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Determination of oxytocin in a dilute IV solution by LC–MS^n

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ABSTRACT

The most common drug prescribed to induce labor in the United States is oxytocin, a peptide hormone composed of nine amino acids. Oxytocin is often reconstituted in intravenous (IV) saline solutions at less than 0.05 units ml^{-1} (125 ng ml^{-1}) to be delivered at 1–4 drops per minute. Existing LC–UV methods for oxytocin do not have sufficient detection limits to quantitate and/or confirm oxytocin in IV solutions without sample concentration. A determinative and confirmatory method for oxytocin was developed using an LC–MS^n ion trap instrument with an electrospray ionization (ESI) interface in positive ion mode. Separation was achieved on a C-18 column using an isocratic elution of water with 50% acetonitrile (v/v) and water with 0.05% formic acid (v/v) at a flow rate of 250 μl min^{-1}. Data was acquired from the selected ion monitoring (SIM) of the precursor ion (m/z 1007.3) and MS^2 scans from the collision induced dissociation of m/z 1007.3 at 30% collision energy. In this method, MS^2 full scans were utilized to obtain three structurally significant ions for the unambiguous identification of oxytocin. Calibration standards, prepared in de-ionized water from 0.006 to 0.046 units ml^{-1}, were linear with an R^2 value of 0.9983. The methods LOD and LOQ were 0.00084 and 0.0029 units ml^{-1} (2 and 7 ng ml^{-1}), respectively. This LC–MS^n method was used to determine the amount of oxytocin in a 0.04 units ml^{-1} clinical sample that was prepared in 0.9% sodium chloride IV solution.

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1. Introduction

1.1. Physiology of oxytocin

The physiological properties of oxytocin from pituitary extracts were first discovered by Sir Henry H. Dale in 1909 [1]. Vincent du Vigneaud began his study of oxytocin thirty years later. By 1953, du Vigneaud had identified the structure of oxytocin, including the biologically important disulfide bond between the two cysteines that creates a 20 member ring in the peptide hormone [2]. The amino acid sequence of oxytocin is shown in Fig. 1. Oxytocin is a biologically important hormone and neurotransmitter. Numerous studies have attempted to forge links between basic and clinical research in the area of neuropeptides and social behavior. Basic research in animals has established the importance of oxytocin in affiliation, including mating, pair bonding and maternal/parenting behaviors. In addition it was also reported to be an important regulator in feeding, grooming and response to stress [3]. The findings from studies of healthy humans parallel those from animal studies and point to the role of oxytocin in stress response and in enhancing social affiliation [4]. Oxytocin coordinates both the causes and effects (e.g. reduced blood pressure) of positive social interactions [5]. In addition to its effect on the central nervous system, oxytocin release from the posterior pituitary and into the circulatory system exerts action on distant target organs. For example, elevated levels of oxytocin in the blood stream induce the uterine contractions necessary for labor and facilitate the release of mother's milk [3].

1.2. Indications for oxytocin

Oxytocin has been prescribed for the augmentation of labor since 1970 as part of the "active management of labor" popularized by Dudley [6]. Since oxytocin is destroyed in the gastrointestinal tract, it is frequently administered by injection. An intravenous (IV) drip solution for labor induction is often prepared at concentrations less than 0.05 units ml^{-1} (125 ng ml^{-1}) [6,7]. Unknowingly administering a sub-potent dose of oxytocin may fail to improve uterine contractions when prescribed for a slow and difficult labor; this may further endanger the mother or fetus. Under-potency may also fail to produce uterine contractions during the 3rd stage of labor

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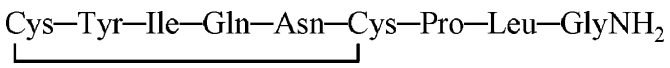

Fig. 1. The peptide oxytocin is represented by the amino acid sequence: cysteine–tyrosine–isoleucine–glutamine–asparagine–cysteine–proline–leucine–glycine.

where it is prescribed to control postpartum bleeding or hemorrhage. An overdosage of oxytocin can lead to tumultuous labor, uterine rupture, fetal hypoxia, or death [6]. Thus, inaccurate IV dosage of oxytocin can lead to serious health complications for both the mother and fetus.

1.3. Analysis of oxytocin

Oxytocin determination is often performed by radioimmunoassay (RIA) because it allows the detection of very low levels (fmol ml^{-1}) of oxytocin present in plasma and cerebral spinal fluid with little sample preparation [8–12]. However, RIA is an indirect method that is subject to interference by specific and non-specific matrix factors present in biological samples [13]. RIA is also sensitive to interference by high salt concentrations, pH variations and antibody binding by divalent cations (e.g. Ca^{2+}, Mg^{2+}) [14,15]. Reliable measurement of oxytocin by RIA requires the use of antibodies with a high affinity for oxytocin and low cross-reactivity with resembling substances (e.g. vasopressin). Even when highly specific antibodies are used, data obtained by RIA should be expressed as analyte–immunoreactivity levels, rather than analyte levels. As a direct measurement approach, a combination of solid-phase extraction, antibody based immunoaffinity column purification and dual channel coulometric detection was used by Kukucka and Misra [15] to enhance the quantitative determination of oxytocin from biological samples. LC–UV methods for oxytocin exist for bulk product in the range 0.8–160 units ml^{-1} (2–400 μg/ml) [16], but solid-phase extraction (SPE) was needed for plasma and IV solutions to concentrate the low levels encountered in these matrices [7,17,18]. Bridges and Marino [14] encountered significant losses in the quantitative recovery of oxytocin when sample cleanup was attempted with SepPak C-18 ODS SPE cartridges (Millipore-Waters) prior to LC. Chaibva and Walker [19] developed a stability indicating method for the quantification of oxytocin in pharmaceutical dosage forms by LC–UV. This method has a linear range between 0.4 and 12 units ml^{-1} (1–30 μg ml^{-1}) and a detection limit of 0.1 unit ml^{-1} (0.25 μg ml^{-1}). By their method, Chaibva and Walker used a 1:5 sample dilution to assay a commercially available 10 units ml^{-1} (25 μg ml^{-1}) oxytocin parenteral preparation. Unfortunately, in our laboratory, we had the need to analyze clinical samples at concentrations (<0.05 units ml^{-1}) below the detection limit of that method. Other LC–UV methods have been developed to determine oxytocin from sample preparations over the range 0.2–20 units ml^{-1} (0.5–50 μg ml^{-1}) [20–22], however, these methods also were not sensitive enough for our clinical sample without first concentrating it by SPE. Sutcliffe and Corran [23] used UV detection for the analysis of neurohypophysial peptides including oxytocin after separation by HPLC and capillary zone electrophoresis (CZE). Laser induced fluorescence (LIF) was found to be a more sensitive detection scheme compared to UV for CZE analysis of oxytocin by Šolínová et al. [24], but this method required analyte derivatization with a fluorescent probe.

Alternatively, liquid chromatography coupled to a mass spectrometric detector (LC–MS) offers some advantages in the analysis of oxytocin at very low concentration levels. Namely, the sensitivity and selectivity of MS often eliminates the need for time consuming sample concentration steps. Numerous peptide amino acid sequences and fragmentation mechanisms have been determined by MS [25–27]. The analysis of peptides by MS has also been the subject of many review articles including those focused on food [28] and biological samples [29]. Recent research in the analysis of oxytocin from biological samples used RIA for quantification with time-of-flight (TOF) or electrospray ionization (ESI)–MS used to identify and confirm the peptide [30,31]. Toll et al. [32] characterized the separation and identification of peptides, including oxytocin, by reverse-phase ion-pair LC–MS, however, ion-pairing reagents represent potential problems for the robustness and sensitivity of the LC–MS interface. Huck et al. [33] studied the degradation of vasotocin, oxytocin and octreotide by thio-disulfide exchange reactions with glutathione (GSH) employing LC–UV, LC–ESI–MS and LC–TOF–MS. The identification of degradation products using MS detection by Huck et al. [33] demonstrates the ability of LC–ESI–MS methods to monitor the stability of oxytocin in pharmaceutical dosage forms.

In this work, we report an LC–MS^n method that was developed to analyze a clinical IV sample. By using LC–MS^n in the determination of oxytocin, the method described herein achieved detection limits an order of magnitude below previous LC–UV methods. The need arose for this sensitive method for oxytocin when a pharmaceutical IV preparation was collected as part of a risk-based enforcement program that monitors sub and super-potent drugs used in sensitive and vulnerable patient populations. The resulting LC–MS^n method is a rapid and sensitive method for the determination of oxytocin in a saline solution without the need for a sample concentration step.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile (ACN) was high purity, HPLC grade purchased from Burdick & Jackson (Muskegon, MI, USA). Oxytocin standard was obtained from the U.S. Pharmacopeia (Rockville, MD, USA). ACS grade glacial acetic acid was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). Sodium chloride was ACS reagent grade purchased from Sigma–Aldrich Chemical Co. Inc. (St. Louis, MO, USA). De-ionized (DI) water was prepared from a Milli-Q Plus system at 18.2 MΩ cm (MilliPore, Bedford, MA, USA).

2.2. Standards

A 9.20 units ml^{-1} (23 μg ml^{-1}) stock standard solution of oxytocin was prepared by quantitatively diluting the contents of one vial of USP Oxytocin (46 units per vial) to 5.00 ml with DI water. This stock standard solution was stored in a refrigerator between 4 and 6 °C. Both the USP standard and the clinical sample referred to oxytocin concentration as units ml^{-1}. For this reason, all reference to oxytocin concentration in this text is given in units ml^{-1}. For reference, there are 2.5 μg of oxytocin per unit [35]. Working standards were prepared fresh daily by making the appropriate dilutions with DI water. Five standards were prepared for the calibration curve using the level of interest as the approximate midpoint of the curve. Calibration standards were prepared in DI water from 0.006 to 0.046 units ml^{-1} (15–115 ng ml^{-1}). Linearity was determined from the coefficient of correlation (R^2). Calibration curves were obtained by plotting the mass to charge ratio (m/z) 1007.3 selective ion monitoring (SIM) chromatogram peak area versus concentration with linear regression analysis.

2.3. Method validation samples

For method validation, oxytocin fortified samples were prepared in 0.45% sodium chloride solution (w/v) to evaluate any

matrix interference or ion suppression. Method validation samples were prepared in triplicate at the following three levels: 0.009, 0.018, and 0.035 units ml^{-1} to determine accuracy and precision. Validation samples were also prepared at 0.003 units ml^{-1} to evaluate the sensitivity of the method. These samples were prepared fresh daily. DI water was used as the reagent blank, and a matrix blank was prepared using a 0.45% sodium chloride solution (w/v) in DI water. Oxytocin recovery was determined by $([C_{calculated}]/[C_{nominal}] \times 100)$. Intra and inter-day precision of oxytocin in saline solution was evaluated by calculating the relative standard deviation (RSD) at three concentration levels with three replicate samples on three different days. Standards were analyzed with each set of samples. Reagent blanks and matrix blanks were analyzed to ensure that there was no carryover or matrix interference.

2.4. Sample preparation

The clinical sample was reported to contain 20 oxytocin units in 500 ml of a 0.9% sodium chloride (w/v) solution to be delivered intravenously. In order to reduce the amount of salt entering the MS, the sample was diluted 1:1 (v/v) with water and transferred to an autosampler vial for analysis by LC–MS^n. A duplicate sample was prepared in the same manner. The IV bag containing the sample was kept refrigerated between 4 and 6 °C.

2.5. LC–MS^n instrument and conditions

Samples were analyzed using an Agilent 1100 capillary LC system (Agilent Technologies, Inc., Santa Clara, CA, USA) coupled to a Thermo Deca XP Plus ion trap mass spectrometer (ThermoFisher Scientific Corp., Waltham, MA, USA). Sample and standard solutions were separated on an Agilent Zorbax SB-C18 5 μm, 2.1 mm × 150 mm column at 25 °C. The mobile phase used was a mixture of water with 50% acetonitrile (v/v) (B) and water with 0.05% formic acid (v/v) (A) set to a flow rate of 250 μl min^{-1}. Isocratic elution was employed using 36% B with a 7 min run time. The injection volume was 20 μl. The LC flow was diverted away from the MS for the first 3 min. The flow was directed to the MS from 3 to 5.5 min, and the flow was again diverted away from the MS from 5.5 to 7 min. The ion transfer capillary was operated at a temperature of 350 °C. The sheath gas was nitrogen set at 60 (arbitrary units). The auxiliary gas flow of nitrogen was set to 55 (arbitrary units). The spray voltage was 3.5 kV and the capillary voltage was 24.0 V. In the tune file, the MS SIM parameters were set to a prescan of 5 and a maximum inject time of 200 ms. The MS^2 parameters used a prescan of 2 with a maximum inject time of 400 ms. The isolation width was set to m/z 3.0 for all MS^n transitions. Using the ion trap, MS and MS^2 scans were performed according to the following program:

Time segment 1: 3–5.5 min

- Scan event 1: (+) MS SIM [m/z 1007.3].
- Scan event 2: (+) MS^2 m/z 1007.3 with 30% collision energy scanning for product ions [m/z 350.0–1100.0].

Table 1
Average recoveries and repeatability of oxytocin at three levels of spiking in 0.45% saline solution

	Recovery of spikes			Average (%)	RSD (%)
0.009 units ml^{-1}					
Day 1	107.1	107.4	104.7	106.4	1.4
Day 2	116.3	119.4	119.8	118.5	1.6
Day 3	131.5	122.1	121.4	125.0	4.5
Interday				116.6	7.5
0.018 units ml^{-1}					
Day 1	103.1	102.9	103.6	103.2	0.4
Day 2	121.5	118.2	115.0	118.2	2.7
Day 3	121.0	123.1	124.8	123.0	1.6
Interday				114.8	7.9
0.035 units ml^{-1}					
Day 1	93.5	95.1	89.9	92.8	2.9
Day 2	103.0	111.3	98.8	104.4	6.1
Day 3	106.5	111.4	103.9	107.3	3.6
Interday				101.5	7.6

3. Results

3.1. Method development

The MS analysis of oxytocin was developed using the tune function of the instrument. The oxytocin USP standard (9.2 units ml^{-1} in de-ionized water) was pumped through a syringe pump at 10 μl min^{-1} and then introduced into the LC mobile phase conditions stated above at a flow of 250 μl min^{-1} via a "T" before entering the MS source. By tuning on the $[M+H]^+$ ion m/z 1007.3, capillary and lens voltages were ramped until the response for oxytocin was optimized. A collision energy setting of 30% was found sufficient to fragment oxytocin into structurally specific product ions. For quantification, an MS SIM scan was performed on the $[M+H]^+$ ion, m/z 1007.3. Analyte confirmation was accomplished with an MS^2 full scan fragmenting m/z 1007.3 with 30% collision energy and observing the product ions from m/z 350.0 to 1100.0. Three significant product ions were used to positively identify oxytocin. In order to be confirmed, the retention time of oxytocin found in samples had to

Table 2
Confirmation data of oxytocin showing retention times and the relative abundance ratios of standards, spiked samples, and IV samples

Sample	RT	Relative abundance m/z 1007.3 → m/z		
		723.1	973.2	933.2
0.046 unit ml^{-1} standard	4.31	100	36.8	20.4
0.018 unit ml^{-1} standard	4.33	100	35.9	21.0
0.009 unit ml^{-1} standard	4.29	100	30.2	20.3
0.006 unit ml^{-1} standard	4.29	100	21.6	25.2
Saline blank	ND[a]	–	–	–
Clinical IV sample	4.39	100	35.8	32.1
Clinical IV sample	4.38	100	36.8	19.2
0.003 unit ml^{-1} spike	4.35	100	37.1	28.5
0.009 unit ml^{-1} spike	4.35	100	31.9	23.0
0.018 unit ml^{-1} spike	4.39	100	38.5	23.1
0.035 unit ml^{-1} spike	4.32	100	36.1	20.4

There are 2.5 μg of oxytocin per unit.
[a] ND = none detected.

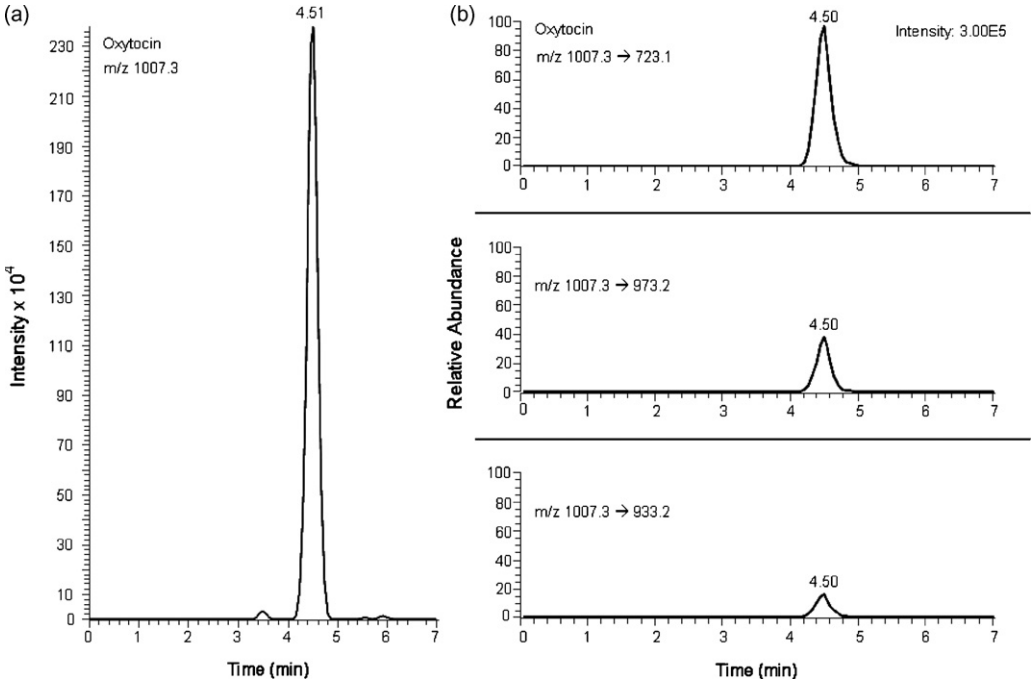

Fig. 2. Selected ion chromatograms of a clinical IV saline sample containing oxytocin analyzed by (a) MS SIM scan of the *m*/*z* 1007.3 precursor ion and (b) MS^2 of *m*/*z* 1007.3 with 30% collision energy showing the product ions *m*/*z* 723.1, 973.2 and 933.2.

match within 5% of that for standards analyzed on the same day, and the relative abundance of the three transitions (*m*/*z* 1007.3 → 973.2, 933.2, 723.1) had to match within ±20% of that for the standards [35,36].

3.2. Linearity, accuracy and precision

Calibration standards, 0.006–0.046 units ml^{-1}, were linear with an R^2 value of 0.9983 using results from the SIM chromatograms of *m*/*z* 1007.3. Standards were run with each set of samples. Reagent and matrix blanks proved to be free of oxytocin or interference. Spiked samples compared favorably with the calibration standards demonstrating that there was no matrix interference. The average recovery of oxytocin from fortified samples at 0.009, 0.018, and 0.035 units ml^{-1} is shown in Table 1. The overall recovery was 111.0% (±9.6% RSD, $n = 27$). Intra and inter-day precision of oxytocin in saline solution (Table 1) was evaluated at three concentration levels with three replicate samples on three different days. At all three levels, both intra-day and inter-day variability was less than 10% (RSD). All sample retention times were within ±5% of the average observed for standards analyzed on the same day (Table 2).

3.3. Sensitivity

The limit of detection (LOD) was determined by multiplying the 99% confidence level Student's *t*-test with the standard deviation of the 0.003 units ml^{-1} spike level. The limit of quantification (LOQ) was established as 10 times the standard deviation at the 0.003 units ml^{-1} spike level. The method LOD and LOQ were 0.00084 and 0.00291 units ml^{-1}, respectively. This is equivalent to 2.1 and 7.3 ng ml^{-1}.

3.4. Determination of oxytocin in IV solution

Oxytocin concentrations in IV fluid are typically 0.04–0.05 units ml^{-1} (100–125 ng ml^{-1}). This method was used to determine the amount of oxytocin in a 0.04 units ml^{-1} clinical sample that was prepared as a 0.9% sodium chloride IV solution. The MS SIM scan of *m*/*z* 1007.3 and the three significant product ions resulting from the MS^2 scan for the clinical IV sample is shown in Fig. 2. Analysis of this sample in duplicate revealed 0.0375 and 0.0370 units ml^{-1}, respectively. At 93.7% of the declared amount, the sample was within the USP-29 limits that state each ml of

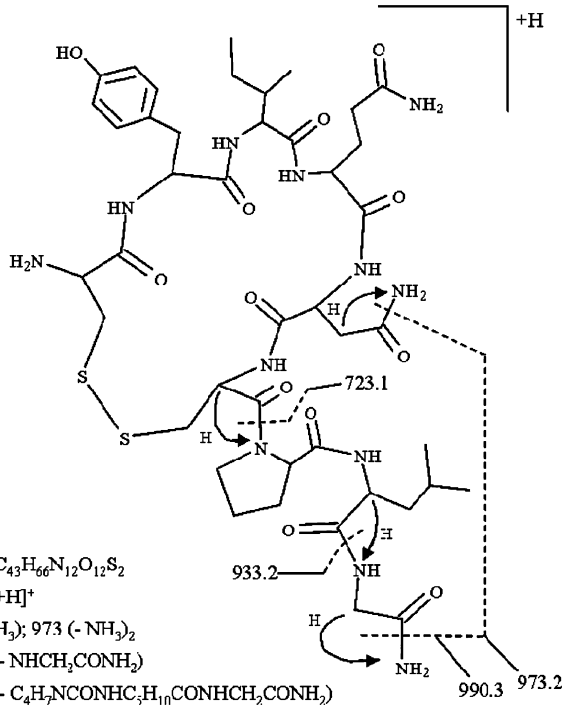

Fig. 3. Chemical structure of oxytocin with possible fragmentation mechanisms.

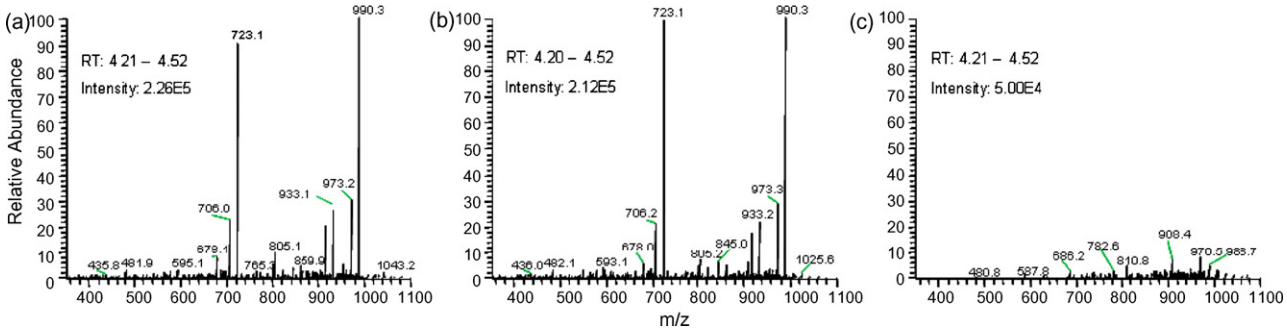

Fig. 4. MS^2 full scan spectra showing CID of m/z 1007.3 with 30% collision energy using ESI in the positive ion mode for (a) an IV solution containing oxytocin, (b) 0.018 units ml^{-1} oxytocin in saline, and (c) a saline blank sample.

oxytocin injection contain 90.0–110.0% of the oxytocin declared value listed on the label [34].

4. Discussion

Since this method was developed to determine oxytocin in a saline solution, the samples were expected to be free of co-extractants that might interfere with the assay. However, sufficient retention of oxytocin is required to separate it from the salt in solution which could interfere with the MS analysis. Oxytocin had a retention time of 4.3 min on the Zorbax SB-C18 column using an isocratic elution of aqueous acetonitrile and formic acid as described above. In addition to diluting the sample to reduce the salt content, the LC flow was diverted from the MS for 3 min. This prevented the salt from reaching the ionization source where it could interfere with sample analysis.

Analytical results showing that each millilitre of oxytocin injection contains less than 90.0% of the oxytocin declared value listed on the label [34], would indicate improper preparation of the oxytocin IV solution or degradation of the oxytocin due to improper handling and/or storage conditions. Research by Huck et al. [33] showed that structural elucidation of oxytocin degradation conjugates was also possible using LC–ESI–MS analysis. While the method presented in our paper was developed for the quantification and confirmation of oxytocin in clinical samples, the method likely could be expanded to include the detection of the degradation products described by Huck et al. by including a full MS scan from m/z 200.0–2050.0.

Oxytocin has a molecular mass of 1006.0 g mol^{-1}. The precursor ion used for MS/MS analysis of oxytocin is m/z 1007.3 $[M+H]^+$, which initially loses a terminal amine group (m/z 990.3). Loss of two terminal amine groups results in the product ion m/z 973.2 ($-2\times$ NH_3). Oxytocin, m/z 1007.3 $[M+H]^+$, also has a primary loss to m/z 933.2 when it loses the protonated Gly–NH_2 terminal group ($-NHCH_2CONH_2$). Collision induced dissociation (CID) of the m/z 1007.3 precursor ion of oxytocin also leads to m/z 723.1 from the loss of the Pro–Leu–Gly–NH_2 tripeptide from the cyclic polypeptide. Under the conditions stated in the method parameters, the 20 member ring was very stable and remained largely intact. The chemical structure of oxytocin with these fragmentation mechanisms is shown in Fig. 3.

MS^2 full scans were utilized to obtain three structurally significant ions for unambiguous identification of oxytocin. Full scan sample spectra should visually match the spectrum obtained from a standard with a general correspondence between relative abundances obtained for sample and standard [36]. MS^2 spectra of oxytocin, averaged across the chromatographic peak of oxytocin, are shown in Fig. 4. This figure shows that the two predominant product ions from fragmentation of m/z 1007.3 were m/z 990.3 (100% average relative abundance) and m/z 723.1 (99% average relative abundance). Because the m/z 723.1 ion is more structurally specific compared to the m/z 990.3 ion, the relative abundance ratios were calculated relative to the m/z 723.1 ion. Table 2 shows selected confirmation data of oxytocin based on the relative abundance ratios of the ions m/z 723.1, 973.2, and 933.2. The average relative abundance ratios of the m/z 1007.3 → 973.2 and 933.2 transitions were 31.1% and 21.7%, respectively. All samples met the ±20% confirmation criteria with absolute ratios ranging from 27.5% to 43.6% for m/z 973.2/723.1 and 15.1% to 32.1% for m/z 933.2/723.1.

5. Conclusions

In this work, we report an LC–MS^n method that was developed and validated to analyze a clinical IV sample. The resulting LC–MS^n method provides structurally significant ions for concentration determination and identity confirmation of oxytocin in saline IV samples formulated at levels below 0.05 units ml^{-1}. Taking advantage of the greater sensitivity of the MS, the clinical IV sample was diluted to reduce matrix effects rather than undergoing clean-up and concentration by SPE, providing increased sample throughput over previously described LC–UV methods for the analysis clinical IV samples below 0.4 units ml^{-1} (1 μg ml^{-1}). The method described herein has a linear range of 0.006–0.046 units ml^{-1} (15–115 ng ml^{-1}). In the analysis of sample preparations below 0.05 units ml^{-1} (125 ng ml^{-1}), this LC–MS^n method represents a significant improvement over previously described LC–UV methods which are capable of determining oxytocin from sample preparations over the range 0.1–20 units ml^{-1} (0.25–50 μg ml^{-1}). To our knowledge, this paper represents the first method to use LC–MS^n for the quantification of oxytocin in saline IV sample preparations at concentrations below 0.05 units ml^{-1} (125 ng ml^{-1}).

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