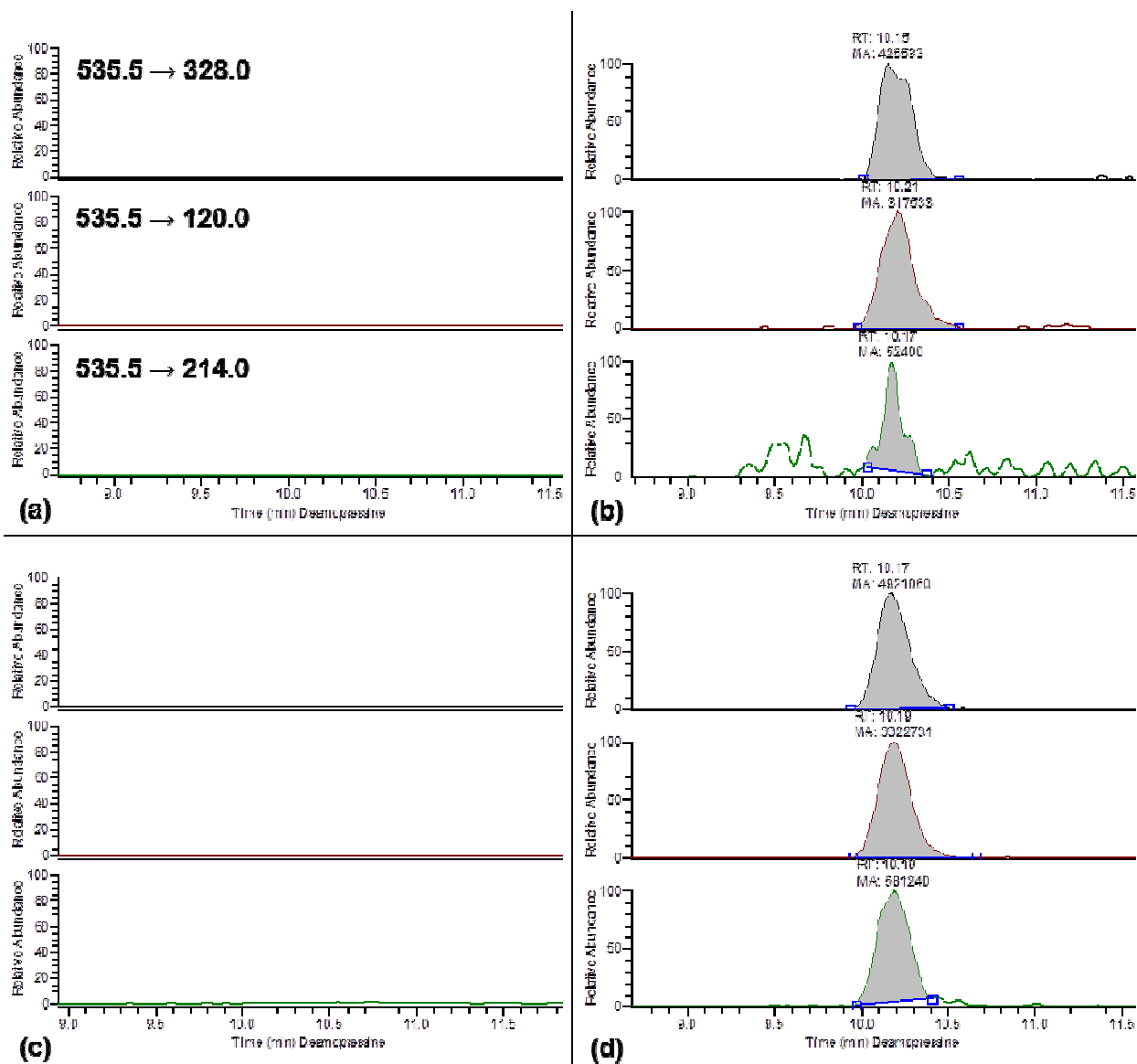
Figure 2

Figure 3

Figure 4

Table 1

Peptide	MW (Da)	Amino acid sequence	tR	Precursor ion charge state	Precursor ion m/z	Production (m/z)	Collision E (eV)	Abundance %
Arginine vasopressin	1083,4378	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH₂	3,63	[M+H]²⁺	542,7	120,0	37	100
						328,0	18	99
						757,3	14	24
Oxytocin	1006,4364	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂	9,88	[M+H]²⁺	504,3	85,9	19	100
						294,9	37	62
						136,0	40	60
Desmopressin	1068,4269	Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-DArg-Gly-NH₂	10,07	[M+H]²⁺	535,5	328,0	16	100
						120,0	35	70
						214,0	16	13
Lys-vasopressin	1056,2200	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH₂	3,50	[M+H]²⁺	528,9	120,0	35	100
						128,9	34	35
						226,0	33	32
Terlipressin	1226,4961	Gly-Gly-Gly-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH₂	3,76	[M+H]²⁺	614,5	120,0	34	100
						128,9	36	99
						226,0	28	93
[deamino-Cys1, Val4, d-Arg8]-vasopressin (ISTD)	1039,4367	Mpa-Tyr-Phe-Val-Asn-Cys-Pro-DArg-Gly-NH₂	10,75	[M+H]²⁺	529,8	328,0	16	100
						120,0	34	67
						924,5	12	12

Table 2

Patient	Route of administration	Dose (μg)	Time of sample collection post-dosing (h)	Detection
1	oral	200	2.00	No
2	intranasal	25	3.30	No
3	intranasal	25	3.00	No
4	intranasal	25	3.00	No
5	intranasal	25	2.00	No
6	intravenous	20	0.30	Yes
7	oral	200	0.30	No