Standardization of HPTLC method for the estimation of oxytocin in edibles

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Abstract Adulteration in food stuff has been regarded as a major social evil and is a mind-boggling problem in society. In this study, a rapid, reliable and cost effective High Performance thin layer Chromatography (HPTLC) has been established for the estimation of oxytocin (adulterant) in vegetables, fruits and milk samples. Oxytocin is one of the most frequently used adulterant added in vegetables and fruits for increasing the growth rate and also to enhance milk production from lactating animals. The standardization of the method was based on simulation parameters of mobile phase, stationary phase and saturation time. The mobile phase used was MeOH: Ammonia (pH 6.8), optimized stationary phase was silica gel and saturation time of 5 min. The method was validated by testing its linearity, accuracy, precision, repeatability and limits of detection and quantification. Thus, the proposed method is simple, rapid and specific and was successfully employed for quality and quantity monitoring of oxytocin content in edible products.

Keywords Adulteration · Oxytocin · HPTLC · Validation

Introduction

Adulteration and contamination both have been practiced since ancient times and has become more sophisticated in the recent past. Various adulterants are used in the food products like urea, formaldehyde, sodium benzoate, etc. in milk (Renny et al. 2005; Lou et al. 2011; Attia et al. 2011), pesticides, heavy metals in soft drinks (Malve et al. 2008) and estrogen, oxytocin, etc. in fruits and vegetables (Sharma et al.

2009; Prasad and Sharma 2010). Among norfloxacin, dichlorofinac sodium, estrogen and oxytocin, later, is a hormonal adulterant used in the form of injections for increasing the growth rate of vegetables/fruits (Lundegardh and Uvnas-Moberg 2006; Siddiqui 2009) and production of milk from animals (Kavitha and Kumar 2010). Milk obtained from milk producing animals does not contain oxytocin naturally but when injected with hormones like oxytocin with the range of dose as 0.1–20 IU (Graf 1969, 1970; Nostrand et al. 1991; Ballou et al. 1993; Lollivier et al. 2002; Ijaz and Aleem 2006), there is over production of milk and traces of oxytocin can be found in the same. When excess of oxytocin is found to be present in edibles it may cause headache, nausea, abdominal pain, drowsiness, etc. to the consumer (MacDonald et al. 2011; Satpathy et al. 2011). Oxytocin is a Schedule H drug banned in India for use on animals. In local parlance it has got many names starting from ‘cocin’ and ‘paani’ to ‘dawai’ and is easily available in almost all general stores. The use of this illegal drug (oxytocin) in fruits and vegetables for early ripening has been highlighted in daily news papers. (Trivedi 2010).

Oxytocin is chemically hemi-cystinyl-tyrosyl-isoleucyl-glutamyl-asparaginyl hemi-cystinyl-propyl-leucyl glycylamide and also known as alpha-hypophamine. It is a 20 membered ring consisting of nine amino acids - cysteine, tyrosine, isoleucine, glutamine, asparagine, cysteine, proline, leucine and glycylamide (Kavitha and Kumar 2010). Although, oxytocin is a birth hormone showing uterotonic and galactogenic activity which occurs naturally in animals, but when it is injected into plant part such as stem, it functions as growth promoter. It stimulates the plant hormones such as cytokinin that invariably causes cell division which results into biomass accumulation and hence growth of fruits and vegetables.

Analytical methods such as HPLC, LC-MS and Capillary electrophoresis have been reported for the determination of drug residues in milk (Jamal and Kalantari 2004). No method

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Table 1 Percentage recovery

S. No.	Amount applied	Amount recovered	% Recovery
1	12 ng	11.6 ng	96.6 %
2	14 ng	13 ng	92.8 %
3	16 ng	15.7 ng	98.1 %
Mean	Mean=95.8±0.7		

has been developed for its estimation in fruits and vegetables. So far, high performance thin layer chromatography, a modified version of conventional thin layer chromatography, a modern contemporary of analytical techniques and is considered highly economical, fast method for analysis (Variyar et al. 2011).

The present piece of work is aimed to develop, standardize and validate a HPTLC based analytical

method for the estimation of oxytocin in milk, vegetables and fruits.

Experimental

Instrumentation

HPTLC system (Camag, Muttanz, Switzerland) consisted of an auto sample applicator Linomat V using 100 μ L and 500 μ L syringes (Hamilton, Bonaduz, Switzerland), connected to a nitrogen cylinder in order to maintain the constant flow rate while loading samples; a TLC scanner which is connected to a PC running win-CATS version 4.4.1 and used for densitometric evaluation and a TLC Visualizer, camag twin trough chambers to develop the plates. In

Fig. 1 **a** Figure showing no peak in blank solution. **b** Peak of the standard oxytocin obtained at 10 ng concentration with mobile phase methanol: ammonia (9:1 v/v) without any interference indicating its purity

addition to this Mettler Toledo electronic balance, microliter syringes were used for the analysis.

Materials and reagents

Silica gel F₂₅₄ 'HPTLC' plates (E. Merck, Darmstadt, Germany); standard oxytocin (Sigma Aldrich Chemicals Pvt. Ltd., New Delhi); methanol, acetone and ammonia (Sigma Aldrich Chemicals Pvt. Ltd., New Delhi) were used for the present analysis. All the chemicals used were of AR grade.

Standard and sample preparation

Standard stock solution of oxytocin was prepared by dissolving 1.0 mg of oxytocin 100 ml MeOH. Stock solution (5.0 ml) was diluted to 50 ml to prepare working solution which was used for further analysis. Five samples (5 kg each) of vegetables (brinjal and bottle gourd), fruits (watermelon and papaya) and a litre of milk samples were collected from the open market only once at five different sites of Agra, India. In case of vegetables and fruits samples, the extraction of oxytocin was carried out by cold percolation (methanol) and sonicated for 60 min. The samples were chopped into small pieces, dipped in methanol (1000 ml) and left them overnight for extraction. Obtained extracts were concentrated by evaporating methanol up to a volume of 20 ml using rotavapor. Milk sample (10 ml) was diluted to twice its volume and used for the estimation of oxytocin in them.

Chromatographic analysis

Each HPTLC plate contains 10 tracks of samples and standards under following conditions: band width 6 mm; distance between tracks 3 mm; application volume of standard oxytocin 2,

4, 6, 8, 10, 12, 14, 16, 18, 20 μL in the concentration range (4–24 ng). UV scanner was set for maximum light optimization with the following settings: slit dimension, 4.00 mm $\times$ 0.30 mm, micro; scanning speed, 20 mm/s; data resolution, 100 μm /step. Remaining parameters were left as default settings. Regression analysis and statistical data were automatically generated by the win-CATS software. The developed plates were examined for qualitative and quantitative analysis in TLC scanner 3 camag HPTLC system. The plate images were recorded using HPTLC vizualizer at 254 and 366 nm. The method was validated by testing parameters like linearity, accuracy, precision and repeatability as per ICH guidelines (ICH guidelines 1999).

Method validation

In order to check the linearity, standard solution of oxytocin was applied on HPTLC plates with volume 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 μL in the concentration range of 4–24 ng. Each sample (10 μL) was loaded on the same plate. The calibration curve was constructed by plotting the peak area versus concentration with the help of win-CATS software. For accuracy, pre analyzed samples (bottle guard, watermelon and milk) were spiked with standard working solution of oxytocin (2–6 μL). The mean percentage recovery was determined and given in Table 1. The precision of the procedure is expressed in terms of variance, standard deviation or coefficient of variation of a series of measurements. Intraday precision was evaluated at three different concentration 12 ng, 14 ng and 16 ng and the results obtained are reported in terms of CV. For, interday precision same experimental protocol was repeated for five consecutive days and measurements are taken whereas for intraday precision a single selected track is scanned a number of times to get the changes in the CV values. The limit of detection (LOD) and limit of quantification (LOQ)

Fig. 2 UV-Visible spectra of standard oxytocin and samples analyzed at λ_{max} 200–800 nm, indicating oxytocin contamination of the samples

Table 2 Linearity parameters

Parameters	Values
Range	4–24 ng
Slope	68.215
Intercept	501.863
SD*	2.76 %
R ² *	0.99459

SD standard deviation
R² regression coefficient

were also determined using the formulas $3.3 \cdot (SD/Slope)$ and $10 \cdot (SD/Slope)$ (Srivastava 2011).

Results and discussion

HPTLC method development

For HPTLC analysis, initially several preliminary tests were performed with different stationary phases, mobile phases and saturation time of the solvent chamber, in attempt to obtain the best separation and resolution for oxytocin. The mobile phase consisting of methanol: ammonia (9:1 v/v) adjusted to pH 6.8 with Sodium hydroxide and sodium bicarbonate buffer gave the satisfactory separation over silica gel as stationary phase with saturation time of the solvent chamber being 5 min, at which a single peak for oxytocin appeared at the R_f value of 0.71 ± 0.02 .

Validation

Method validation is the process of verifying that a procedure yields acceptable results. The developed analytical

method was validated as per ICH guidelines using following parameters:

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the method was appraised for the purity of the target compound. The developed HPTLC method was found to be specific, since, there is no interference of the standard oxytocin peak with that of others as shown in Fig. 1.

The contaminated samples were confirmed for the presence of oxytocin in them by recording their UV-Visible spectra in the range of 200–800 nm as shown in Fig. 2. However, all the samples don't contain oxytocin in them. Peaks of oxytocin present in the sample appear at the same position (λ_{max}), where the peaks of standard oxytocin appeared.

Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test result which are directly proportional to the concentration (amount) of analyte in the sample. Every analysis was done in triplicate and the linearity parameters generated are shown in Table 2. Samples are analyzed for oxytocin content in them and the results found are given in Table 3.

Accuracy

Accuracy is the closeness of agreement between the conventional true value or an accepted reference value and the

Table 3 Oxytocin content present in samples

S. No.	Sample name	Oxytocin content over HPTLC plate (ng/10 μ l)	Oxytocin content (μ g/kg)	Average oxytocin content
1	Bottle gourd	S _A *=12.60 S _B *=13.07 S _C *=12.00 S _D *=11.65 S _E *=11.22	S _A *=50.40 S _B *=52.30 S _C *=48.00 S _D *=46.60 S _E *=44.90	48.4 $\pm$ 2.45 (μ g/kg)
2	Brinjal	Not Detected	Not Detected	Not Detected
3	Watermelon	S _A *=18.31 S _B *=19.35 S _C *=not found S _D *=19.03 S _E *=18.63	S _A *=73.25 S _B *=77.43 S _C *=not found S _D *=76.12 S _E *=74.54	75.25 $\pm$ 2.67 (μ g/kg)
4	Papaya	Not Detected	Not Detected	Not Detected
5	Milk	S _A *=10.22 S _B *=9.55 S _C *=9.77 S _D *=not found S _E *=10.23	S _A *=40.89 S _B *=38.20 S _C *=39.09 S _D *=not found S _E *=40.92	39.10 $\pm$ 2.20 (μ g/L)

*- S_A=Dayalbagh, S_B=New Agra, S_C=Sikandra, S_D=Khandari, S_E=Bodhla

found value. The accuracy of the method was determined by standard addition method (Vial and Jardi 2005). Pre analyzed samples (watermelon, bottle guard and milk samples) were spiked with known amount of the standard added in increasing order of concentration and after developing the plate, amount of oxytocin recovered on the plate was determined.

Precision

Precision of an analytical procedure is the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The CV for intraday precision was in the range of 0.71–1.62 %. Interday precision of the method were determined by estimating the corresponding CV values in five different days over a period of 1 week. The CV of the method was found in the range of 0.91–1.76 %.

Repeatability

Repeatability is the closeness of agreement between mutually independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time. During every scan, we get the similar value for peak area of the analyte, which shows the repeatability of the method.

Limit of detection and quantification

The limit of detection (LOD) is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified, whereas limit of quantitation (LOQ) of an individual analytical procedure can be termed as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ value of oxytocin were calculated and found to be 133 and 404 picograms, respectively indicating the adequate sensitivity of the method.

Conclusion

Radio immune assay and enzyme immune assay techniques are about 1,000 folds more sensitive than HPTLC method and used to measure physiological levels in blood, tissues of animals and humans. However, such methods are associated with the limitations, in general, high cost and the non availability of radioactive technique. Therefore, the present piece of work explores the possibility of measurement of hormones like oxytocin in fruits and vegetables using

HPTLC method. Further, the preconcentration of the oxytocin content in samples, has been attempted to bring oxytocin content in the detection limit of HPTLC system. Among the vegetables, fruits and milk samples studied, maximum adulteration was found in vegetables while maximum amount of oxytocin was found to be in watermelon (75.25 µg/kg) whereas milk sample contain lowest (39.10 µg/kg) oxytocin concentration. However, no adulteration was found in the samples of papaya and brinjal. The present analysis shows that the proposed method is simple, rapid, economical, specific and could be applied for quality and quantity routine analysis of oxytocin content in edibles in absence of conventional established methods.

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