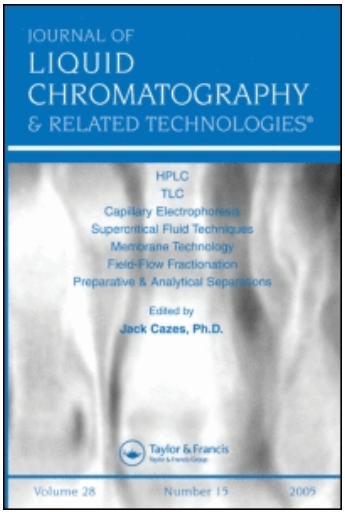

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Vipul Kumar[a]; Rajanikanth Madabushi[a]; Hartmut Derendorf[a]; Lisa A. Boothby[b]; Burnis D. Breland[b]; Randy C. Hatton[c]; Paul L. Doering[c]
[a] Department of Pharmaceutics, College of Pharmacy, University of Florida, Gainesville, FL, USA [b] Drug Information Services, Columbus Regional Healthcare System, Columbus, Georgia, USA [c] SHANDS Drug Information and Pharmacy Resource Center, College of Pharmacy, University of Florida, Gainesville, Florida, USA

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Vipul Kumar, Rajanikanth Madabushi, and Hartmut Derendorf
Department of Pharmaceutics, College of Pharmacy, University of Florida, Gainesville, FL, USA

Lisa A. Boothby and Burnis D. Breland
Drug Information Services, Columbus Regional Healthcare System, Columbus, Georgia, USA

Randy C. Hatton and Paul L. Doering
SHANDS Drug Information and Pharmacy Resource Center, College of Pharmacy, University of Florida, Gainesville, Florida, USA

Abstract: Oxytocin is an important therapeutic peptide; it is commonly used for the induction and augmentation of the first stage of labor. A simple, isocratic, reproducible, and selective HPLC method was developed and validated to study oxytocin stability in Ringer's Lactate. A C_8 column with mobile phase of acetonitrile and potassium dihydrogen orthophosphate buffer 0.05 M, pH-7.0 (20:80, v/v) at a flow rate of 1.25 mL/min was used. The detection wavelength was 220 nm and 100 μL of sample was injected. Ethyl p-aminobenzoate was used as the internal standard (I.S.). The retention time for oxytocin and I.S were 8.5 and 17 min, respectively, with a total run time of 20 min. Solid phase extraction was used and a lower limit of quantitation (LLOQ) of 0.0075 USP units/mL could be achieved. The method showed excellent linearity in the range 0.0075–0.9 USP units/mL. Oxytocin recovery ranged from 80–99%. Precision and accuracy were within the acceptable limits. The method was used to estimate oxytocin concentrations in Ringer's Lactate over an extended period of time.

Keywords: Oxytocin, HPLC, Validation, Ringer's lactate, Stability

Address correspondence to Prof. Hartmut Derendorf, Department of Pharmaceutics, College of Pharmacy, University of Florida, P.O. Box-100 494, Gainesville, FL 32610, USA. E-mail: hartmut@cop.ufl.edu

INTRODUCTION

Oxytocin is a peptide hormone that has the distinction of being the first peptide hormone to be sequenced and synthesized.[1] Today, synthetic oxytocin is used clinically for the induction and augmentation of the first stage of labor, mid trimester abortion, and the cessation or prevention of postpartum hemorrhage.[2] It is estimated that 30% of all newborn deliveries in the United States used oxytocin to induce or augment labor.[2] In clinical practice, oxytocin parenteral admixtures are often compounded in a sterile laminar flow hood, and stored at both room temperature and under refrigeration (4°C) until needed. However, there remains sparse information on the oxytocin stability in commonly used intravenous (IV) solutions at room temperature and at 4°C.[3]

The present work was aimed at investigating the stability of oxytocin in Ringer's lactate up to one month. A prerequisite for such an investigation is a precise and accurate analytical method with low sensitivity. A literature survey indicated many reported HPLC assays for oxytocin.[3–13] However, most of the methods were for biological samples with typical sample preparation steps involving radioimmunoassay with extraction or gradient elution or with long analysis time. The official USP monograph method utilizes gradient elution and does not have adequate sensitivity for our work. The required assay sensitivity for our work was at least 0.01 USP units/mL. A recently reported method with low sensitivity was tried, but the method was not reproducible and was non-specific.[3] No chromatographic resolution could be achieved after solid phase extraction (concentrating the sample ~8 fold) between oxytocin and an interfering peak when the method was tried in our laboratory.[3] Therefore, it was decided to first develop a simple, isocratic, sensitive, and reproducible analytical method for accurate estimation of oxytocin in Ringer's Lactate. The present work describes the development and validation of the HPLC assay and its utility for the stability analysis of oxytocin at two concentrations (0.06 and 0.02 USP units/mL) at room temperature and at 4°C for a one month period.

EXPERIMENTAL

Chemicals

Reference oxytocin acetate salt hydrate was purchased from Sigma, USA. Oxytocin injection (10 USP units/mL) was procured from American Pharmaceutical Partners Inc., USA. Ethyl p-aminobenzoate (Internal Standard) and chlorobutanol were procured from Sigma, USA. Ringer's Lactate solution (500 mL and 1 L) bags were obtained from Baxter Healthcare Corporation, USA. Acetonitrile (HPLC Grade), acetic acid (Analytical grade), and potassium dihydrogen phosphate (Analytical Grade) were purchased from Fisher Chemicals, USA. Solid phase extraction cartridges (C_{18}, 3 mL

capacity Bond Elut®) were obtained from Varian, USA. Triple distilled water used in analysis was prepared at an in-house distillation unit.

Equipment and Chromatographic Conditions

The HPLC system consisted of a pump LC-10AT $_{VP}$ (Shimadzu, Japan), an auto sampler Perkin Elmer Series 200 (Perkin Elmer, USA), UV detector SPD-10A $_{VP}$ (Shimadzu, Japan), and a 3396 Series-III integrator (Agilent, USA). Chromatographic separation was achieved on a Supelco C_8 column (150 mm × 4.6 mm, 5 μm) attached with a pellicular guard column. The mobile phase was composed of acetonitrile:potassium dihydrogen orthophosphate buffer 0.05M, pH-7.0 (20:80, v/v) at a flow rate of 1.25 mL/min. The mobile phase was degassed for 20 minutes in a sonicator before use. A 100 μL volume of the clear supernatant was injected into the HPLC system, and peaks were analysed at 220 nm. The chromatography was performed at room temperature.

Preparation of Standard Solutions and Diluent

The stock solution of oxytocin (60 USP units/mL) was prepared with 5% acetonitrile in distilled water. Further working stock solutions, WS-1 (0.3 USP units/mL), WS-2 (0.6 USP units/mL), WS-3 (1.8 USP units/mL), and WS-4 (2.4 USP units/mL) were prepared from the initial stock solution in mobile phase. The working stocks were used to prepare mobile phase standards with concentrations of 0.015, 0.02, 0.03, 0.06, 0.012, and 1.8 USP units/mL. To each mobile phase standard, ethyl p-aminobenzoate (I.S) stock solution (20 μg/mL in acetonitrile) was added to achieve a concentration of 400 ng/mL. All stock and standard solutions were stored at 4°C.

The calibration standards (0.0075, 0.01, 0.015, 0.03, 0.06, and 0.09 USP units/mL) and quality control samples [low (0.01 USP units/mL), medium (0.03 USP units/mL), and high (0.09 USP units/mL)] in Ringer's Lactate were prepared by spiking appropriate volumes of WS-1 to WS-4 in 6 mL of Ringer's Lactate solution.

The commercial oxytocin preparations are diluted in a diluent containing chlorobutanol as stabilizer. The diluent for the analysis was prepared by taking 500 mg of chlorobutanol, 0.5 mL acetic acid, and making up the volume to 100 mL with distilled water. The diluent solution was used as a blank to establish specificity of the assay.

Sample Preparation

The samples were processed using C_{18} solid phase extraction cartridges. The cartridges were preconditioned with 3 mL of acetonitrile and washed with

1.5 mL distilled water, followed by 1.5 mL potassium dihydrogen orthophosphate buffer (0.05M, pH-7.0). The samples (3 mL) were loaded into the cartridge and eluted with 1.5 mL elution solvent (acetonitrile:buffer, 22:78, v/v). IS (30 μL of 20 μg/mL) was added, samples were vortexed, centrifuged at 5000 rpm for 5 min, and 100 μL was injected into the HPLC.

Method Validation

The method was validated for five days in terms of the HPLC system reproducibility and specificity, lower limit of quantitation (LLOQ), and limit of detection (LOD), linearity, recovery, accuracy, and precision.[14]

HPLC System Reproducibility and Specificity

The HPLC system reproducibility was checked with five replicate injections of each analytical standard. The variations in the peak heights of oxytocin were reported as %CV. The specificity of the assay method is defined as non-interference in the region of interest. This aids in the accurate determination of analyte concentration. The specificity of the method was checked by comparing a sample containing oxytocin against the blank sample containing diluent, under normal condition and under thermal stress, by subjecting blank diluent and oxytocin samples to a temperature of 65°C for two hours. The absence of any interference in the oxytocin's region was used to determine the specificity of the method.

LLOQ and LOD

The limit of detection (LOD) for oxytocin was defined as the concentration in Ringer's Lactate solution after sample clean up that corresponds to three times the baseline noise ($S/N > 3$). The LLOQ was defined as the concentration of oxytocin in the sample that can be quantified with $<20\%$ deviation.[14]

Linearity

Linearity for calibration standards (6 point curve) was assessed by subjecting the spiked concentrations and the respective peak height ratios to a least square linear regression analysis with and without intercepts. A proper calibration model was chosen based on residuals and the coefficient of determination.[15]

Recovery

Spiked quality control (QC) samples at low (0.01 USP units/mL), medium (0.03 USP units/mL), and high (0.09 USP units/mL) concentrations of oxytocin were used for calculating the recovery of oxytocin. The samples

were processed as described in the sample preparation, and the concentration of oxytocin was determined from the mobile phase standard curve. The recovery was calculated by comparing the observed concentration with the spiked concentration.

Accuracy and Precision

For determination of accuracy and precision, QC samples at low, medium, and high concentrations were analysed in triplicates on five different days. Intra- and inter-batch accuracy was determined by calculating the % bias from the theoretical concentration using the following equation:

$$\%\text{Bias} = \frac{\text{Observed concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} \times 100$$

Inter- and intra-batch precision in terms of % RSD was obtained by subjecting the data to one-way analysis of variance (ANOVA).

Data Analysis

The peak height response of analyte and I.S. was taken and ratios were plotted against concentrations. Linear regression was performed with $y = mx+c$ to draw the calibration curve. Unknown sample concentrations were estimated using the standard curve.

Application: Oxytocin Determination in Ringer's Lactate Solution Over Extended Storage Under Different Conditions

Four bags of Ringer's Lactate solution (each containing 1000 mL + overfill) were taken, and 2 mL of oxytocin injection (10 USP units/mL) was aseptically transferred into each bag to achieve a concentration of 0.02 USP units/mL. Two bags were stored in a cold room (4–4.8°C) and two bags were stored at room temperature. Similarly, four bags of Ringer's Lactate solution (each containing 500 mL) were taken, and 3 mL of oxytocin injection (10 USP units/mL) was aseptically transferred into each bag to achieve a concentration of 0.06 USP units/mL. The bags were stored in a cold room and room temperature as described for the 0.02 USP units/mL concentrations. Samples (6 mL each) were withdrawn at 0, 0.5, 1, 2, 3, 5, 7, 10, 14, 17, 21, 25, and 31 days and were analyzed for oxytocin concentrations using the validated method.

RESULTS AND DISCUSSION

LC Optimisation

Based upon the available literature, it was observed that the most common columns for previously described oxytocin analyses were either C_{18} or C_8.[3–13] Thus, development of the method was initiated with a Lichrocart® C-18 column (100 mm × 4.6 mm, 5 μm) with acetonitrile and potassium dihydrogen orthophosphate buffer 0.05M, pH-7.0 in 50:50, v/v as the mobile phase at 1 mL/min. Under these conditions, the oxytocin peak merged with the solvent front. Oxytocin was found to be sensitive to the organic concentration in the mobile phase, and an increase in acetonitrile percentage reduced the retention time significantly. An optimised organic concentration of 20%, at a flow rate of 1.25 mL/min, yielded a chromatographic separation for oxytocin within a run time of 8 min. The LLOQ, under these conditions, was 0.02 USP units/mL. The present work required a sensitivity of at least 0.01 USP units/mL in Ringer's Lactate and, therefore, it was decided to use a 2-fold concentration in Ringer's Lactate, using solid phase extraction to achieve the required sensitivity. A solid phase extraction, as described in the sample preparation, was used; however, interference was observed in the region of oxytocin as the detection wavelength ($\lambda = 220$ nm) is in a non-specific region. No chromatographic resolution could be achieved by manipulating the mobile phase composition between the interference and oxytocin. A backwash for blank and spiked oxytocin in Ringer's Lactate with diethyl ether (3 mL) was tried. Ether wash removed the impurity; however, no consistent recoveries could be observed. One possible explanation for this observation is that ether is a polar solvent and, consequently, it extracted some oxytocin as well, in a concentration dependant manner, yielding inconsistent recoveries. It was, therefore, decided to replace the stationary phase with a C_8 (150 mm × 4.6 mm, 5 μm) column under similar conditions. A clear separation between the interfering peak and oxytocin was achieved with the C_8 column. Good linearity was observed in calibration standards of oxytocin in Ringer's Lactate solution. Under these conditions, different analogues of benzoic acid were tried as the internal standard, and ethyl p-aminobenzoate was found suitable with a total analytical run time of 18 min. Thus, the optimised method was validated and used for the estimation of oxytocin concentrations over and extended time period.

Method Validation

HPLC System Reproducibility and Specificity

The HPLC system reproducibility was checked with five replicate injections of each analytical standard. The variations in peak heights of oxytocin

were $<5\%$ at all concentration levels indicating that the system yields reproducible data.

Specificity of the assay method is defined as non-interference in the regions of the compound of interest with impurities. A clean chromatogram with no interfering peak for oxytocin was observed in the diluent blank and oxytocin sample. For thermally induced degradation samples, oxytocin peak height decreased by about 23% when kept at a temperature of 65°C for two hours. However, no interference was observed for oxytocin, which suggests the stability indicating nature of the HPLC method. Typical chromatograms of oxytocin in mobile phase standard, solid phase extracted samples of diluent blank, oxytocin test samples in Ringer's Lactate are shown in Figures 1, 2, and 3, respectively. Chromatograms of solid phase extracted samples of diluent blank and oxytocin standard under thermal stress are shown in 4 and 5, respectively.

Linearity, LLOQ, and LOD

Calibration curves obtained in Ringer's Lactate were found to be linear over a range of 0.0075–0.09 USP units/mL. An unweighted linear regression scheme ($y = mx + c$) was used for calibration standards. The values for slope, intercept, and coefficient of determination are given in Table 1. LOD

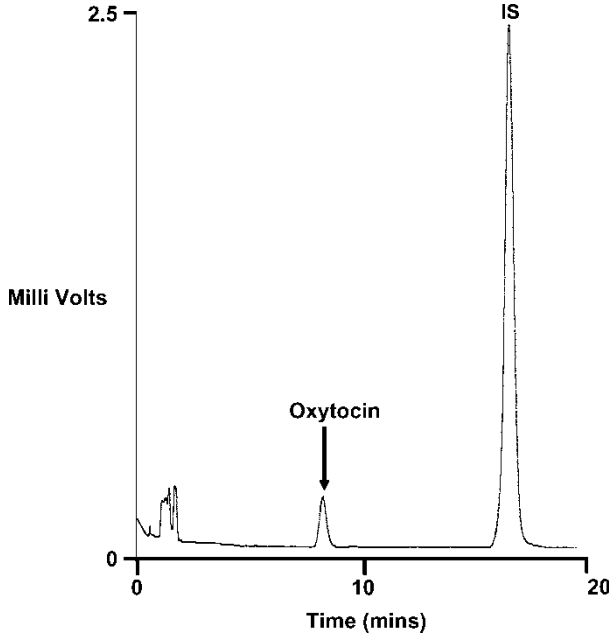

Figure 1. Representative chromatogram of Oxytocin (0.06 USP units/mL) in mobile phase standard.

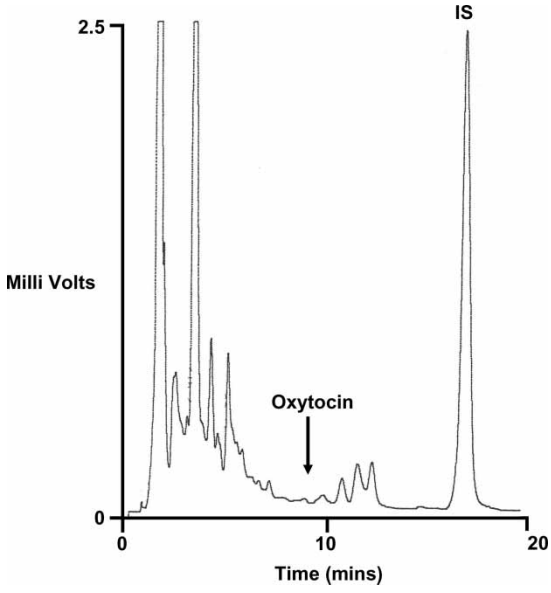

Figure 2. Representative chromatogram of diluent blank in Ringer's Lactate after extraction.

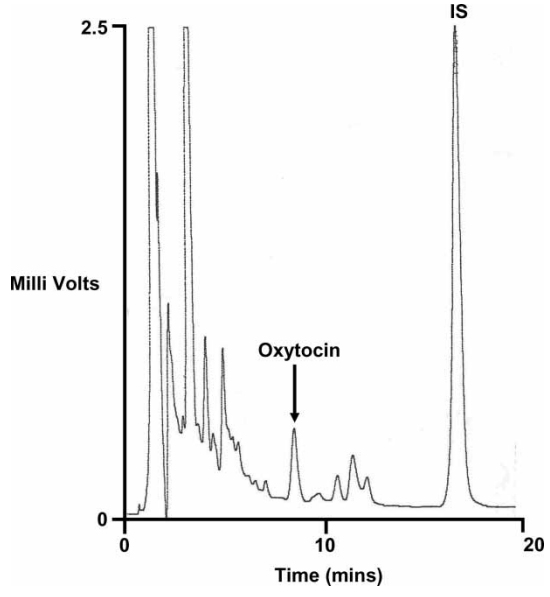

Figure 3. Representative chromatogram of oxytocin test sample (0.06 USP units/mL, concentrated two times) in Ringer's Lactate after extraction.

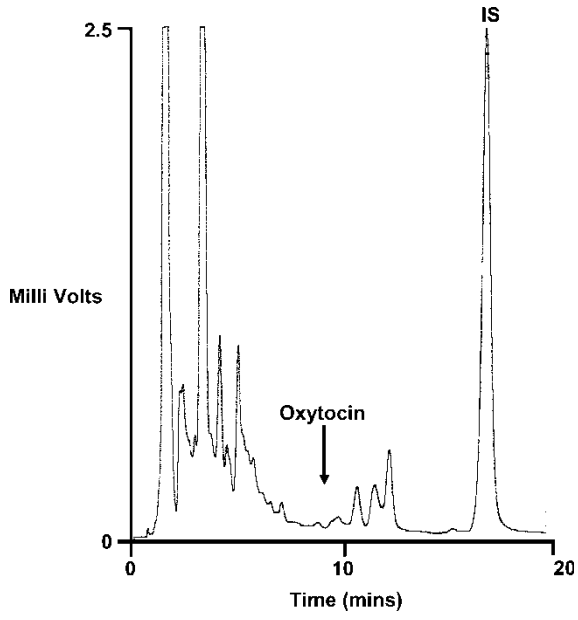

Figure 4. Representative chromatogram of blank diluent under thermal stress after extraction.

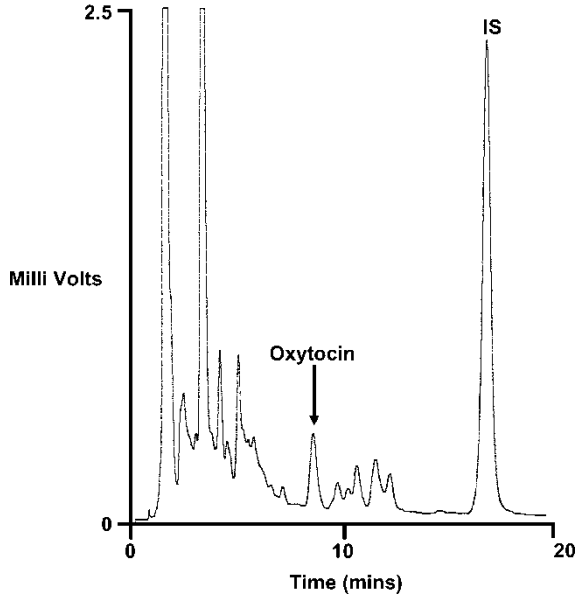

Figure 5. Representative chromatogram of oxytocin (0.06 USP units/mL, concentrated two times) in Ringer's Lactate under thermal stress after extraction.

Table 1. Calibration curve parameters summary ($y = mx + c$) for oxytocin during validation

Day	Slope	Intercept	Coefficient of determination
1	4.7962	0.0017	0.9985
2	4.9256	0.0013	0.9979
3	4.9220	0.0011	0.9975
4	4.7428	0.0058	0.9999
5	5.0749	0.0007	0.9994

and LLOQ for oxytocin in Ringer's Lactate were 0.005 and 0.0075 USP units/mL, respectively.

Recovery

The recoveries of oxytocin at the 3 QC concentrations in triplicate for 5 different days in Ringer's Lactate ranged from 90 to 99% (Table 2).

Accuracy and Precision

The parameters obtained for accuracy and precision for oxytocin in Ringer's Lactate are listed in Tables 3 and 4. The results obtained show that the method was precise and accurate with intra and inter-batch variation, within acceptable limits of $\pm 20\%$ at low and $\pm 15\%$ at other concentration levels.[14]

Table 2. Recovery % (Mean $\pm$ S.D) of oxytocin

QC samples (USP units/mL)	Recovery (Mean $\pm$ S.D)
0.01	90.25 ± 9.91
0.03	90.67 ± 3.65
0.09	92.66 ± 3.30

Table 3. Intra and inter day accuracy of oxytocin

QC samples (USP units/mL)	Inter-Batch (%)	Intra-Batch (%)
0.01	6.2	6.8
0.03	−5.5	−5.5
0.09	−1.9	−2.2

Table 4. Intra and inter day precision of oxytocin

QC samples (USP units/mL)	Inter-batch (%)	Intra-batch (%)
0.01	16.6	3.5
0.03	0.8	2.5
0.09	5.0	1.7

Application: Oxytocin Estimation in Ringer's Lactate Solution Over Extended Storage Period

The method described here was successfully applied to study the oxytocin concentration profile (0.02 USP/mL and 0.06 USP/mL) at room temperature and 4°C for one-month. The profile of oxytocin concentration over a one month period is shown in Figure. 6.

CONCLUSIONS

A simple, accurate, and reproducible isocratic HPLC-UV method for the estimation of oxytocin concentrations in Ringer's Lactate was developed and

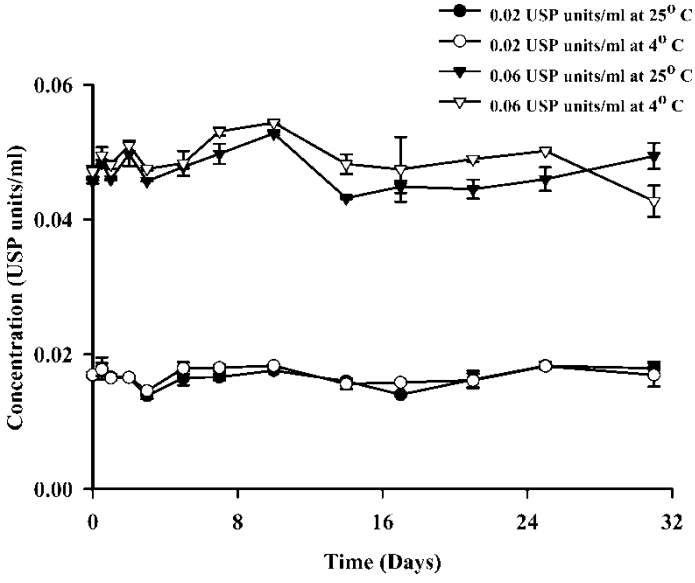

Figure 6. Oxytocin stability profile up to 31 days in Ringer's Lactate solution at two concentrations.

validated. The method was within the acceptable validation limits for accuracy and precision. The calibration curves were linear over the concentration range of 0.0075 to 0.09 USP units/mL. The stability indicating method was successfully used for determining oxytocin stability in Ringer's Lactate solution over an extended storage period.

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