A High Sensitivity Dual Solid Phase Extraction LC-MS/MS Assay for the Determination of the Therapeutic Peptide Desmopressin in Human Plasma

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Overview

This white paper describes a sensitive and selective dual mechanism SPE LC-MS/MS method for the quantitation of the small therapeutic peptide Desmopressin in human plasma. Extract cleanliness, LC focusing and a novel MRM summation approach combine to give enhanced sensitivity and overall method performance allowing a lower limit of quantitation of 2.00 pg/mL.

Introduction

Desmopressin (1-desamino-8-D-arginine vasopressin) is a synthetic replacement for the antidiuretic hormone Arginine-Vasopressin, a peptide containing nine amino acids. It may be taken nasally, intravenously, or orally in tablet form. Typical doses of 0.200 to 1.20 mg per day may result in very low plasma concentrations. A highly sensitive and selective method for the determination of therapeutic levels during clinical studies is therefore required.

Radioimmunoassay (RIA) has been the technique of choice for quantitation of such peptides in the past due to its favorable sensitivity. However, this technique may suffer from cross reactivity issues which can compromise selectivity. Additionally sample throughput and sample volume requirements may be an issue when supporting larger clinical studies. A sensitive and selective dual mechanism SPE LC-MS/MS method for the quantitation of Desmopressin in human plasma (EDTA) down to 2.00 pg/mL was established and validated in our laboratory.

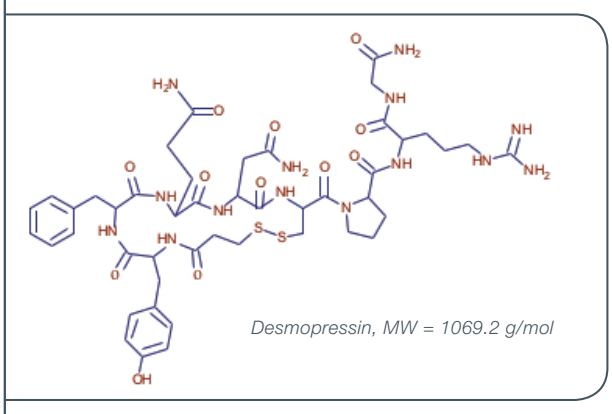

Figure 1. *Molecular structure of Desmopressin*

Method

Human plasma (EDTA) samples were determined at an analytical range of 2.00 – 250 pg/mL using a two-dimensional SPE combined with LC-MS/MS. A 1 mL sample aliquot was mixed with 100 µL of Acetonitrile/water containing an appropriate concentration of labeled internal standard 3-Mercaptopropionyl-Tyr-[C13, N15] Phe-Gln-Asn-Cys-Pro-D-Arg-Gly-NH2.

The whole volume was loaded onto Varian Bond Elut® CN-E (100 mg) extraction plate followed by several washing steps using neutral aqueous and organic solvents. Elution was performed in Methanol/Millipore water/Formic acid, (50:50:0.2, v/v/v). Samples were then pH adjusted by the addition of 0.25% Ammonium hydroxide solution (aq.) and further loaded onto a Varian Bond Elut® CBA (100 mg) extraction plate. After further wash steps samples were eluted in Methanol/Formic acid, (98:2, v/v). Samples were evaporated under nitrogen at 40°C and reconstituted in 100 µL of Methanol/Millipore water/Formic acid, (20:80:0.1, v/v/v). The whole extraction procedure could be automated in 96-deep well format using a Tomtec Quadra 96 pipette robot equipped with a vacuum manifold.

100 µL of reconstituted sample was trapped on a YMC-Pack ODS-AQ (12 nm, S-3µm, 10x2.1mm) precolumn to achieve a sharp peak and therefore maximum intensity. Further analytical separation was performed on an Agilent Zorbax 300 SB-C18 (3.5 µm, 50x2.1mm) with gradient elution using a Methanol/Millipore water/Formic acid system. The gradient was optimized to achieve

separation from small interfering peaks. Detection was achieved on an AB SCIEX API 4000 LC-MS/MS system using electrospray in positive mode. MRM transitions measured were 535.5/328.2 amu for Desmopressin and 540.5/328.2 amu for its labelled IS (both at unit/unit resolution). The precursor ion at 535.5 amu could be related to the double charged Desmopressin (Figure 2), whilst its corresponding product ion at 328.2 amu was identified as single charged Gly-Arg-Pro (Figure 3). Single and triple charged precursor ions could not be detected (Figure 2). Example chromatograms of representative extracted LLOQ and blank samples are shown in Figure 4.

Analyte and IS transitions were measured as separate experiments. The number of measured MRM transitions and dwell time was optimized. For the analyte 15 MRM, transitions were measured at a dwell time of 40 ms for each. For the IS, the number of MRM transitions was reduced to 5 at a dwell time of 40 ms to keep the total scan cycle time minimal. This allows the measurement of sufficient data points across the peak thereby maintaining method precision and performance. Quantitation was performed by area ratio using TIC (total ion count) for summation of all MRM transitions. A more detailed discussion about MRM transition summation can be found in the results section.

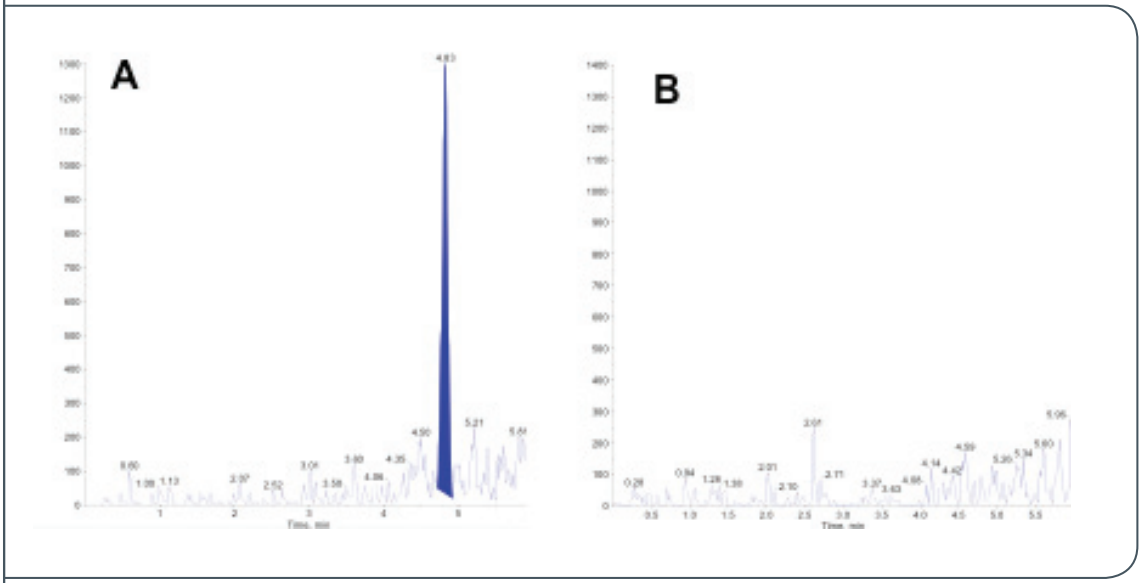

Figure 2. *Example chromatograms*
A: LLOQ, 2.00 pg/mL of Desmopressin in extracted human plasma (EDTA)
B: Blank, extracted human plasma (EDTA)

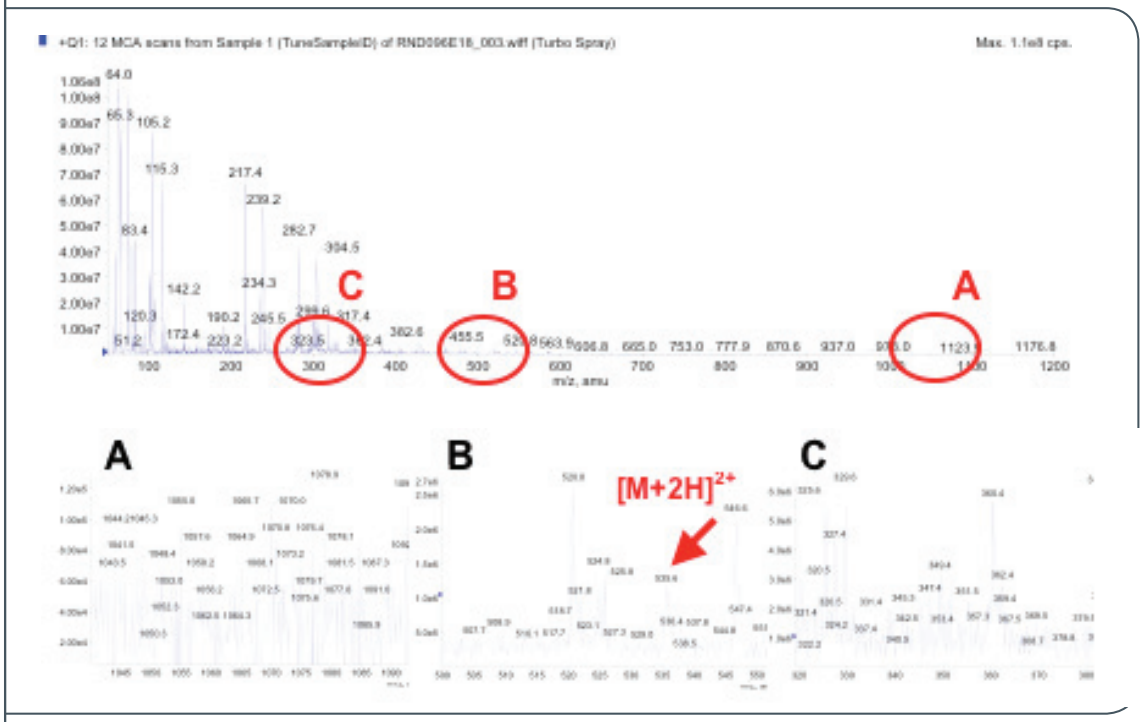

Figure 3. Precursor ion scan of Desmopressin, 100 ng/mL in Methanol / Water / Formic acid (50:50:0.1, v/v/v)
A: Region of suspected [M+H]+ precursor ion
B: Region of [M+2H]2+ precursor ion, note sodium adduct at 546.6 amu
C: Region of suspected [M+3H]3+ precursor ion

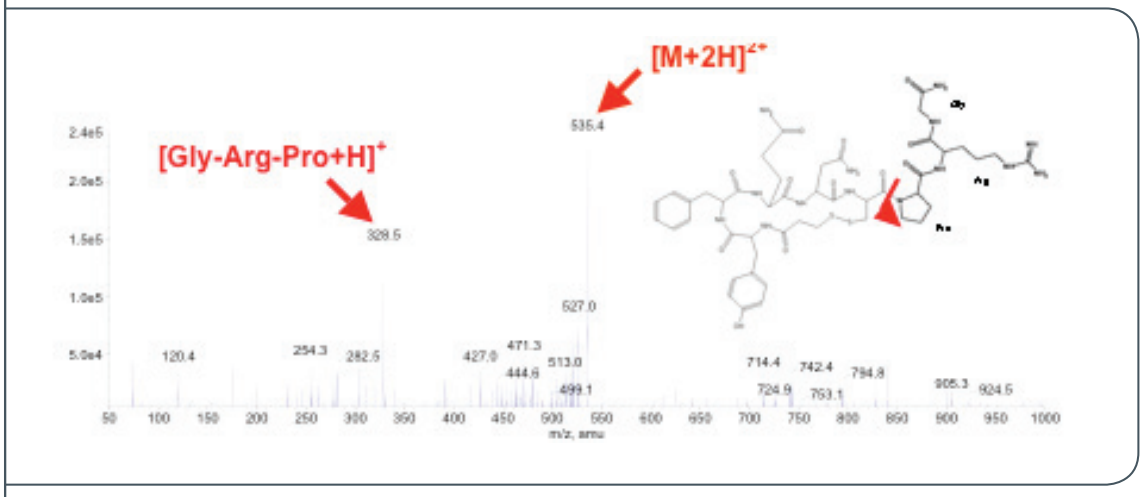

Figure 4. Product ion scan of Desmopressin, 100 ng/mL in Methanol / Water / Formic acid (50:50:0.1, v/v/v) at collision energy of 20 using 535.5 amu as precursor ion

Influence of dwell time and number of MRM transitions

The influence of the dwell time and the number of MRM transitions on the sensitivity was investigated. The signal to noise ratio (S/N) and the precision (CV%) was determined using pure solutions, equivalent to the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ).

By increasing the dwell time on a single MRM, the number of data points across the peak decreases. Due to reduction of the noise with increase in the dwell time (effective data smoothing) the S/N improves without compromising precision (see Table 1).

The summation of identical MRMs was shown to increase S/N. A possible explanation for this is that due to the randomized noise around the peak of interest, the gain in peak signal is greater than the gain in noise (Figure 5). No significant influence on the precision was observed using this summation approach.

The best compromise between S/N and numbers of data points across the peak was reached by using summation of 15 MRMs and a dwell time of 40ms.

	Data points across peak	S/N at LLOQ	CV LLOQ, area ratio (n=3)	CV ULOQ, area ratio (n=3)
1 MRM 400ms	11	35:1	6.7%	2.6%
1 MRM 150ms	30	30:1	4.6%	2.8%
1 MRM 40ms	100	25:1	6.4%	1.0%
15 MRM 40ms	10	40:1	9.4%	2.2%
25 MRM 40ms	6	45:1	2.5%	1.5%

Table 1. *Comparison of S/N on different dwell times and numbers of MRM transitions*

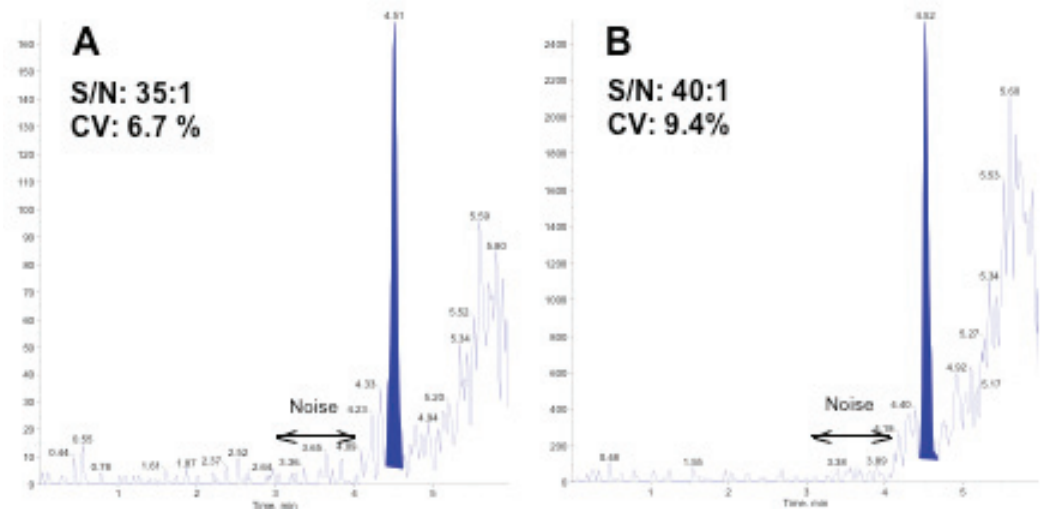

Figure 5. *Example chromatograms on different dwell times and numbers of MRM transitions*
A: 1 MRM transition at a dwell time of 400 ms.
B: Summation of 15 identical MRM transitions at a dwell time of 40 ms.

Method validation

The LC-MS/MS method was validated to all current guidelines for bioanalytical method validation. Validation results are summarized in Table 2. All validation criteria in respect to accuracy, precision, selectivity, specificity and stability were met. The method was shown to be robust between instrumentation and to allow a relatively high throughput without intervention.

Validation Parameter	Result (inc LLOQ)
Intra day precision	<7% (<12%)
Inter day precision	<7% (<12%)
Selectivity	In at least 10 plasma lots
Matrix effect	Absence in at least 10 plasma lots
Plasma freeze thaw stability	3 additional cycles
Plasma room temperature stability	24 hours
Recovery	80%
Extract stability	5 days

Table 2. *Validation results*

Incurred sample evaluation

In order to assess the suitability of the LC-MS/MS method incurred samples were analyzed by both the original RIA and new LC-MS/MS methods. Data was then compared using ISR (Incurred sample reproducibility) procedure and criteria. Incurred samples from a clinical study (human plasma EDTA) were selected based on previous RIA data in order to obtain a varied sample set with respect to subject, timepoint and measured concentration. A single aliquot was analysed for each sample for each method. In order to compare results sample batches were required to be valid analytically. Valid sample results from both methods were then compared and the percentage difference calculated using the following equation:

% Difference = 100 (RIA result – LC-MS/MS result/Mean of RIA and LC-MS/MS results)

Results were considered to be matching pairs if the percentage difference was less than or equal to 30 (this value is used routinely for ligand binding assays). For overall methodologies to be considered consistent at least 75% of result pairs should match. 47 out of 48 sample pairs were in agreement and of these 15 were measurable results for both techniques (the remaining pairs were less than method LLOQ for one or both techniques and therefore considered to be matching pairs). Results are tabulated in Table 3 and measured results shown graphically in Figure 6.

SAMPLE ID NUMBER	LC/MS/MS CONC. pg/mL	RIA CONC. pg/mL	% DIFFERENCE	INTER-TECHNIQUE ISR CRITERIA MET
1	<2.00	<6.27	NR	✓
2	21.3	18.9	**11.9**	✓
3	3.04	<4.16	NR	✓
4	<2.00	<7.05	NR	✓
5	18.1	20	**-10.0**	✓
6	1.27	<6.2	NR	✓
7	2.12	<7.93	NR	✓
8	30.7	27	**12.8**	✓
9	8.38	<6.91	NR	✓
10	<2.00	<6.09	NR	✓
11	17.5	10.9	**46.5**	X
12	1.08	<4.71	NR	✓
13	<2.00	<5.38	NR	✓
14	21.4	25.2	**-16.3**	✓
15	0.584	<5.71	NR	✓
16	<2.00	<5.41	NR	✓
17	32.4	24.9	**26.2**	✓
18	1.77	<3.84	NR	✓
19	<2.00	<6.73	NR	✓
20	39.5	33.8	**15.6**	✓
21	1.56	<6.27	NR	✓
22	<2.00	<7.93	NR	✓
23	50.8	60	**-16.6**	✓
24	2.84	<5.88	NR	✓
25	<2.00	<4.28	NR	✓
26	42.6	40.8	**4.3**	✓
27	2.42	<3.69	NR	✓
28	<2.00	<5.98	NR	✓
29	64.3	72.8	**-12.4**	✓
30	3.56	<5.53	NR	✓
31	<2.00	<5.27	NR	✓
32	53.3	48.9	**8.6**	✓
33	3.82	<4.06	NR	✓
34	<2.00	<5.68	NR	✓
35	27.3	23.8	**13.7**	✓
36	1.69	<5.53	NR	✓
37	<2.00	<6.23	NR	✓
38	64.5	53.8	**18.1**	✓
39	3.47	<4.07	NR	✓
40	<2.00	<4.43	NR	✓
41	36.2	29.3	**21.1**	✓
42	2.35	<3.77	NR	✓
43	<2.00	<4.47	NR	✓
44	50	39.2	**24.2**	✓
45	2.44	<3.8	NR	✓
46	<2.00	<4.78	NR	✓
47	48.1	46.7	**3.0**	✓
48	7.00	<4.99	NR	✓

Table 3. *Incurred sample comparison*

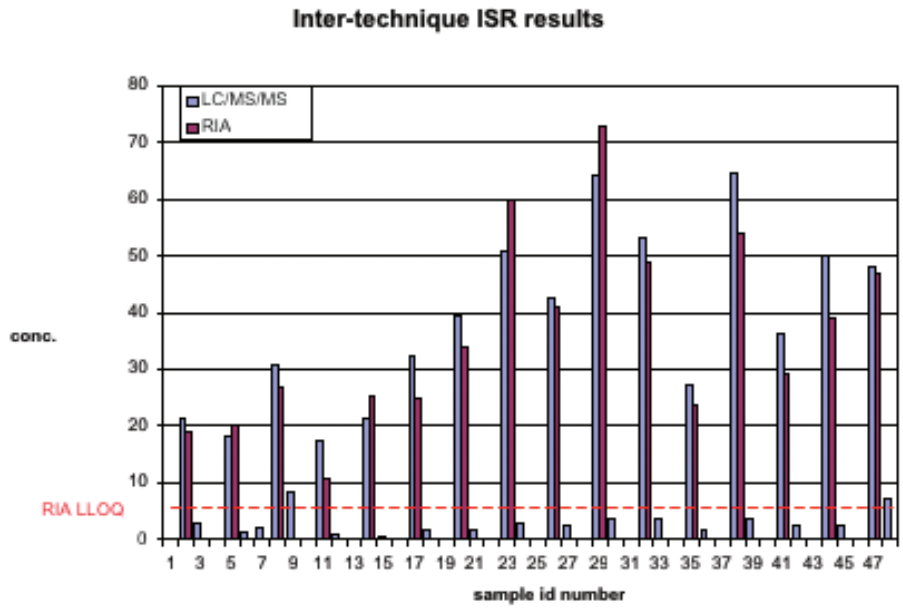

Figure 6. *Inter-technique incurred sample results*

CONCLUSION

The results presented here demonstrate the successful development and validation of a sensitive, selective and robust LC-MS/MS method for the determination of the small peptide Desmopressin in human plasma. The combination of extract cleanliness, LC peak focusing and use of an MRM summation approach provide excellent sensitivity which addresses the low limits of quantitation required for this therapeutic peptide. Results generated with incurred samples illustrate the LC-MS/MS method is very comparable with RIA (the previous method of choice for this type of compound). In addition LC-MS/MS offers additional benefits of improved sensitivity, robustness and throughput and reduced sample volume.