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Liquid chromatographic analysis of oxytocin and its related substances

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ABSTRACT

A selective gradient liquid chromatographic (LC) method for the determination of oxytocin (OT) and its related substances in bulk drugs has been developed. The method uses a reversed-phase C18 column (25 cm × 4.0 mm i.d.), 5 μm kept at 40 °C. The mobile phases consist of acetonitrile, dihydrogen phosphate solution pH 4.4 and water. The flow rate is 1.0 ml/min. UV detection is performed at 220 nm. A system suitability test (SST) was developed to govern the quality of the separation. The separation towards OT components was investigated on different C18 columns. The developed method was further validated with respect to robustness, precision, sensitivity and linearity. A central composite design was applied to examine the robustness of the method. The method shows good precision, sensitivity, linearity and robustness. Two commercial OT samples were examined using this method. Furthermore, the method proved to be successful when applied to analyze a marketed OT formulation for injection.

1. Introduction

Oxytocin (OT) is a synthetic cyclic nonapeptide having the structure of the hormone produced by the posterior lobe of the pituitary gland that stimulates contraction of the uterus and milk ejection in mammals [1]. The chemical structures of OT and two of its related substances are shown in Fig. 1. It possesses a cyclic portion linked by a disulphide bridge between the two cysteine residues. The main use of OT in clinical practice is the induction and the augmentation of labour, control of post-partum haemorrhage and uterine hypotonicity in the third stage of labour. It is also used to stimulate lactation [2].

To our knowledge, there is no paper describing a liquid chromatographic (LC) method that allows the separation of OT and its impurities in bulk drugs. Some articles exist on isocratic LC methods for the determination of OT in dosage forms [3,4]. However, these isocratic methods use short columns and are suitable for assay only since they focus on the main peak. Applying method [3] to the separation of impurities in a bulk OT sample gave poor separation of the impurities. The methods described for the determination of related substances and assay of OT in official compendia [5,6] were also not sufficiently selective as they do not separate all the impurities of OT. Hence, the objective of this study was to develop a more selective LC method for the analysis of OT and its related substances. It should be robust, precise, selective, sensitive and linear with a reasonable total analysis time. Furthermore, the developed method should be applicable for the analysis of the drug substance as well as commercially available drug products containing OT. The suitability of different C18 columns will also be investigated towards the separation of OT and its impurities.

2. Experimental

2.1. Reagents and chemicals

HPLC gradient grade acetonitrile (ACN) and sulphuric acid were purchased from Fisher Scientific (Leicester, UK). Phosphoric acid was purchased from Riedel-de Haën (Seelze, Germany). Sodium dihydrogen phosphate dihydrate and sodium monohydrogen phosphate anhydrous were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Demineralized water was further purified by filtering through Milli-Q (Millipore, Milford, MA, USA). OT commercial samples, OT injections at concentrations of 10 IU/ml and 5 IU/ml, OT unpurified sample, acetyloxytocin, carbamido oxytocin, α-dimer and β-dimer were all donated by the WHO (World Health Organization, Geneva, Switzerland).

2.2. Preparation of standard solutions

For the investigation of the separation of OT and its impurities on the different columns, for repeatability and intermediate precision studies, a 0.50 mg/ml (~300 IU/ml) OT solution was prepared in mobile phase A (Section 2.4). For method optimization and robustness studies, the OT solution was mixed with the unpurified OT sample to prepare a solution with sufficient impurities. For linearity studies, a stock solution containing 0.625 mg/ml OT was prepared in mobile phase A and diluted with the same solvent to yield solutions in the concentration range of 0.1 μg/ml

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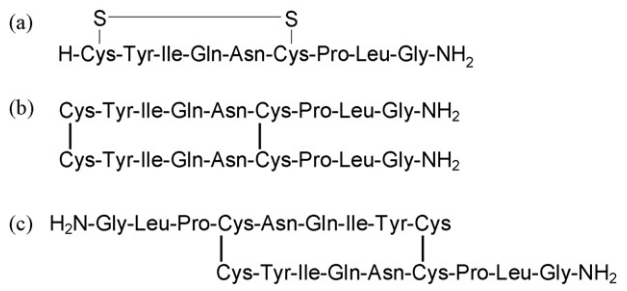

Fig. 1. Chemical structures of oxytocin (a), α-dimer (b) and β-dimer (c).

to 0.625 mg/ml, corresponding to 0.020% to 125%. The solutions were prepared and analyzed in triplicate. For the analysis of related substances, the OT test sample solution was prepared at a concentration of 0.50 mg/ml (100%) and dilutions were made to obtain 5.0 μg/ml (1%) solutions used as reference to quantify the impurity peaks in the test sample. The OT injections were used as such without further preparation. The solution enriched with impurities for method optimization and robustness studies was stored in the refrigerator. Fresh solutions were made daily for quantification experiments.

2.3. Instrumentation and liquid chromatographic conditions

LC equipment I (LaChrom, Merck Hitachi, Darmstadt, Germany) consisted of an L-7100 pump, an L-7200 autosampler, an L-7400 UV detector set at a wavelength of 220 nm and a D-7000 interface. EZChrome Elite 4.0 (Merck Hitachi) software was used for data acquisition. A Eurospher 100-5 C18 column (25 cm × 4.0 mm i.d.), 5 μm (KNAUER, Berlin, Germany) was used. The column was kept in a water bath at 40 °C and the temperature was controlled using an EC Julabo thermostat (Seelbach, Germany). The flow rate was 1.0 ml/min and the injection volume 50 μl.

For intermediate precision studies, analyses were performed by a second analyst using a different Eurospher 100-5 C18 column and LC equipment II (LaChrom Elite, Merck Hitachi) consisting of an L-2130 pump, an L-2200 autosampler and an L-2400 UV detector. Other conditions such as detection wavelength, column temperature, flow rate and injection volume were set the same.

2.4. Mobile phases

Mobile phase A consisted of 15 volumes of ACN, 15 volumes of dihydrogen phosphate solution pH 4.4 and 70 volumes of water. Mobile phase B consisted of 70 volumes of ACN, 15 volumes of dihydrogen phosphate solution pH 4.4 and 15 volumes of water. The gradient used is given in Table 1. The dihydrogen phosphate solution pH 4.4 was prepared by dissolving 31.2 g of sodium dihydrogen phosphate dihydrate in 1000 ml of purified water (0.2 M). The pH of this solution was measured as 4.4 and was used as such without adjustment.

Table 1
Gradient program proposed for the analysis of OT and its related substances.

Time (min)	Mobile phase A (%, V/V)	Mobile phase B (%, V/V)	
0–5	100	0	Isocratic
5–20	100 to 94	0 to 6	Linear gradient
20–50	94 to 60	6 to 40	Linear gradient
50–51	60 to 100	40 to 0	Linear gradient
51–65	100	0	Isocratic re-equilibration

2.5. Selection of a set of C18 columns

In order to evaluate different stationary phases for selectivity, different columns available in our laboratory were examined. The columns were chosen based on a column ranking system developed in our laboratory [7–9] and which is also freely accessible on our website [10]. In this system, characterisation of a column is performed by determining 4 chromatographic parameters. Columns are ranked according to their *F*-values, calculated versus a reference column (in this case, a Eurospher 100-5 C18 was taken) and based on the differences observed between the 4 characteristic column parameters. So, the column with the highest *F*-value deviates most from the reference column. The C18 columns (250 mm × 4.6 mm i.d., 5 μm) examined in this study include: Alltima ($F = 0.612$), Exsil ($F = 1.065$), Prevail ($F = 1.132$), Lichrospher ($F = 2.251$) and Prontosil AQ plus ($F = 5.100$).

3. Results and discussion

3.1. Method development

The method described in the European Pharmacopoeia (Ph. Eur.) [5] was used as starting point for further development. Compared to the Ph. Eur. method, a longer column (25 cm instead of 12.5 cm) was used in order to improve the overall separation and the mobile phase composition was adapted in order to ensure that the same phosphate concentration is maintained throughout the gradient: ACN/dihydrogen phosphate solution/water (A, 15:15:70) and (B, 70:15:15). UV detection was performed at 220 nm. The flow rate was 1.0 ml/min and the column was kept at 30 °C. The gradient applied was: 0–5 min, 100% A; 5–35 min, linear increase of mobile phase B from 0% to 40%; 35–36 min, return to the initial conditions; 36–55 min, re-equilibration with 100% A. With these conditions, no good separation was achieved, especially the impurity eluting after the principal peak was not separated from OT. To improve this, different combinations of mobile phases A and B with various gradient programs, column temperatures (30, 35 and 40 °C) and phosphate solution pHs (2.0, 4.4 and 6.0) were tried. Among the various combinations, dihydrogen phosphate solution pH 4.4, 40 °C column temperature and the gradient program given in Table 1 were found to give the best separation. A typical chromatogram of OT obtained with the final conditions is shown in Fig. 2. As can be seen from the chromatogram, the method is capable of separating OT and ten of its impurities. Peak number 2 is identified as acetic acid. The identities of four other impurities were confirmed by spiking the OT solution with the four available reference impurities. It was found that peaks 3, 9, 10 and 11 correspond to carbamido

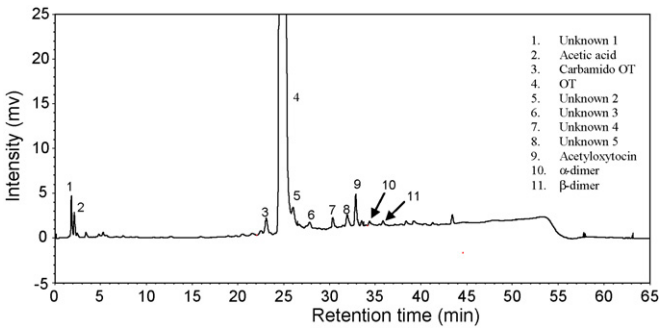

Fig. 2. Typical chromatogram of a 0.5 mg/ml OT solution (Sample No. 1) using the final optimized method (column: Eurospher 100-5 C18 (25 cm × 4.0 mm i.d.), 5 μm kept at 40 °C, mobile phases: ACN/dihydrogen phosphate solution/water (A, 15:15:70) and (B, 70:15:15), flow rate: 1.0 ml/min, injection volume: 50 μl, UV detection: 220 nm. Gradient: 0–5 min, 100% A; 5–20 min, from 0% to 6% B; 20–50 min, from 6% to 40% B; 50–51 min, from 40% to 0% B; 51–65 min, 100% A).

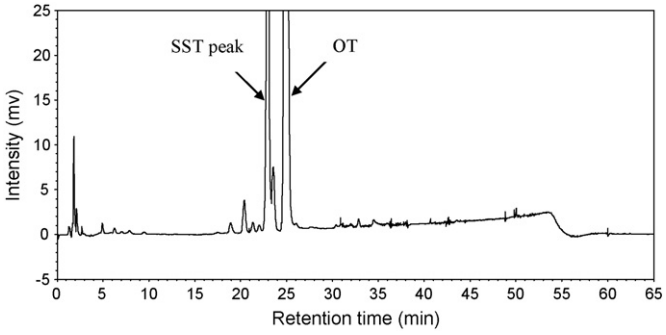

Fig. 3. Typical chromatogram of the system suitability test solution prepared by mixing 3.0 ml of a 0.50 mg/ml OT solution with 2.0 ml of 0.1 M sulphuric acid and heating in a boiling water bath for 20 min. Chromatographic conditions are the same as for Fig. 2.

oxytocin, acetyloxytocin, α-dimer and β-dimer, respectively. The peaks eluted after 36 min are mainly blank peaks (peaks originating from the chromatographic system: mobile phase and column and not from the sample). Other peaks that are not numbered are below the disregard limit, which was set at 0.10%.

3.2. System suitability test

In a pharmacopoeial LC method, a system suitability test (SST) is suggested to check its separation quality [11]. To avoid the use of additional (reference) substances, the SST was developed by degrading a 0.50 mg/ml OT sample solution. To the latter solution, various quantities of 0.1 M sulphuric acid were added and the mixture was exposed to heat in a boiling water bath for different time periods. When 2.0 ml of 0.1 M sulphuric acid was added to 3.0 ml of the OT sample solution and heated in boiling water for 20 min, a degradation peak with a retention time of approximately 23 min (relative retention: 0.9) was seen to increase in size over time. It was observed by spiking of solutions that the degradation product does not correspond to carbamido oxytocin since both peaks are not co-eluted, but partly overlap. The resolution (2.6) between the OT peak and the degradation peak can be used as SST. A typical chromatogram for the SST is shown in Fig. 3.

3.3. Evaluation of different stationary phases for different selectivity

The Eurospher 100-5 column was chosen as a reference column to calculate the *F*-value. This value is automatically calculated on the website when the 4 column parameters are inserted. A list of all the columns in the database is given, ranked by their *F*-value. From this list, five columns were selected to evaluate their performance towards the analysis of OT using the optimized method. Three of the columns are high ranked (*F*-values < 2) and are expected to yield a similar separation as the reference column. The remaining two columns are of the intermediate group, with *F*-values between 2 and 6. They should rather give rather different results compared to the reference column.

The Alltima column had the lowest *F*-value (0.612) and should thus give a similar separation compared to the Eurospher 100-5 column. This was found to be true as can be seen from Fig. 4(a). The retention time of OT was approximately the same and the separation of the impurities was also comparable. The Exsil column (*F* = 1.065) also gave a similar retention time for OT. The separation before the main peak is a little different since an extra peak is eluted before the carbamido oxytocin peak. Also less impurities are detected between the OT peak and the acetyloxytocin peak (Fig. 4(b)).

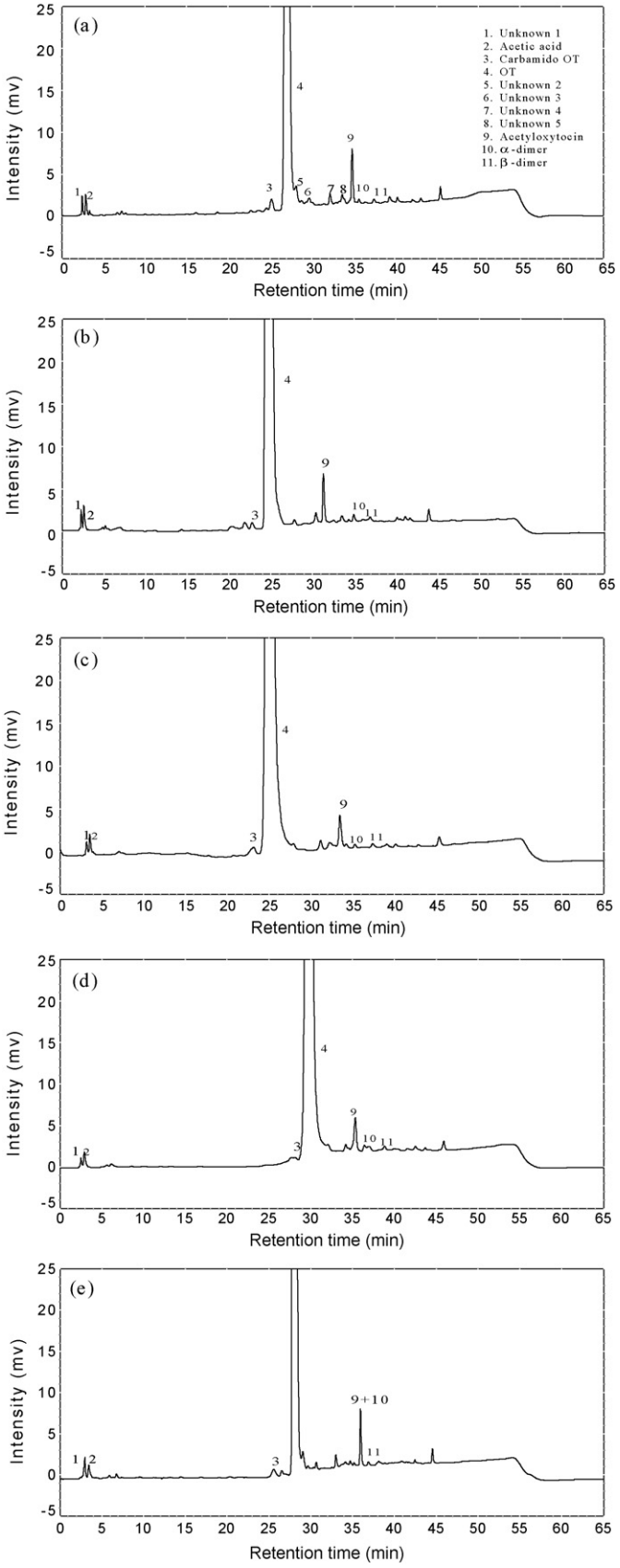

Fig. 4. Chromatograms for separation of OT obtained on different columns, Alltima AQ; *F* = 0.612 (a), Exsil ODS; *F* = 1.065 (b), Prevail; *F* = 1.132 (c), Lichrospher; *F* = 2.251 (d) and Prontosil 120-5; *F* = 5.100 (e). Chromatographic conditions are the same as for Fig. 2.

Table 2
Chromatographic parameter setting applied in the robustness investigation, corresponding to low (−), central (0) and high (+) levels.

Parameter	Low value (−)	Central value (0)	High value (+)
Acetonitrile (%)	65	70	75
Amount of dihydrogen phosphate solution	13	15	17
pH	4.2	4.4	4.6
Temperature (°C)	37	40	43

The third column is the Prevail column ($F = 1.132$). Separation is similar until the main peak is eluted (Fig. 4(c)). There is a shoulder on the main peak that could be due to an unknown peak. Only three peaks appear between the OT peak and the acetyloxytocin peak. In general, less impurities are separated on this column. Although the results on the different columns are not identical, all columns with *F*-values <2 could separate the known impurities.

The next two columns examined have *F*-values greater than 2. So, separation could be different compared to the Eurospher 100-5 column. The Lichrospher column ($F = 2.251$) gave a retention time around 30 min for the main peak (Fig. 4(d)). The carbamido oxytocin and the α-dimer are not fully separated from other impurities that have similar retention times. Only two peaks are eluted between the main peak and the acetyloxytocin.

Finally, the Prontosil AQ plus column ($F = 5.100$) gave a separation that looks very good at first sight (Fig. 4(e)). An extra peak is eluted between the carbamido oxytocin peak and the OT peak. After spiking, it was found that the α-dimer is co-eluted with acetyloxytocin. Both columns with *F*-values >2 gave overlapping or co-elution of the known impurities.

3.4. Method validation

3.4.1. Robustness study

The influence of four (k) chromatographic parameters on the separation was investigated. The parameters examined were the amount of ACN in mobile phase B, the pH of the dihydrogen phosphate solution, the amount of dihydrogen phosphate solution in the mobile phases and the column temperature (°C). Their effects on the resolution of different peak pairs (SST peak–OT, OT–unknown 2, OT–acetyloxytocin, acetyloxytocin–α-dimer and α-dimer–β-dimer) were evaluated by means of an experimental design and multivariate data analysis using Modde 5.0 statistical graphic software (Umetrics, Umea, Sweden). The chromatographic parameter settings in the experimental design are shown in Table 2.

The regression coefficient plots of the resolution between the different peak pairs are given in Fig. 5. It was observed that, under the conditions examined, the pH has a significant effect on the

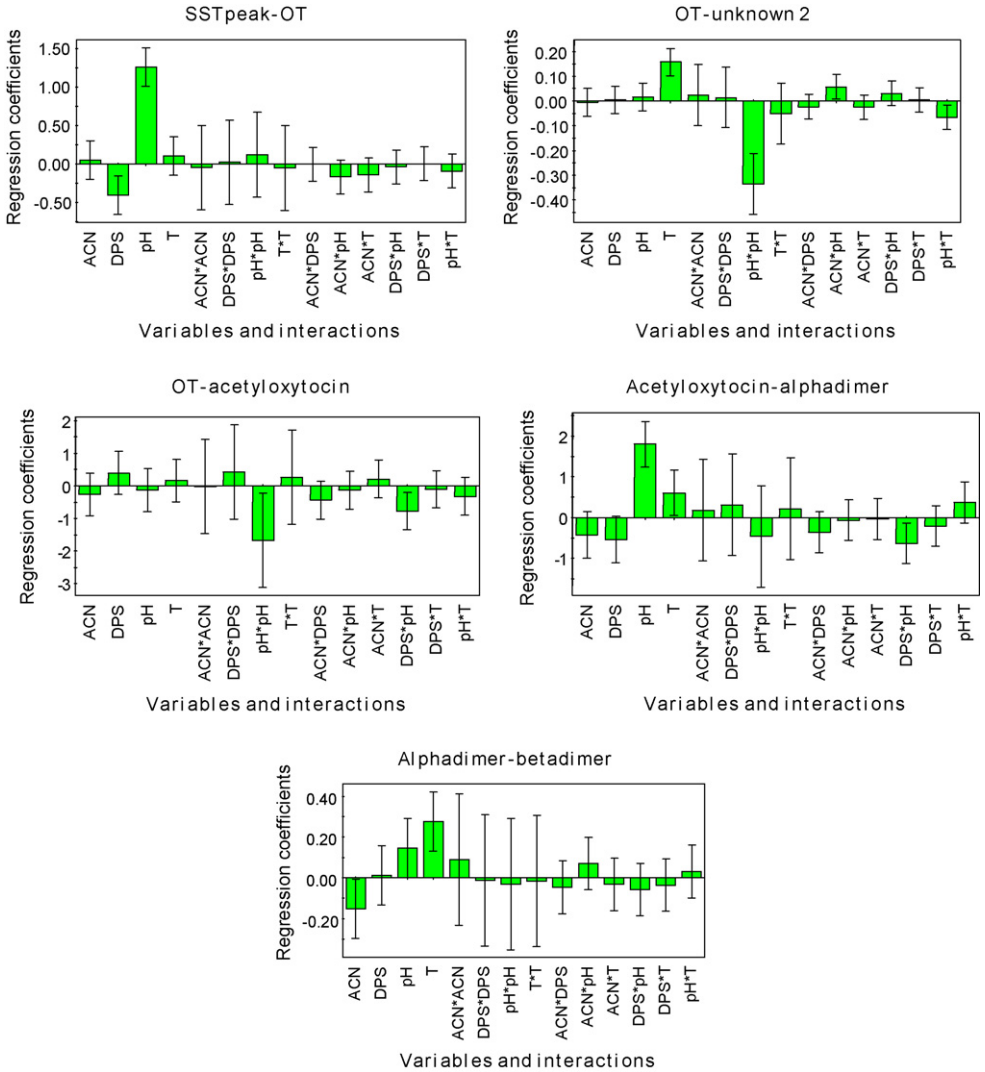

Fig. 5. Experimental design: regression coefficient plots of the resolution between the peak pairs SST peak–OT, OT–unknown 2, OT–acetyloxytocin, acetyloxytocin–α-dimer and α-dimer–β-dimer (ACN = acetonitrile; DPS = dihydrogen phosphate solution; pH = pH of dihydrogen phosphate solution; T = column temperature).

resolution of the peak pairs SST peak–OT and acetyloxytocin–α-dimer. The effect is positive for both peak pairs which means that increasing the pH leads to an increased resolution. Temperature has a significant positive effect on the resolution of the peak pairs OT–unknown 2, acetyloxytocin–α-dimer and α-dimer–β-dimer. The negative effect of the amount of dihydrogen phosphate solution for peak pair SST peak–OT indicates that the resolution decreases as the amount of dihydrogen phosphate solution increases. In other cases, the amount of dihydrogen phosphate solution has no effect on the separation.

There is a negative quadratic effect of the pH on the OT–unknown 2 and OT–acetyloxytocin peak pairs.

Some parameters interact with each other. For peak pairs OT–acetyloxytocin and acetyloxytocin–α-dimer, there is a small interaction between the amount and pH of the dihydrogen phosphate solution used. Similarly, small interactions were observed between temperature & pH and ACN & pH for peak pair OT–unknown 2.

Within the domain examined, the method can be considered as robust, except for the pH which has quite some influence and has to be set carefully.

3.4.2. Precision

The method precision was assessed using multiple preparations of a single commercial sample. Three different preparations of the same OT sample, each 0.50 mg/ml, were analyzed in triplicate on the same day. New solutions were prepared and analyzed on each of three successive days. RSD values obtained for the peak areas of carbamido oxytocin, acetyloxytocin and α-dimer on a single day (day 1, $n = 9$) are 9.4, 7.1, and 3.7%, respectively. RSD values on triplicate injections on three successive days (days 1–3, $n = 27$) are 10.6, 8.3 and 8.1%, respectively. Intermediate precision studies were performed using another Eurospher 100-5 C18 column (25 cm × 4.0 mm i.d.) and a different LC system (LC equipment II). The RSD values were of the same magnitude as above. The RSD values for the main peak were all below 1%. RSD values for the impurities were higher, but still acceptable since they were present in only small amounts (0.19–0.35%).

3.4.3. Sensitivity

The limit of quantitation (LOQ) for OT corresponding to a signal-to-noise ratio of 10 was 0.020% ($n = 6$; RSD = 7.15%). The limit of detection (LOD) corresponding to a signal-to-noise ratio of 3 was 0.006%. The percentages were calculated with respect to the main component nominal value (0.50 mg/ml = 100%, 50 μl injected).

3.4.4. Linearity

The linearity was checked by analyzing OT in the concentration range of 0.020–125% (0.5 mg/ml = 100%, 50 μl injected). Seven different concentrations (0.02%, 1%, 25%, 50%, 75%, 100% and 125%) were prepared and each concentration was injected three times. The following results were obtained: $y = 63881840x + 181332$; $r^2 = 0.9999$ and $S_{y,x} = 276119$, where y = peak area, x = concentration of OT solution expressed as percentage; r^2 = coefficient of determination and $S_{y,x}$ = standard error of estimate. The results indicate that the method is linear over the concentration range studied.

Table 3
Percentage of impurities in two OT commercial samples.

Peaks	Ret. time (min)	Sample No. 1 Impurity (%)	Sample No. 2 Impurity (%)
1	1.8	0.28	0.26
2	2.1	0.19	0.20
3	23	0.27	0.44
4 = OT	25		
5	26.8	0.61	0.21
6	28.4	0.18	0.18
7	30.5	0.14	0.18
8	32.4	0.18	–
9	32.9	0.35	0.52
10	34.7	0.19	0.26
Sum of impurities (%)		2.39	2.25

–: below LOQ.

3.5. Analysis of commercial bulk samples

Two bulk samples of OT were analyzed to determine the related substances. The results obtained are summarized in Table 3. All the impurities are expressed as OT, using a 1% dilution (5.0 μg/ml) of the sample examined as reference.

3.6. Applicability of the developed method to marketed OT injections

Two commercially available OT injections (Oxytocinum 10 IU/ml and Oxytocinum 5 IU/ml) were analyzed using the developed method. The preservative chlorbutol is eluted at about 38 min whereas the other components (acetate and acetic acid) are eluted earlier. Therefore, the method is specific for OT and hence can be used for the assay of OT injections. The percentage content, taking one of the bulk samples as 100% reference, was 107.2% and 106.7% for Oxytocinum 10 IU/ml and Oxytocinum 5 IU/ml, respectively.

4. Conclusions

The gradient LC method developed shows a good separation of OT from its impurities present in bulk drug substances. The robustness study indicated that mainly the pH of the dihydrogen phosphate solution should be monitored carefully to ensure the best separation as this has a significant effect on the separation. The method was shown to be selective, precise, sensitive and linear. The method can be used for the determination of related substances and assay of OT and may be applied for the analysis of drug substances and drug products.

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