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Quantification of oxolamine phosphate in human plasma by LC-MS/MS

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ABSTRACT

A simple, sensitive and specific liquid chromatography–electrospray ionization-mass spectrometry method was developed for the quantitative determination of Oxolamine phosphate in human plasma. Sample preparation involved simple liquid–liquid extraction. Bromhexine was used as internal standard. The separation of the analyte, internal standard and possible endogenous compounds were accomplished on a Shim-pack ODS column (150 mm×4.6 mm i.d., 5 µm) with methanol–water (98:2, v/v) as mobile phase. A Turbo-Ion spray source was interfaced between the HPLC and triple quadrupole mass spectrometer (MDS Sciex API 4000). The precursor-product ion m/z was 246.3 → 86.1 m/z and 377.3 → 263.9 m/z were used for quantification of an analyte and its IS. The method was validated over the range of 0.5–60 ng/mL and the results were acceptable. The method could offer the advantages of shorter run time (5.0 min) and lower LLOQ (0.5 ng/mL) with a decreased plasma volume requirement (250µL).

KEY WORDS: Oxolamine phosphate, LC-MS/MS, Human Plasma.

1. INTRODUCTION

Oxolamine phosphate (OXP) is chemically known as N,N-diethyl-2-(3-phenyl-1,2,4-oxadiazol-5-yl) ethanamine phosphoric acid with molecular weight 343.31g/mol and the molecular formula was $C_{14}H_{22}N_3O_5P$. Oxolamine is a compound having respiratory tract, anti-inflammatory, bronchospasmolytic and antitussive activity.

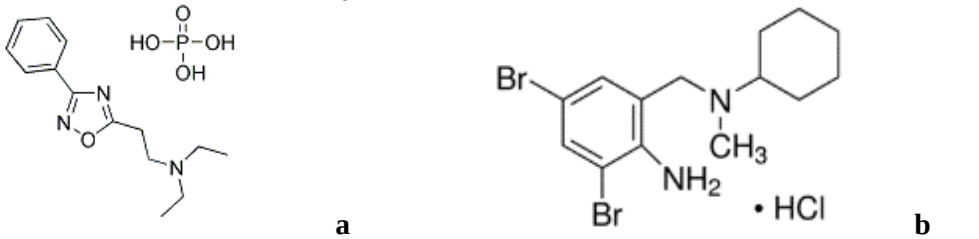
In the literature review, different methods are reported that simultaneous quantification of acetaminophen, chlorpheniramine and phenylephrine (Garcia, 2003; Heydari, 2008; Olmo, 2005; Senyuva and Ozden, 2002) in the rapid determination of three active compounds by using a Bondapak CN column in different pharmaceutical dosage forms. HPLC (Das Gupta and Heble, 1984; Marin, 2002), RP-HPLC (Kanumula, 2001) Liquid Chromatography (Krieger, 1987). Gradient elution methods are not suitable because these increases baseline disturbances and the column re-equilibration time. In the literature, no one reported LC-MS/MS method applied to medication containing a Oxolamine phosphate.

The aim of the present research work was to develop a LC-MS/MS method for determination of Oxolamine phosphate in biological matrix (Venkata Suresh, 2011). The developed method was validated as per FDA guidelines (2001).

2. MATERIAL AND METHODS

Instrumentation: The Agilent 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany) connected to the API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) with turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

Reagents / Materials: Oxolamine phosphate (Fig. 1A) and Bromhexine hydrochloride (BH) (Fig. 1B) was obtained from Lambda Therapeutic Research Limited, Gota, Ahmedabad, India. Potassium dihydrogen ortho phosphate, water (HPLC Grade) was purchased from Merck, Mumbai, India. Methanol (HPLC Grade), Cyclohexane (HPLC grade) was obtained from J.T. Baker, USA. Human plasma was procured from Navjeevan Blood Blank, Hyderabad. Milli Q water was taken from the in-house Milli-Q system.



a **b**

Fig.1. Chemical Structure a) Oxolamine phosphate, b) Bromhexine hydrochloride

Chromatographic and detection conditions: Chromatographic separation was performed on a Shim-pack ODS column (150 mm×4.6 mm i.d., 5 µm) protected by a Phenomenex ODS guard column (4.0 mm×3.0 mm i.d., 5 µm). The mobile phase of methanol–water (98:2, v/v) was filtered and degassed. A constant flow-rate of 1.0 mL/min was maintained and the temperature of column was set at 35°C. BH (Internal standard-IS) was used as IS in terms of chromatography and extractability. OXP and BH were eluted at 1.9 and 0.9 min, for each sample the total run time was 5 min.

Detection was done by turboionspray (API) positive mode with unit resolution. Quantification was by MRM, where the acquired masses for OXP were 246.3 → 86.1 m/z and for BH were 377.3 → 263.9 m/z (Fig. 2 & 3).

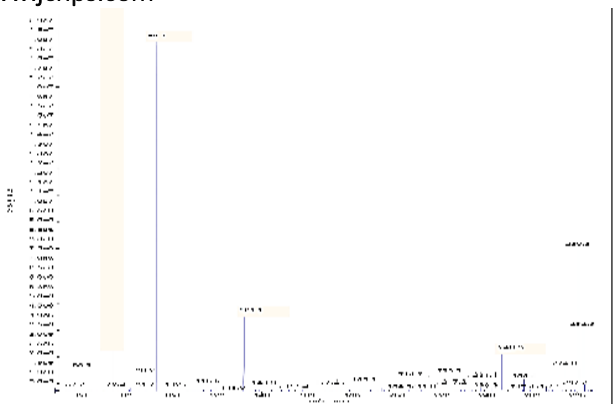


Figure.2. Mass scan spectrum of Oxolamine phosphate (OXP)

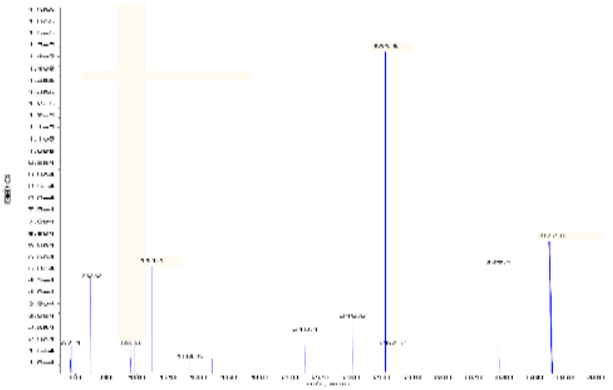


Figure.3. Mass scan spectrum of Bromhexine hydrochloride (BH)

Preparation of stock and working solutions: Stock solutions of OXP and BH were prepared individually by dissolving the accurately weighed reference compounds in methanol to get a final concentration of approximate 500 µg/mL and the working solutions for OXP at the concentrations of 250, 50 and 5 ng/mL and for IS at that of 50 ng/mL were obtained further by gradually diluting the stock solutions with methanol. All the solutions were stored at -20°C when not in use.

Preparation of standards and quality control samples: Calibration samples were prepared by spiking control plasma samples (250 µg/mL) with OXP at 0.5, 1, 5, 10, 20, 30, 40 and 60 ng/mL and IS at 6 ng/mL on the day of sample work-up. A calibration curve covering with the unknown samples, the whole analytical working range was run in duplicate in each batch of sample. The calibration curve was constructed by plotting the peak area ratios of OXP to the IS versus the concentrations of OXP. At the concentrations of 1.5, 25 and 50 ng/mL Quality control (QC) samples were prepared in the same method as calibration standards based on an independent weighing of reference substance. Along with the unknown samples, each analytical batch was analyzed.

Sample preparation: A 250 µL aliquot of human plasma was introduced into a 10 mL glass centrifuge tube followed by 30 µL IS working solution. After briefly vortex-mixing, the mixture of 250 µL of phosphate buffer (pH 5.8) and 3 mL of cyclohexane were added, vortex-mixed for about 3 min, and centrifuged for 10 min at 4000 rpm. With a gentle stream of nitrogen, the upper organic layer was transferred and dried at 40°C for evaporation. These dry residues were dissolved in 100 µL mobile phase and 20 µL of reconstituted sample was injected into the LC–MS/MS system.

3. RESULTS AND DISCUSSIONS

Selection of LC and MS conditions: According to the results of MS scan in positive or negative mode, the peak intensity got from positive mode was higher than that from negative one. Scanning in the range of m/z 50–400 showed that the positive ion with m/z 86.1 was of most abundant. Considering that it was produced from OXP molecular ion and selected for the determination of OXP.

The composition of mobile phase was explored by several trials to achieve high sensitivity, good resolution, symmetrical peak shape, as well as short analytical time. Acetonitrile, methanol and water were mixed at different ratios. Addition of mobile phase modifiers such as ammonium acetate was also tested. The results showed that a mixture of methanol and water (98:2, v/v) could satisfy the peak shapes, acquirement of resolution and analytical efficiency.

Selection of IS: The IS included fenfluramine hydrochloride, benzhydramine, sibutramine hydrochloride, lovastatin and bromhexine hydrochloride. On the bases of extract and chromatographic character of each compound, bromhexine hydrochloride was selected as IS. The retention time of IS and OXP were approximately 1.9 and 0.9 min, respectively as selected above the chromatographic conditions. The HPLC run time was 5.0 min for each sample.

Optimization of sample preparation: Extraction solvents including hexane, cyclohexane, cyclohexane–ethyl acetate (3:2, v/v) and cyclohexane–ethyl acetate (9:1, v/v) were tested. These solvents are used alone or combination with phosphate buffer (pH 5.8). Combination of cyclohexane and phosphate buffer (pH 5.8) was chosen after the analysis of recovery. The volumes of cyclohexane and phosphate buffer (pH 5.8) were adjusted to 3 and 0.25 mL, respectively.

Method Validation: To confirm the reliability of the proposed method, validation assays were fulfilled according to the U.S. Food and Drug Administration (FDA, 2001) Guidance for Industry (Bio analytical Method Validation).

Selectivity: It was essential to certify that the signals measured were related only to the analytes. Selectivity of the method was examined by analyzing the blank human plasma from six different sources using the selected extraction procedure and LC–MS/MS conditions. Chromatograms showed that the method was able to discriminate the analytes

from all potential interfering substances. Chromatograms obtained from plasma spiked with OXP and BH was depicted in (Fig.4 a & b).

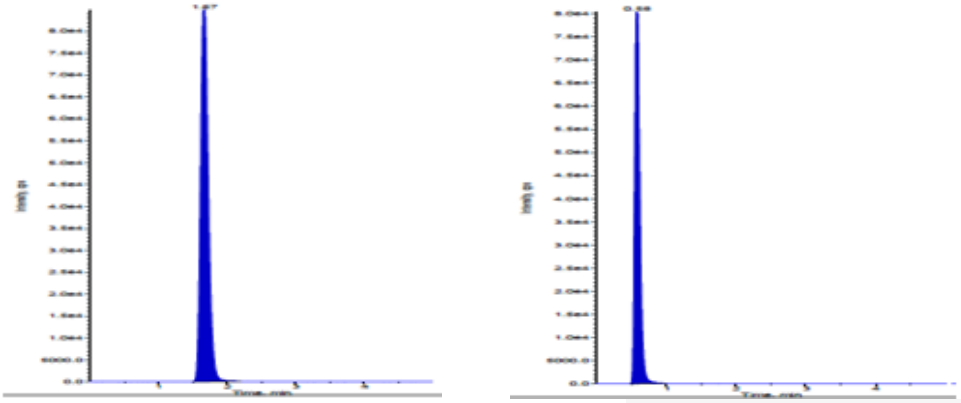


Fig.4. Chromatograms a) Oxolamine phosphate b) Bromhexine hydrochloride

Linearity and lower limit of quantification: Calibration curve was determined by plotting the peak area ratios of OXP to IS versus the nominal concentrations of OXP. Good linearity was observed over the range of 0.5–60 ng/mL. Typical calibration curve had a coefficient correlation of 0.9996.

The lower limit of quantification (LLOQ) of OXP was defined as the lowest concentration of the calibration curve and it was determined to be 0.5 ng/mL in human plasma using five samples independent of calibration curves. The precision and accuracy at LLOQ was within 80–120% at LLOQ in this method.

Precision and accuracy: To evaluate the accuracy and precision, the samples of spiked plasma were used in three consecutive runs. Three replicate samples consists of known quantity of bromhexine (1.5, 25, and 50 ng/mL in plasma) were prepared and analyzed along with each calibration curve. Concentrations were determined by back-calculation of peak area ratios from the corresponding calibration curve. Accuracy was measured by the formula: accuracy (%) = (measured concentration)/ (nominal concentration) ×100. Precision was expressed by relative standard deviation (R.S.D %). The results were presented in Table 1.

Table.1. Precision and accuracy of the Oxolamine phosphate QC samples

Nominal concentration (ng/ml)	Mean measured concentration (ng/ml) ± S.D	Intra-run RSD (%)	Inter-run RSD (%)	Accuracy (%)
1.5	1.59 ± 0.14	9.1	12.3	111.3
25.0	25.53 ± 0.60	5.5	6.3	95.3
50.0	50.30 ± 1.90	3.0	4.7	100.8

Extraction recovery: The extraction recovery was estimated at the concentrations of 1.5, 25 and 50 ng/mL by analyzing the peak areas of OXP extracted from plasma samples with those from standards. The extraction recovery was 95.5%, 97.9% and 98.3% respectively at three concentrations. The results exhibited both good extraction efficiency and repeatability.

Matrix effect: To resolve whether a significant matrix effect (ME) remained, the post extraction addition technique was used. The concentrations of 1.5, 25, and 50 ng/mL were developed from two groups of samples as follow, in group A, from different sources blank plasma were extracted, solutions of analytes were added and vaporized to dryness, then the residues were resolved in 100 µL/mL mobile phase, the peak areas of analytes in group A were defined as A; in group B, analytes were vaporized to dryness and resolved in 100 µL/mL mobile phase, the peak areas were defined as B. Samples were tested at each concentration and the matrix effect was calculated by the formula: ME= A/B ×100.

IS was tested at the concentration of 6 ng/mL. No interferences were detected and the ME values were within the range of 85–115%. It demonstrated that no co-eluting components interfering with the ionization of the analytes.

Stability: Stability studies were conducted at the concentrations of 1.5, 25, and 50ng/mL with three replicate samples for each level. For the freeze and thaw stability, spiked plasma samples were placed at -20ºC for 24 h and thawed at room temperature and refrozen at -20ºC for 24 h. After repetition of the freeze–thaw cycles second cycle, samples were tested on the third cycle. Spiked plasma samples were defrosted and kept at room temperature for 12 h for stability. After storage of spiked plasma samples at -20ºC for 5 days the long-term stability was determined. The stability was determined by comparing the results of stored samples with freshly prepared samples. The results were summarized in Table 2 which confirmed that OXP was stable under the investigated handling and storage conditions, and no stability related problems would be expected during routine analysis for clinical trial samples. The stock solutions stability was tested at room temperature and the results exhibited reliable stability behaviour.

Table.2. Stability of Oxolamine phosphate under various storage conditions

Nominal concentration (ng/ml)	nominal concentration (ng/ml) ± S.D	Mean measured concentration (ng/ml) ± S.D
Freeze and thaw stability at -20°C	1.5	1.53 ± 0.01
	25.0	25.90 ± 0.81
	50.0	51.31 ± 1.85
Room temperature Stability for 12 h	1.5	1.50 ± 0.14
	25.0	25.70 ± 0.05
	50.0	50.74 ± 1.62
Long term stability for 5 days at -20°C	1.5	1.52 ± 0.02
	25.0	25.10 ± 0.02
	50.0	51.05 ± 1.17

4. CONCLUSION

The determination of Oxolamine phosphate by bioanalytical method in human plasma was developed. A LLOQ of 0.5ng/mL was achieved based on 250 µL of plasma samples. This method exhibited good sensitivity and accuracy over the range of 0.5–60 ng/mL. No significant matrix effect and interference caused by endogenous compounds or other drug were observed. A LC–MS/MS run time of 5.0 min per sample contrived the method a pragmatic one in bioanalysis of OXP.

5. ACKNOWLEDGEMENTS

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