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Liquid Chromatography-Mass Spectrometry Determination of Oxytocin in Cow Milk

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Oxytocin is a cyclic octapeptide hormone released by the posterior pituitary and having uterotonic and galctagenic activity in mammals. In cows oxytocin is injected to stimulate uterine contractions and allow for easy flow of milk. This study was carried out to validate a method for the determination of oxytocin in cow milk by liquid chromatography-mass spectrometric (LC/MS/MS system). The method was in-house validated in term of detection limit (LOD), quantification limit (LOQ), linearity, sensitivity, accuracy and recovery in milk. Oxytocin was isolated from milk by solid phase extraction followed by extraction with HPLC. Oxytocin was determined by mass spectrometer using turbo ion spray source in the negative mode. The multiple reaction monitoring procedure has been applied and the ion transitions were: m/z 1005 > 939 (for quantification) and 1005 > 489 (for qualification). It was found out that oxytocin can be quantitated and confirmed at the 1 IU/L level in milk. At these levels trueness ranged between 85 to 101 % and reproducibility was lower than 15 % expressed as RSD. The limit of detection (LOD) and limit of quantification of this method were 1.0 IU/L, 2.0 IU/L respectively.

Key Words: Liquid chromatography, Mass spectrometry, Oxytocin, Cow milk.

INTRODUCTION

Milk is initially secreted into small sacs within the mammary gland called alveoli, from which it must be ejected for consumption or harvesting. Mammary alveoli are surrounded by smooth muscle (myoepithelial) cells, which are a prominent target cell for oxytocin. Oxytocin stimulates contraction of myoepithelial cells, causing milk to be ejected into the ducts and cisterns.

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Oxytocin is also known as nonapeptide. Its 20-membered ring is composed of five amino acids-cystine, tyrosine, isoleucice, glutamine and aspargine. The side chain contains a further 3 amino acids -proline, leucine and glycinamide. All the optically active amino acids belong to the L-series[1].

Injection of oxytocin in cows results in the uterine contractions and hence easy flow of milk occurs. Oxytocin does not increase the amount of milk but merely makes it flow faster. It causes the equivalent of labour pains in the cow twice a day and destroys her reproductive system prematurely. She grows sterile in 4 years and is then abandoned onto the street or else slaughtered.

The methods for the determination of oxytocin were proposed by liquid-chromatography with photodiode-array detector[2]. In liquid-chromatography with photodiode-array detector or UV detector methods, the conformation of oxytocin was on the basis of retention time. In very low quantity of oxytocin, the confirmation of oxytocin peak in the matrix is very critical job because a lot of interference is come by the matrix. Suppose we analyze a milk sample then we will find many peaks due to the matrix interference in the chromatogram. So we proposed a method for the determination of oxytocin in milk by liquid chromatography mass spectrometer with lower detection. In this technique, the points of confirmation of oxytocin in milk are as below-

(i) On the basis of retention time.

(ii) On the basis of atomic mass (parent ion) of oxytocin.

(iii) On the basis of ion ratio of the daughter ion of oxytocin.

The API 3000™ LC/MS/MS system gives a significant edge with sensitivity, selectivity and reproducibility across a wide range of applications and sample types. Innovative ion optics and pumping technology ensure the lowest limits of detection and quantitation, even on the most difficult matrices. Revolutionary LINAC™ collision cell technology provides fast scan times without compromising sensitivity or mass spectral quality for superior MS/MS performance. Robust ion sources and proven Curtain Gas™ interface technology ensure reliability and uptime. Advanced software applications, including automated method development for the routine analysis of hundreds of samples per day, deliver greater throughput. Information dependent acquisition (IDA) enables unattended, automated MS to MS/MS acquisition providing better structural information. With the LINAC™ collision cell, we can analyze multiple components simultaneously. Analyst™ Software provides complete system control of data acquisition and post processing for the API 3000 system[3].

EXPERIMENTAL

Methanol, acetonitrile (HPLC grade purity) were purchased from Merck. Analytical reagent grade HCl, formic acids were obtained from Merck. During the process purified-water (Millipore) was used.

Oxytocin was obtained the analytical stock solution of 200 IU/mL. Stock solution was stored at 4 ºC.

All preparation was carried out at room temperature. Dilute the standard from 200 IU/mL with mobile phase A. The homogenized milk (2 mL) was transferred into the centrifugation tube (15 mL) and 3 mL of 0.1 M HCl was added and mixed. Centrifuge the solution at 3000 rpm for 10 min, then, the supernatant liquid was transferred into another test tube. A C-18 (RIDA C 18 column 100 mg/1 mL, R-Biopharm AG) SPE cartridge was conditioned successively with methanol (2 mL) and distilled water (2 mL). The previous extract was loaded on to the cartridge and after penetration the column was washed with distilled water (2 mL) and dried by nitrogen. The analyte was finally eluted with methanol (3 mL). The eluate was evaporated to dryness under nitrogen at 40 ºC. Dissolve the residue with 1 mL of mobile phase A. The resulting solution was filtered through 0.45 µ nylon filter paper in to the vial and inject on LC.MS/MS.

The LC/MS/MS system consists of a series 200 pump and auto sampler (Perkin Elmer) coupled to an API 3000 triple quadruple mass spectrometer (Applied Biosystems/MDS SCIEX).

HPLC elution was performed on a C-18 column (Purospher® STAR RP-18 (3 µm), 55 mm) held at a temperature of 30 ºC using a gradient solvent system. Mobile phase A was 0.3 % formic acid and mobile phase B was 0.3 % formic acid in methanol. The flow rate was 250 µL/min.

MS/MS determination was performed by operating the mass spectrometer in negative mode with a Turbo Ion Spray source, heated with 400 ºC. Capillary voltage was set at *ca.* 4.0 KV. The collision energy was separately optimized for the two selective ion transitions. Data were acquired according to the multiple reaction monitoring (MRM) approach. The optimum determined conditions by operating the mass spectrometer in negative (ESI-) mode of the interface.

RESULTS AND DISCUSSION

The infusion of a 0.1 IU/mL solution of the analyte into the mass spectrometer optimized the parameters of the mass spectrometer. The chromatographic method developed in this work allowed to achieve both a good retention of oxytocin in a reasonable time and separation of oxytocin from matrix constituents ensuring at the same time mass spectrometer compatibility.

Preliminary trial were performed by analysis of standard solution and sample extracted in reverse phase partitioning mode using a C-18 column and element made up of 0.3 % (v/v) aqueous solution of formic acid and 0.3 % (v/v) methanolic solution of formic acid with a percentage of aqueous phase varying between 70 to 90 %. Optimal conditions were obtained in corresponding to the use of an aqueous solution/organic solvent ratio of (90:10) in the mobile phase with gradient.

Method validation was carried out accordingly to in house validation procedure. The parameters for method validation are-specificity, linearity, accuracy, precession (repeatability and reproducibility), limit of detection (LOD), limit of quantification (LOQ), recovery and ruggedness. The accuracy and precision was determined by analyzing 8-point standard of different concentration and five injection of each standard. The accuracy was expressed in term of recovery and precession as relative standard deviation. Limit of detection & limit of quantification was declared on the basis of signal to noise ratio (calculated by software analyst 1.4.1).

Specificity: The ability of method to measure and differentiate the analyte in the presence of components that may be expected to be present. Samples of blank milk and spiked milk were analyzed as per optimal condition (Figs. 1 and 2).

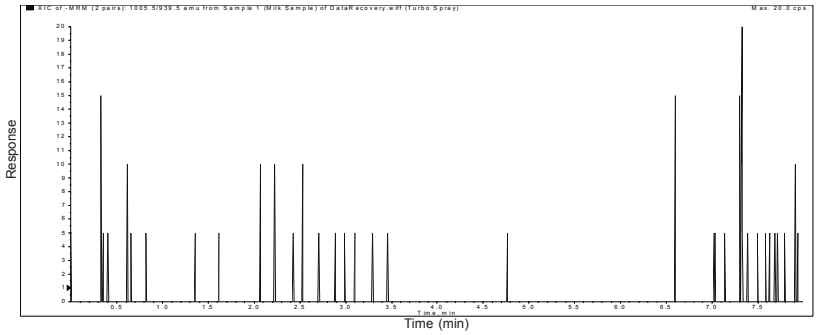

Fig. 1. Chromatogram of blank milk sample

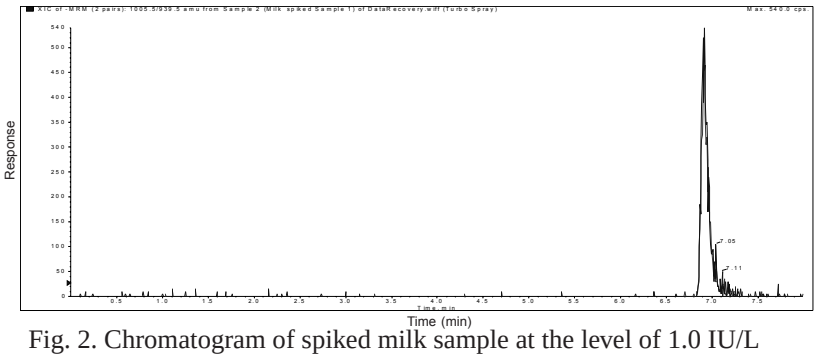

Fig. 2. Chromatogram of spiked milk sample at the level of 1.0 IU/L

Linearity: The linearity of the method was demonstrated for standard solutions in the range were prepared and analyzed as per the method. Calibration curves were found the linear in the range of 1.0 to 10.0 IU/L for LC/MS/MS.

Recovery: After adding oxytocin to milk at a concentration 1.0, 2.0 and 3.0 IU/L, the oxytocin were extracted and cleaned up by solid-phase extraction and the sample solutions were analyzed by LC/MS/MS under the optimal conditions. TIC profiles are shown in Fig. 2. The oxytocin peak is clearly observed in TIC profiles indicating the determination of oxytocin in milk. The average recoveries and relative standard deviation of oxytocin from milk fortified at 1.0, 2.0 and 3.0 IU/L are 90.06, 98.89 and 97.63 %, respectively in Tables 1 and 2.

TABLE-1
RECOVERY OF THE PRESENT METHOD

Analyte	Fortified level (IU/L)	Raw milk			Inter-day
		Intra-day			
		Day-1	Day-2	Day-3	
Oxytocin	1.0	82.00	92.50	94.00	
	1.0	87.50	88.00	92.50	
	1.0	85.50	100.50	88.00	
	Mean	85.00	93.67	91.50	90.06
	Std. Dev.	2.78	6.33	3.12	5.45
	RSD (%)	3.28	6.76	3.41	6.05
	2.0	98.50	109.00	100.00	
	2.0	83.50	95.25	91.75	
	2.0	104.75	100.25	107.00	
	Mean	95.58	101.50	99.58	98.89
	Std. Dev.	10.92	6.96	7.63	7.96
	RSD (%)	11.43	6.86	7.67	8.05
	3.0	93.17	100.33	103.50	
	3.0	97.33	107.17	96.67	
	3.0	94.33	93.33	92.83	
	Mean	94.94	100.28	97.67	97.63
	Std. Dev.	2.15	6.92	5.40	5.08
	RSD (%)	2.26	6.90	5.53	5.20

Precision and accuracy: The accuracy of the ESI-LC/MS/MS method was tested both in the term of precision and trueness. Under both intra-day repeatability (RSD between 2 to 11 %) and intermediate precision (5 to 8 % RSD) for milk in Table-2. Method accuracy was determined by calculating from the calibration curves that were constructed by peak area of the oxytocin and were linear over the range of 0.5 to 10.0 IU/L with correlation coefficients over 0.995. The recovery from varies sample fortified at levels of 1.0, 2.0

and 3.0 IU/L and the coefficient of variation (CV) for both the intra and inter assay precision are listed in Tables 1 and 2. And for the intra day precision was determined by duplicate assays of fortified with oxytocin at 1.0, 2.0 and 3.0 IU/L. The average recoveries and % RSD of samples were 90, 98, 97 % and 6.05, 8.05, 5.20, respectively in Table-2.

TABLE-2
INTRA-DAY AND INTER-DAY PRECISION OF THE PRESENT METHOD

Analyte	Fortified level (IU/L)	Raw milk			Inter-day
		Intra-day			
		Day-1	Day-2	Day-3	
Oxytocin	1.0	85.00 (3.28)	93.67 (6.76)	91.50 (3.41)	90.06 (6.05)
	2.0	95.58 (11.43)	101.50 (6.86)	99.58 (7.67)	98.89 (8.05)
	3.0	94.94 (2.26)	100.28 (6.90)	97.67 (5.53)	97.63 (5.20)

Limit of detection and limit of quantification: Limit of detection (LOD) and limit of quantification (LOQ) of oxytocin in cow milk are calculated on the basis of signal to noise ratio. The limit of detection of oxytocin in cow milk is 1.0 IU/L (signal to noise ratio = 31) and limit of quantification of oxytocin in milk is 2.0 IU/L (signal to noise ratio = 83).

Over all results indicates that the present method is sensitive, accurate and precise enough for monitoring of the oxytocin in milk.

Conclusion

An LC/MS/MS method that is rapid, reliable and sensitive has been developed for the identification and quantification of oxytocin in milk. The method clearly demostated with good accuracy and the ability to quantify the presence of residue. The method has been validated with a routine sensitivity limit of 1.0 to 10.0 IU/L in milk.

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