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Development and validation of a method for the enantioseparation of oxybutynin hydrochloride by HPTLC

P.Sitadevi[1]*, P.L.K.M.Rao[2]

[1]Analytical Chemistry Division, Indian Institute of Chemical Technology, Tarnaka, Hyderabad - 500 007, (INDIA)

[2]SLC's College of Pharmacy, Survey No 17/2, Piglipur Village, Hayatnagar Mandal, Near Ramoji Film City, Hyderabad - 501 512, (INDIA)

E-mail : sitadevi@iict.res.in; sdevipotturi@yahoo.co.in

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ABSTRACT

A specific chiral HPTLC method has been developed for the separation and quantitative determination of the enontiomers of oxybutynin hydrochloride without any prior derivatization and sample preparation. A sample mobile phase such as toluene: acetone: methanol (8:1:1 v/v) facilitated good separation of the enantiomers and the findings are supported by on-line UV analysis and Mass spectrometric data. Further, a simple method validation has been carried out with the total drug molecule on normal phase silica gel plates (precoated with 60 F_{254}). The results suggest that the method is specific, accurate and can be used for routine quality assurance evaluation.

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KEYWORDS

Enantiomers;
Derivatization;
Thin layer chromatography;
Densitometry.

INTRODUCTION

Oxybutynin is an anti –muscarinic drug that is widely used as a racemate in the treatment of unstable bladder[1]. It is an anti-cholinergic drug chemically named as (4-diethyl amino)-2-butynyl-α-phenyl cyclohexane glycolate hydrochloride. It is relatively a new drug presently available as a neurogenic bladder antagonist. According to USFDA, suitable qualitative analytical assays are mandatory for the evaluation of individual enantiomers because most of these drugs are marketed as racemates, though the enantiomers possess different pharmacological activity and often demands a specific analytical procedure involving chiral columns for the pharmacokinetic studies. Essentially, chiral compounds cannot be separated by conventional TLC or HPLC. But very compatible chiral stationary phases which can offer selective separations are now available that facilitate direct separation of the enantiomers without derivatization[2]. Thus, the two optical isomers of oxybutynin hydrochloride that showed different pharmacological properties as reported[3,4] are resolved on a preprative (250×10mm I.D) chiralpak Ad column by earlier workers[5]. Also achiral assays using electrochemical detection has been reported[6] where in the minimal detectable concentrations of oxybutynin and N-desethylebutynin are 0.5 and 5μg/ml respectively (at a S/N ratio of 3:1). Further chiral bioanalysis by normal phase HPLC-atmospheric pressure ionization tandem mass spectrometry has been developed for the separation and determination of chiral drugs and their metabolites[1]. However, as known and in our own experience solvent consumption, elaborate sample preparation methodologies and time taken for a single analytical run

are certain undesirable parameters in other chromatographic methods though they are often used for selective applications. Nevertheless, chiral TLC offered an absolutely clean separation of the two optical isomers of OBC without resorting to any elaborate sample preparation or derivatization methods. The findings are subsequently supported by spectral studies. Further, this simple but novel analytical approach facilitates a rapid screening of the drug for routine impurity profiling studies as well. We describe here in, a chiral HPTLC method with a simple mobile phase where separation and quantification have been accomplished in a single analytical run. The method appears to be highly sensitive and specific and finds potential application in day-to-day quality control evaluation as TLC is rapid, economical and solvent consumption is minimum with a very simple operation.

EXPERIMENTAL

Materials

Racemic-oxybutynin chloride (OBC) was USP standard. Chiral TLC plates are produced from Merck (0.20mμ, Darmstadt, Germany). Cystran-5 (label claim 1mg per tablet, Intas pharmaceuticals ltd, Ahmedabad, India) was purchased from local pharmacy. All chemicals and reagents used are of analytical grade and are purchased from Merck Chemicals, India.

Standard solution and calibration plots

A stock solution of OBC was prepared by dissolving the authentic total drug sample in methanol to obtain a concentration of 1mg/ml. This solution was used to prepare a standard solution of OBC (100μg/μl) in methanol and this was used to construct a calibration plot by applying 0.5, 1, 1.5, 2, 2.5, 3 & 3.5μl in the concentration range of 50-350μg per spot. The data of peak area versus drug concentration are treated by linear least- square regression analysis.

HPTLC instrumentation

Chiral TLC

HPTLC system (CAMAG, Switzerland) was used for analytical and micro preparative separations using chiral TLC plates. TLC was performed on 10x10 cm precoated chiral plates (E.Merck, Darmstadt, Germany). Different volumes of the standard solutions (1-8μl, each μl = 50μg per spot) were applied on to the plate in the form of bands of 6mm width using Linomat-V sample applicator (CAMAG, Switzerland) up to a distance of 8.5cm using a mobile phase containing toluene: acetone: methanol (8:1:1 v/v). The spots were visualized in a UV visualization chamber. The plate was densitometrically scanned at λ = 254nm by means of a TLC Scanner-3 (CAMAG, Switzerland) in Absorption/Reflection mode with a slit width of 5×0.45mm. The scanned data were procured by means of on-line CAMAG win CATS software loaded in a personal computer .The configuration and R_f values were assigned in comparison with Mass spectra and UV obtained for individual isomers by micro preparative TLC.

Normal phase TLC

TLC was performed on 10×10cm HPTLC plates pre coated with 60 F_{254} (With 0.25mm thickness; Merck, Darmstadt, Germany) and the plates are washed with methanol before use. The samples were potted in the form of bands of 6mm width using Linomat V applicator (Muttenz, Switzerland, supplied by Anchrom technologists, Mumbai) equipped with 100μl syringe. A constant application rate of 6μl/sec was employed and the space between two bands was 10mm. The slit dimension was kept at 5×0.45mm and a scanning speed of 20mm/sec was employed. A common mobile phase consisted of toluene: acetone: methanol (8:1:1 v/v) and 10 ml of mobile phase was used for chromatography. Linear ascending development was carried out in 10×10cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 20 min. at room temperature. The length of chromatogram run was 8.5cm and subsequent to the development, the TLC plates were dried in a current air with the help of a dryer in wooden chamber with adequate ventilation. Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode at 254nm and operated by WINCATS software (Camag) resident in the system. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400nm and concentrations of the compound chromatographed were determined from the intensity

of diffusely reflected light.

Micro preparative TLC

Preparative chromatography when used for the separation of 2-5mg of compound, it was called Micro preparative TLC. The purpose of preparative chromatography was to obtain pure compounds for further chromatographic or spectrometric analysis or to investigate chemical/biological properties.

Sample solutions were prepared using methanol as solvent. Chromatography was performed on 10cm×10cm chiral TLC plates (Merck, Darmstadt, Germany). The plates were prewashed with Methanol and activated at 110°C for 5minutes. Since it was micro preparative TLC, sample was applied by streaking across the full width of the plate instead of spotting to achieve higher loading capacity of the sample. The sample was applied as a single band of 170mm wide, at a constant application rate of 6µl/sec. This was eluted using 20ml toluene: acetone: methanol (8:1:1 v/v) as mobile phase. After development of the TLC plates, the zones containing separated compounds (Figure1) were detected, marked under UV light and scrapped from the plate backing. The compounds were eluted with methanol to obtain the isolated compounds, concentrated by evaporating the solvent on a rotovapour at a temperature of 35-40ºC and the isolated pure compounds thus obtained were subjected to mass spectrometry for characterization.

UV spectroscopy

Conventional UV spectroscopy has also been employed to confirm the findings where solutions in hexane of rac-OBC and standard reference materials were analysed using a double beam UV-spectrometer (Shimadzu, Kyoto, Japan) and the results were compared with those obtained by on-line in-situ UV spectra of HPTLC.

Method validation

The method was validated in accordance with ICH guidelines on the validation of analytical methods[7,8]. For linearity, different volumes of stock solution (0.5, 1, 1.5, 2, 2.5, 3 and 3.5µl) were applied to a TLC plate to give 50, 100, 150, 200, 250, 300 and 350µg per spot respectively. Peak-area and drug-concentration data were treated by linear least-square regression

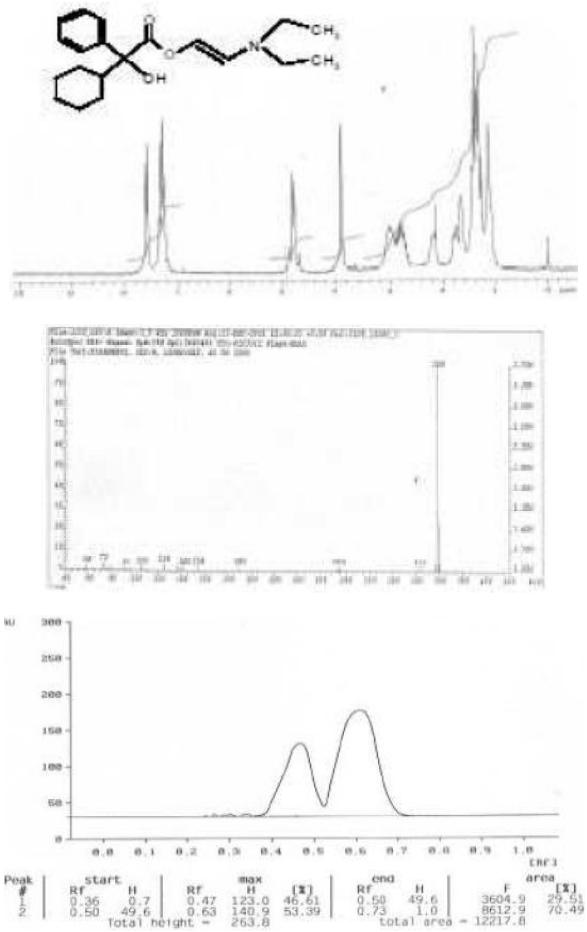

Figure 1 : Densitogram of oxybutynin chloride enantiomers along with mass and NMR spectra

analysis and the calibration plot was generated. The limit of detection (LOD) and quantification (LOQ) were calculated on the basis of the slope (s) of the calibration plot and the standard deviation of the response (SD) using the formula LOD = 3.3×SD/s and LOQ = 10×SD/s. The precision of the method was studied with the help of repeatability and intermediate precision analysis. Repeatability was performed by analyzing three different concentrations (100µg, 200µg, 300µg/spot) of the drug for six times on the same day while the intermediate precision of the method was checked by separation studies on the three different days. Also robustness was evaluated by an analysis of sample solutions after making small changes to mobile phase composition and development distance. Also the mobile phase composition toluene: acetone: methanol solution as 8:1:1 and 7.5:1.5:1 (v/v) were also tried along with two run lengths 8.5cm and 9cm. Robustness of the method was carried out at three different concentration levels of 150,

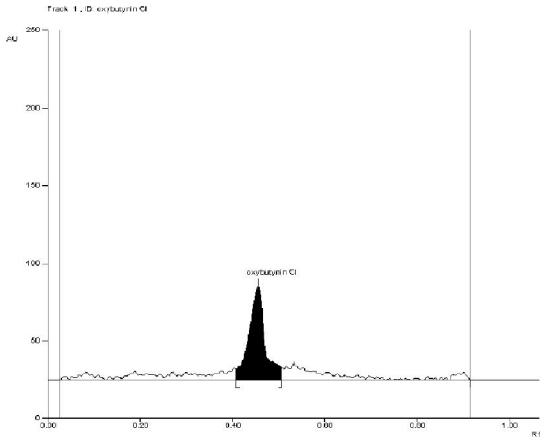

Figure 2 : A typical densitogram of oxybutynin chloride Rf 0.46±0.03 at 254nm using toluene-acetone -methanol solution (8:1:1 v/v)

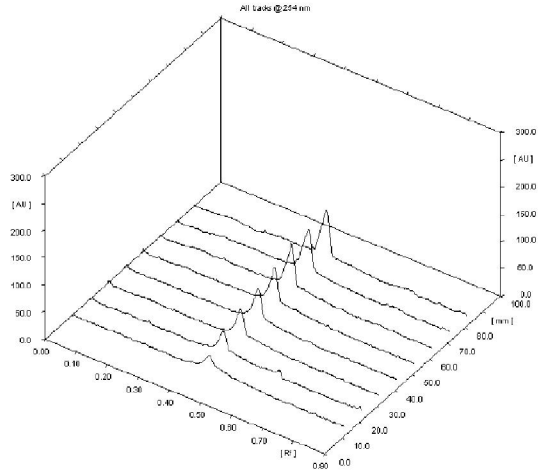

Figure 3 : Densitogram of standard oxbutynin chloride in different concentrations at 254nm

TABLE 1 : Linear regression data for the calibration curves (n = 3)

Parameters	TLC densitometry
Linear range	50-350 µg/spot
Correlation coefficient (r) ±SD	0.9992±0.00217
Slope±SD	4.55±0.059
Intercept±SD	449.6±3.25
LOD(µg/spot)	11.12
LOQ(µg/spot)	35.3

TABLE 2 : Intra and inter-day precision by HPTLC method (n = 6)

Amount (µg/spot	Repeatability		Intermediate precision	
	Mean area (AU)±SD	RSD	Mean area (AU) ±SD	%RSD
100	883.24±3.38	0.38	889.73±8.82	0.99
200	1353.1±3.91	0.28	1349.4±7.36	0.54
300	1796.3±4.15	0.23	1794.9±12.23	0.68

200 and 350µg per spot. Futher, the recovery study was performed by standard addition method. Analyzed samples were spiked with additional 50, 100 and 150µg of standard OBC and the samples were reanalyzed by the proposed method in triplicate. The specificity of the method was also determined by analyzing standard drug and test samples. The spots for OBC and the sample were confirmed by comparing the R_f and spectrum of the spot with that of the standard. The peak purity of OBC was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E).

RESULTS AND DISCUSSION

Scope of the work

It is well known that even in TLC, chiral stationary phases offer very discrete separations of enantiomers from a recemic mixture. The unique advantage in TLC is the recemic mixture can be directly analysed and the enantiomers were separated as such without any derivatization or prior sample treatment. However, the TLC plates with chiral stationary phases were now not available. Therefore, the present work has been carried out with a single plate gifted by (Anchrom, Bombay) exclusively for the separation of the enantiomers, but the validation studies were carried out by ordinary silica gel plates 60 F_{254} (Merck, Darmstadt Germany). This study has been undertaken only to ascertain that unlike in HPLC where enantiomers are further converted to diastereiomers and separated on a chiral column, the recemic mixture can directly be analysed on chiral plates. However, the non-availability of the plates was the greatest limitation and our attempts to procure them from various companies have been futile. But encouraged by the initial positive results our observations and the analytical details of the present study undertaken for separation of OBC by chiral TLC and the validation of the method using the racemic mixture by TLC have been presented here in.

Selection of the optimum mobile phase

The best separations were obtained with $CHCl_3$: MeoH, but as halogenated solvents were always dis-

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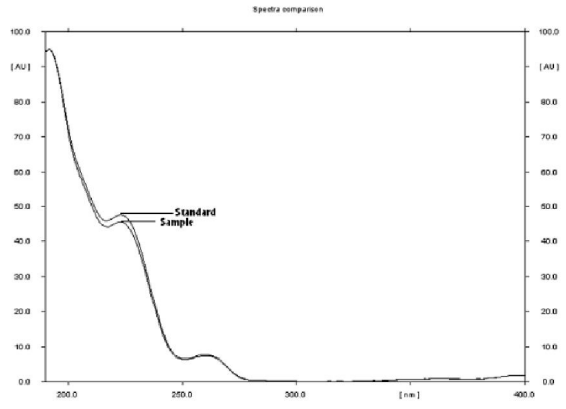

Figure 4 : Insitu UV spectra of oxbutynin chloride standard and oxbutynin chloride from sample

couraged, varying concentrations of toluene-acetone-methanol were attempted. However, toluene-acetone-methanol solution in a ratio of (8:1:1 v/v) only offered a well-resolved separation of individual isomers of OBC. Figure 1 shows a typical densitogram of the two enontiomers of OBC and the inset shows the mass spectrum that was obtained from the isolated samples of individual isomers by micro TLC. This particular study was a part of our routine quality control work and required only a very short time to confirm and conclude the separation and identification.

It was evident from the chromatographic study that though the bands were close (R_f 0.47 and 0.63) they were well resolved and facilitated a subtle but distinguishable separation and isolation of individual isomers by micro preparative TLC. Incidentally, satisfactory results could be accomplished using a very simple mobile phase like toluene-acetone-methanol and the development time was absolutely minimum. An on-line UV spectrum along with densitometric scanning resembled well that of the UV spectrum obtained from a conventional UV spectrometer with authentic samples. However, the individual bands obtained by micro TLC are separately analyzed by LSISMS/FAB and (Figure 1) m/z 358 confirmed identify of oxybutynin chloride or both the isomers.

Method validation

The method validation was carried out with the total drug molecule on 10×10cm silica gel plates (precoated with 60 F_{254}) and toluene-acetone-methanol solution in a ratio of (8:1:1 v/v) offered a sharp and well-defined peak of OBC at R_f value of 0.47± 0.03

TABLE 3 : Recovery studies of oxybutynin chloride (n = 3)

Excess of drug added to the analyte (%)	Theoretical content (µg)	Amount of drug found (µg) (%)	Recovery %	% R.S.D.
0	100	98.24	98.24	0.22
50	150	152.8	101.3	0.36
100	200	200.6	100.3	0.54
150	250	253.7	101.7	0.27

TABLE 4 : Robustness of the method

Amount [ng/ spot]	Mobile phase composition T-A-M:[a]		Plate run length	
	8+1+1	7.5+1.5+1	8.5 cm	9 cm
150	0.31	0.48	0.42	0.38
250	0.54	0.92	0.73	0.81
350	0.49	0.57	0.61	0.52

(Figure 2 and 3). Linear regression data for the calibration plots (n = 3), as given in TABLE 1 was indicative of a good linear relationship (r = 0.9993) between peak area and amount of OBC over the range of 50-350µg per spot. A significant difference was observed in the slopes of the plots if a different concentration range was used (ANOVA, $P< 0.05$). Limit of detection and limit of quantification were calculated by the method described in the experimental section and found to be 11.12µg/spot and 35.33µg/spot TABLE 1 respectively. The results of the repeatability and intermediate precision experiments were shown in TABLE 2. The developed method was found to be precise as the RSD values for repeatability and intermediate precision respectively were around 2% as recommended by ICH guidelines. The low values of %RSD obtained after the small changes of mobile phase composition and development distance (TABLE 3) were indicative of the robustness of the method. To check the degree of accuracy of the method, recovery studies were performed in triplicate by standard addition method at 50%, 100% and 150%. Recovery of added OBC was 98.24-101.7, as listed in TABLE 4. The specificity of the method was ascertained by analyzing the standard drug and sample. The spot for OBC in sample was confirmed by comparing the R_f and spectra of the spot with those of the standard, the sample and they were presented in figure 4.

CONCLUSIONS

Chiral HPTLC is a rapid, simple but specific meth-

odology that can be successfully employed for selective separation, isolation and quantitative determination of enantiomers without derivatization of diasteromers. The method can be routinely used not only for process development but also for quality control studies.

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