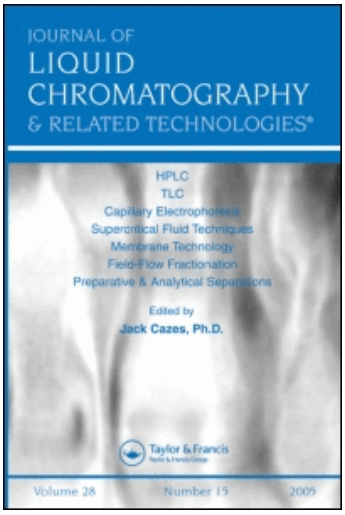

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DEVELOPMENT OF A VALIDATED STABILITY INDICATING LC METHOD FOR QUANTITATIVE DETERMINATION OF AN ANTISPASMODIC DRUG AND ITS RELATED SUBSTANCES

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□ *Present article describes development and validation of stability indicating liquid chromatographic method for quantitative determination of α-cyclohexyl-α-hydroxy-benzeneacetic acid-4-(diethylamino)-2-butynyl ester hydrochloride a potential antispasmodic and anticholinergic drug and its four impurities (related substances) using a Zorbax SB-Cyano column and mobile phase consisting of aqueous buffer (0.20% triethylamine) and acetonitrile in the ratio 49:51 and with a pH 6.3. The developed LC method was validated with respect to linearity, specificity, accuracy, precision, limit of detection, and limit of quantitation. For the quantitative determination of the impurities, a relative response factor approach was applied with respect to the drug substance. The developed LC method for the related substances and assay determination of drug can be used to evaluate quality of regular production samples. It can also be used to test the stability samples of the drug substance.*

Keywords anticholinergic, antispasmodic, LC method, relative response factor, stability indicating method, validation

INTRODUCTION

The α-cyclohexyl-α-hydroxy-benzeneacetic acid-4-(diethylamino)-2-butynyl ester hydrochloride (OXB HCl) is a potential antispasmodic and anticholinergic drug.[1] It is used to relieve urinary and bladder difficulties and also helps to decrease muscle spasms of the bladder.[2]

In the present article we have used OXB HCl as the abbreviated name of this drug for convenience to the reader in further chapters.

In various literature, High Performance Liquid Chromatographic (HPLC),[3–9] High performance frontal analysis,[10,11] GC-MS,[12,13] and LC-MS[14,15] methods are reported for the estimation of OXB HCL in various matrix systems.

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However, none of these aforementioned reported methods describe determination of related substances of OXB HCl using the relative response factor approach. Hence, a novel HPLC method was developed for the estimation of these related substances in the drug. The method was further validated, as per the requirements of the US Pharmacopeia[17] and ICH guidelines Q2 (R1) with respect to Linearity, Precision (System Precision, Method Precision, and Intermediate Precision), Relative Response Factors (RRF), Accuracy, Robustness, Specificity, Quantitation and Detection Limit, and Stability in the solution.[16]

In this manuscript, we describe development and validation studies of novel stability indicating isocratic reverse phase HPLC method for quantitative determination of the drug and its related substances.

EXPERIMENTAL

Reagents and Chemicals

Reference standard of OXB HCL drug substance and its impurities were prepared in a synthetic laboratory at Chemistry Department, Ruparel College, Mumbai 400016. The potential impurities in OXB HCl are 4-(diethylamino) but-2-ynyl (RS)-2-(cyclohex-3-enyl)-2-cyclohexyl-2-hydroxyacetate (impurity-A), 2- cyclohexyl phenyl glycolic acid (impurity-B), 4-diethylamino 2-butynyl acetate (impurity-C), and 2, cyclohexyl phenyl glycolic acid methyl ester (impurity-D).

The authentic standards of these impurities are prepared in respective lab experiments and characterized using various spectroscopic and chromatographic techniques. The relative response factors are determined to quantify these impurities more accurately.

All the reagents used were of analytical reagent grade unless stated otherwise and were purchased from Merck (Darmstadt, Germany). The LC grade acetonitrile and water were also obtained from Merck (Darmstadt, Germany).

Apparatus

Agilent LC System was equipped with quaternary gradient pump with an autosampler and auto injector connected with a photo diode array detector controlled with Chemstation Data acquisition and integration software (Agilent).

A Shimadzu UVPC spectrophotometer was used for recording the UV spectrum.

Chromatographic Conditions

The separation was achieved using a Zorbax SB-Cyano column. The mobile phase was prepared by mixing aqueous buffer (0.20% triethylamine) and acetonitrile in the ratio 49:51 (*v*/*v*) and of pH 6.3. The aqueous buffer solution was prepared by dissolving triethylamine (2 mL) in sufficient water and diluted further to 1000 mL.

Chromatograms were recorded at 205 nm. The system was equilibrated for 30 minutes and analysis was carried out under isocratic conditions using a flow rate of 1.0 mL/min, injection volume of 20 µL at column temperature of 40°C. and run time of 30 min.

Standard Preparation

The OXB HCl standard solution was prepared by dissolving about 25 mg accurately weighed OXB HCl reference standard in 10 mL of mobile phase with intermittent sonication in a 25 mL volumetric flask that was further made up to the mark using mobile phase. This solution was then diluted using mobile phase to obtain a solution having concentration of about 10.0 µg/mL.

Sample Preparation

For a preparation of sample solution, about 25 mg accurately weighed OXB HCl sample, was dissolved in 10 mL of mobile phase with intermittent sonication in a 25 mL volumetric flask and made up to the mark using mobile phase.

System Suitability Solution

A stock solution of four impurities and OXB HCl was prepared by dissolving, accurately weighed 10 mg each of impurities A,B,C,D, and OXB HCl (reference standard), in 20 mL of mobile phase placed in a 100 mL volumetric flask and further made up to the mark using mobile phase. For each impurity, a solution having a known concentration of about 10.0 µg/mL of the impurity and OXB HCl was obtained by diluting 1 mL of respective stock solution to 10 mL of mobile phase.

System Solubility Parameters

Resolution between OXB HCl and impurity-A from the chromatogram obtained with the system suitability solution was not less than (NLT) 2.0.

The RSD (%) of OXB HCl for replicate injections of system suitability solution should not be more than 10.0%. Theoretical plates and tailing factor for OXB HCl peak should be NLT 2000 and not more than (NMT) 2.0, respectively.

RESULTS AND DISCUSSION

The four related substances of OXB HCl have been described as process raw material, process by-product, and process intermediate. The standard samples of the drug and four related substances were prepared in respective lab experiments; and using various structure elucidation techniques, their structures were established to confirm formation of required compounds.

A novel LC method was developed for quantitative determination of the drug and its four related substances. The method was further validated. The aim of the method validation was to confirm that the present method was suitable for its intended purpose as described in ICH guidelines Q2 (R1) with respect to Linearity, Precision (System Precision, Method Precision, and Intermediate Precision), Relative Response Factors (RRF), Accuracy, Robustness, Specificity, Quantitation and Detection Limit, and Stability in the solution.

Method Validation

The validation of the proposed method for estimation of related substances and assay of OXB HCl was carried out as recommended in US Pharmacopoeia[17] and as per ICH guidelines Q2 (R1).[16]

Specificity

Specificity is ability of analytical method to measure the analyte response in the presence of components that may be expected to present, such as impurities, degradation products, and matrix components.

Stress studies were performed for the drug substance to provide an indication of the stability, indicating property and specificity of the proposed method (Figure 1). Intentional degradation of OXB HCl was attempted by subjection to various stress conditions, such as 1 N acid 1 N Base, aqueous solution, 30% v/v hydrogen peroxide, heat (105°C), UV light, and sunlight (1.2 million lux hours), to evaluate the ability of the proposed method to separate the drug substance from its degradation product.

No degradation was observed in aqueous condition and in dry environmental conditions like sunlight, UV light, and heat (105°C). In alkaline, acidic, and oxidation conditions, unknown degradation products were

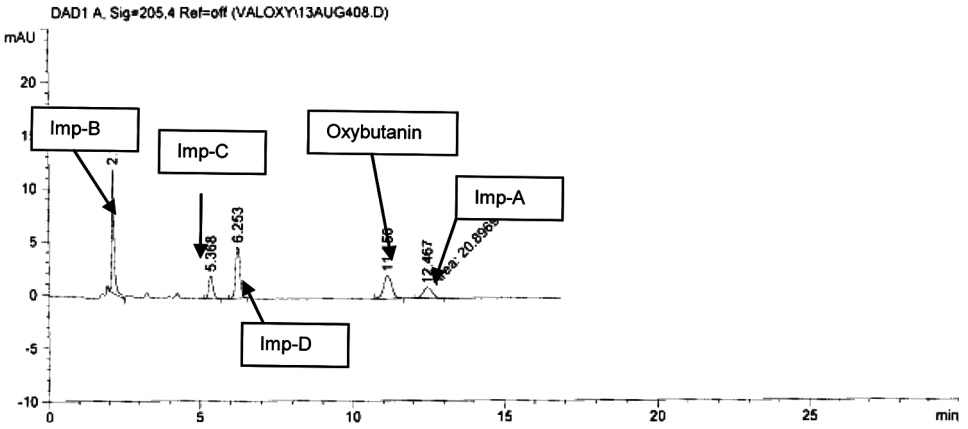

FIGURE 1 Typical chromatogram of Specificity.

observed. A peak purity test was carried out for the drug by using a photodiode array detector. As the main peak of the drug was not found attributable to any other peak, the method was proven to indicate stability.

Selectivity of the method was also performed by separately injecting individual impurities A, B, C, and D. None of the respective impurity peaks interfered with the peak of drug substance. Minimum resolution of 2.4 was observed between the drug and impurity A peak, reflecting the selectivity of the proposed method.

Relative Response Factor. A relative response factor (RRF) and relative retention time (RRT) was determined for all four impurities with respect to the response of the drug at the wavelength of the experimental conditions recommended for the method.

The estimated RRT and RRF of impurities are as under:

Impurity-A: RRT 1.10, RRF 0.42
Impurity-B: RRT 0.19, RRF 1.44
Impurity-C: RRT 0.46, RRF 0.27
Impurity-D: RRT 0.56, RRF 1.16

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were determined by measuring the magnitude of analytical background. The LOD and LOQ were determined from slopes of linear regression curves. The LOD and LOQ for Impurity-A, Impurity-B, Impurity-C, and Impurity-D were determined by injecting a series of dilute solutions with known concentrations. The limit of detection was found to be 0.004% of related substances concentrations for the drug and all four related impurities A, B, C, & D is 0.008%, 0.006%, 0.006%, & 0.006%, respectively.

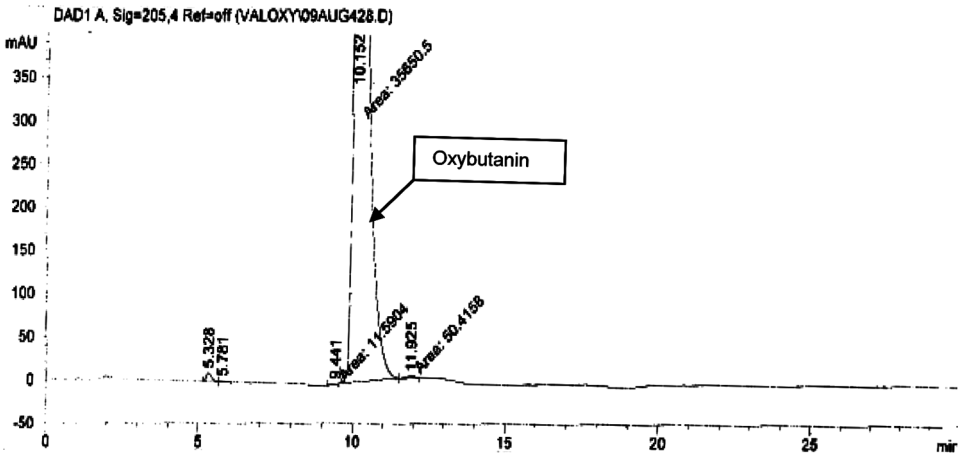

FIGURE 2 Typical chromatogram of Limit of Quantitation.

Limit of quantitation was the lowest amount of analyte in a sample that could be determined with acceptable precision and accuracy under the stated experimental conditions. The limit of quantitation was found to be 0.01% of related substances concentration for the drug and all four related impurities A, B, C, and D is 0.03%, 0.02%, 0.02%, and 0.02%, respectively (Figure 2).

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

Linearity test of impurities was performed using standard solutions of lower concentrations of the drug and impurities A, B, C, and D over concentration range 0.5 ug/ml to 1.50 ug/ml (0.05% to 0.15% of related substance preparation). The data obtained from this analysis was subjected to regression analysis. The method shows linear response for the drug and all four impurities over the aforementioned stated concentration ranges. The correlation coefficient for the drug is 0.9999 and for four related impurities A, B, C, and D is 0.9999, 0.9999, 0.9999, & 0.9999, respectively, as shown in Table 1.

Precision

The precision of an analytical method is the degree of agreement among the individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample.

The precision studies, which include system precision, method precision, and intermediate precision, were conducted (Figure 3).

TABLE 1 Linearity Table of Oxybutynin Hydrochloride & Impurities (Related Substances)

Level	Conc. ug/mL	Mean Area of Oxybutanin	Related Compound A	Related Compound B	Related Compound C	Related Compound D
1	0.50	10.02	20.3	29.77	10.46	25.29
2	0.75	15.07	29.41	47.34	15.13	37.14
3	1.00	20.68	39.19	62.00	20.32	49.90
4	1.25	24.81	49.05	79.48	25.44	62.34
5	1.50	30.10	59.35	95.11	30.45	75.05
	Co-coeff	0.9999	0.9999	0.9999	0.9999	0.9999
	Slope	0.048	0.025	0.015	0.049	0.019
	Intercept	0.036	0.024	0.046	0.014	0.07

The system precision was determined by injecting a set of six replicate injections of impurities. The RSD (%) of retention time for the drug and Impurities A, B, C, and D was observed to be 0.041%, 0.046%, 0.00%, 0.113%, and 0.00%, respectively, which was well within the acceptance criteria of NMT 0.50%.

The RSD (%) of area for the drug and Impurities A, B, C, and D was observed to be 0.634%, 0.211%, 0.454%, 1.88%, and 0.161%, which is well within the acceptance criteria of not more than (NMT) 10.0%.

The method precision was determined by analyzing samples of the drug by accurately weighing six different samples on the same day. While impurities A, B, C, and D were not observed in the sample, RSD (%) of an unknown impurities was calculated, which is well within the acceptance criteria of NMT 10.0%.

Intermediate precision was determined by analyzing drug samples in two sets of six each on different days and on different LC systems. The RSD (%) of unknown impurities for twelve samples was calculated which is well within the acceptance criteria of NMT 10.0%.

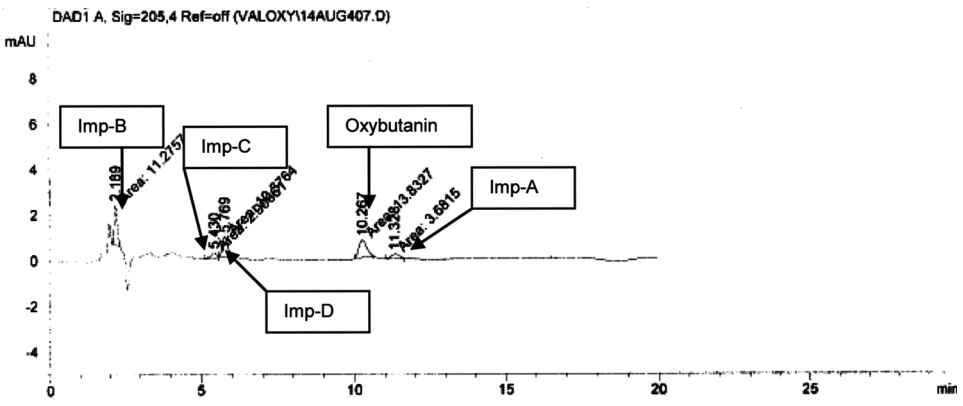

FIGURE 3 Typical chromatogram of Sample preparation.

The aforementioned results show that the method is Rugged and Precise.

Accuracy

The accuracy test was carried out by spiking the drug sample with known amounts of each impurity. Impurities A, B, C, and D were spiked in to the drug sample at 50%, 100%, and 150% of the specified limit concentration. The results were calculated using the calculated RRF. The mean recovery of all the impurities at each level was within 90% to 110% with RSD (%) less than 5 as described in Table 2.

Robustness

The robustness of an analytical method is a measure of its capability to remain unaffected by small but deliberate variations in method parameters. This study provides method reliability during routine use.

The robustness study was carried out by altering the mobile phase composition, pH, column, temperature, and flow rate. Samples of OXB HCl were analyzed for estimation of related substances under these changed experimental conditions. It was observed that the method is unaffected by small changes in experimental conditions.

Stability in Analytical Solution

Stability in an analytical solution study was carried out to establish the stability of the sample in an analytical solution (diluent) over a period of time during routine analysis.

The OXB HCl was stable at a specified concentration in diluent solution for more than 24 hours. No significant increase in impurity levels

TABLE 2 Accuracy Table of Oxybutynin Hydrochloride & Impurities (Related Substances)

Sr. No.	Level	Related Substance-A	Related Substance-B	Related Substance-C	Related Substance-D
1	Level-1 50%	102.5	97.5	101.25	103.75
2		101.25	95.0	102.5	102.5
3		103.75	95.0	100.0	105.0
4	Level-2 100%	102	99.0	110.0	103.0
5		94.0	94.0	108.0	103.0
6		99.0	94.0	98.0	103.0
7	Level-3 150%	99.16	95.83	103.33	104.16
8		97.5	98.33	104.16	103.33
9		95.83	98.33	105.0	105.0
	Mean	99.443	96.33	103.582	103.638
	SD	3.249	1.973	3.770	0.909
	RSD (%)	3.267	2.048	3.640	0.877

was observed when a sample at a related substance concentration was stored at ambient temperature under normal laboratory lighting conditions. The correlation of the standard solution and sample solution after 24 hours was between 98 and 102%.

The difference between 0 hr and 24 hr results were not more than 0.01%, indicating that the sample prepared in diluent was stable for at least 24 hrs.

System Suitability

To ensure that the analytical method was working satisfactorily and capable of giving accurate and precise results, system suitability test was incorporated in the analytical method. A resolution solution was injected into the chromatograph six times. System suitability results obtained are tabulated as follows.

System suitability results obtained for theoretical plates of OXB HCl peak was 9051 (Limit: NLT 2000), tailing factor of OXB HCl peak was 1.13 (Limit: NMT 2.0), RSD (%) of areas of OXB HCl peak was 0.63% (limit: NMT 10.0%), and resolution between OXB HCl and impurity-A peak was 2.4 (limit: NLT 2.0).

CONCLUSION

The isocratic LC method developed for quantitative and related substance determination of α-cyclohexyl-α-hydroxy-benzeneacetic acid-4-(diethylamino)-2-butynyl ester hydrochloride was precise, accurate, rapid, and specific. The method facilitates the separation of four of the known related substances of the drug with a resolution minimum of 2.0. The method was fully validated showing satisfactory data for all the method validation parameters tested. The developed method was stable, and can be conveniently used by quality control departments to determine the related substance and assays in regular production samples of drugs and stability samples.

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