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Development and validation of spectrophotometric, HPTLC and HPLC methods for the determination of Imipramine and Chlordiazepoxide in pharmaceutical dosage forms

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ABSTRACT

Three reliable, rapid and selective methods have been developed and validated for the determination of imipramine and chlordiazepoxide in pharmaceutical dosage forms. The first method is spectrophotometric method includes simultaneous estimation method, first order derivative spectrophotometric method, isoabsorptive method and multiwavelength method. All variables affecting the reaction have been investigated and the conditions were optimized. The second method is based on separation of the cited drugs (imipramine Rf =0.45 and chlordiazepoxide Rf = 0.26) followed by densitometric measurement of the intact drug spots at 288 nm by HPTLC. The separation was carried on silica gel plates using toluene: ethyl acetate: ethanol: diethanolamine (70: 15: 4: 1 v/v/v/v) as a mobile phase. The linearity range was 4-10µg for chlordiazepoxide and 5-9µg for imipramine with mean accuracy 99.99±1.02%. The third method is accurate and sensitive stability-indicating HPLC method based on separation of imipramine and chlordiazepoxide on a reversed phase C18 column, using a mobile phase of methanol at temperature 27±2 °C and UV detection at 275nm in an overall analysis time of about 9 min., based on peak area. The results obtained were analyzed by statistically to assess that no significant difference between each of the three methods. The validation was performed according to ICH guidelines.

Key words: Imipramine; chlordiazepoxide; spectrophotometric; HPTLC; HPLC.

INTRODUCTION

Chlordiazepoxide is the first benzodiazepines to be used clinically as antianxiety drug, it is (7-Chloro-*N*-methyl–5–phenyl-*3H*-1,4–benzodiazepin–2–amine 4–oxide). Imipramine is dibenzazipine tricyclic antidepressant, chemically it is (10, 11-Dihydro-*N*, *N*-dimethyl–5*H*-dibenz [*b*,*f*] azepine–5–propanamine). Some stability indicating HPLC [1-4], GC [5] and TLC [6] methods have been reported for the estimation of chlordiazepoxide in blood plasma and in pharmaceutical dosage forms. HPLC [7], Spectrophotometric [8] and capillary electrophoresis [9] methods have been reported for the estimation of imipramine in blood plasma and in pharmaceutical dosage forms. Although chlordiazepoxide and imipramine are widely prescribed combination and commonly used in dual drug therapy as a potent anti depressant drug, yet no method is so far reported for their simultaneous estimation. A successful attempt has been made to estimate these two drugs simultaneously by spectrophotometrically, HPTLC and HPLC.

MATERIALS AND METHODS

Instrumentation

Shimadzu UV/Vis spectrophotometer 1700 pharmaspec. A Camag HPTLC system (Switzerland) comprising of Camag Linomat V semiautomatic sample applicator, Camag TLC scanner 3, Camag twin trough chamber (10x10 cm), Camag Cats 4 software, Hamilton syringe (100µl) were used during the study. TLC plates (10x10 cm) aluminium plates precoated with 0.25 mm silica gel F254, were purchased from E. Merck. A gradient high-pressure liquid chromatograph (Shimadzu HPLC Class VP series) with two LC-10AT VP pumps, variable wavelength programmable UV/Vis detector SPD-10AVP and C18 LUNA (5 micron 25 cm×4.6 mm) column from Phenomenex was used for separation and quantification.

Materials and Reagents

Imipramine and chlordiazepoxide working standard, was supplied by Pifer Pharma Chandigarh (India), the purity of the sample was found to be 99.12±0.89% for imipramine and 99.24±0.12% for chlordiazepoxide according to the reported specifications. All chemicals are of spectroscopic and chromatographic grade purchase from Merck, India Limited, Mumbai was used in the study. The commercially available marketed tablet containing a combination of chlordiazepoxide 10 mg and imipramine 25mg were procured from local pharmacy.

Standard Solutions

Chlordiazepoxide and imipramine (1mgml^{-1}) were prepared in methanol for spectrophotometric, HPTLC and HPLC methods. The standard solution of both the drugs were subsequently used to prepare working standard solution (2-26 µg ml^{-1}) for spectrophotometric method in methanol and (1-10 µg ml^{-1}) for HPLC method in the mobile phase. All solutions were kept in a refrigerator at 4 °C and were stable for one week.

Sample Preparation

Twenty tablets were accurately weighed and finely powdered. An amount equivalent to 10 mg chlordiazepoxide and 25 mg imipramine was transferred into 100 ml volumetric flask, and dissolved in 50 ml methanol. The solution was stirred with magnetic stirrer for 10 min, filtered and the volume was completed to the mark.

Chromatographic Conditions

For HPTLC method, the plates were developed in toluene: ethyl acetate: ethanol: diethanolamine (70: 15: 4: 1 v/v/v/v) as a mobile phase. For detection and quantification, each of the sample solutions and standard solutions of different concentrations within the linearity range were applied as separate compact spots 12 mm apart and 15 mm from the bottom of the HPTLC plate using Linomat V applicator. The chromatographic tank was saturated with the mobile phase for 20 min before development of the plates. The plates were developed up to 8 cm in the usual ascending way, air-dried, and scanned for chlordiazepoxide and imipramine at 288 nm by using the instrumental parameters mentioned above. For HPLC method, the mobile phase was methanol. It was filtered by using a 0.45µm membrane filter and degassed in an ultrasonic bath before use. The samples were also filtered by using 0.45 µm membrane filters. The flow rate was set at 0.6 ml/min and UV detector at 288 nm. The column was conditioned for 30 min. All determinations were performed at ambient temperature 27±2 °C and the injection volume was 20µl.

Calibration for Spectrophotometric Method

Aliquots of standard solution equivalent to (2-26 µg ml^{-1}) chlordiazepoxide and imipramine were transferred into 10 ml volumetric flasks separately. The volume was completed to the mark with methanol. Absorbance of the aliquots was measured at different wavelengths for different spectrophotometric method (viz: for simultaneous equation method 251nm and 284nm, for Ist order derivative method 283nm and 251 nm, for isoabsorptive method 237.5 nm and 284 nm and for multi wavelength method, difference of absorbance b/w (288.5-275.5nm) and difference of absorbance b/w (244.5-224.5nm). The calibration curve was plotted and the regression equation was recorded.

Calibration for HPTLC Method

Aliquots of standard solution (1mgml^{-1}) equivalent to (4-10 µg of chlordiazepoxide and 5-9 µg of imipramine) was applied to HPTLC plate by Linomat V applicator with the help of micro syringe and developed as described under chromatographic conditions previously mentioned under 'chromatographic conditions.' The plates were visualized at 254 nm and scanned at 288 nm by densitometer. Calibration curve was plotted representing the relationship between the average peak area and concentration and the regression equation was recorded.

Calibration for HPLC Method

Aliquots of standard solution (1mgml^{-1}) equivalent to (1-10µgml^{-1}) of chlordiazepoxide and imipramine were transferred into 10-ml volumetric flasks and the volume was completed to the mark with the mobile phase.

Triplicate 20µl injections were made of each concentration. The average peak areas were calculated and plotted versus concentrations, linear relationship was obtained and the regression equation was recorded.

Application to Tablets
The above producers were applied to the analysis of marketed tablets, using sample preparation as mentioned under sample preparation. The concentration of chlordiazepoxide and imipramine was calculated from the recorded regression equations.

Method Development
Spectrophotometric method
Method I (Based on simultaneous equation method):
Overlain spectra (Fig. 1) of standard solutions of chlordiazepoxide and imipramine were scanned. Chlordiazepoxide shows absorption maxima at 284 nm and imipramine shows at 251 nm. The calibration curves for chlordiazepoxide and imipramine were prepared in the concentration range of 4-18 $\mu g ml^{-1}$ for both the drugs at both the wavelengths i.e. 284 nm and 251 nm. The absorptivity coefficients were determined for both the drugs at both the wavelengths and following equations were made.

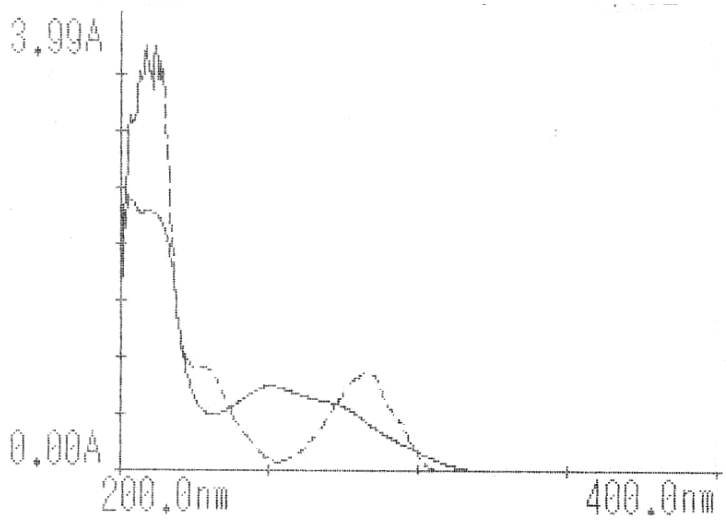

Fig. 1 Overlain spectra of chlordiazepoxide (-------) and imipramine (-)

$$A_1 = 224.56\ C_{Imipra} + 36.76\ C_{Chlord} \text{ ---------- (at } \lambda_{251}) \text{ (1)}$$
$$A_2 = 126.27\ C_{Imipra} + 259.04 C_{Chlord} \text{ ---------- (at } \lambda_{284}) \text{ (2)}$$

A_1 and A_2 are absorbances at 251 nm and 254 nm respectively and C_{Imipra} and C_{Chlord} are concentrations of imipramine and chlordiazepoxide respectively. The concentrations of both the drugs in the mixture were determined by eqs. (1) and (2).

Method II Derivative spectrophotometric method
Upon examining the first-derivative spectra of the two drugs, it can be noticed that chlordiazepoxide can be determined at 251 nm (Fig. 2) where imipramine has no contribution and imipramine can be determined at 283 nm (Fig. 3) where chlordiazepoxide shows a zero crossing.

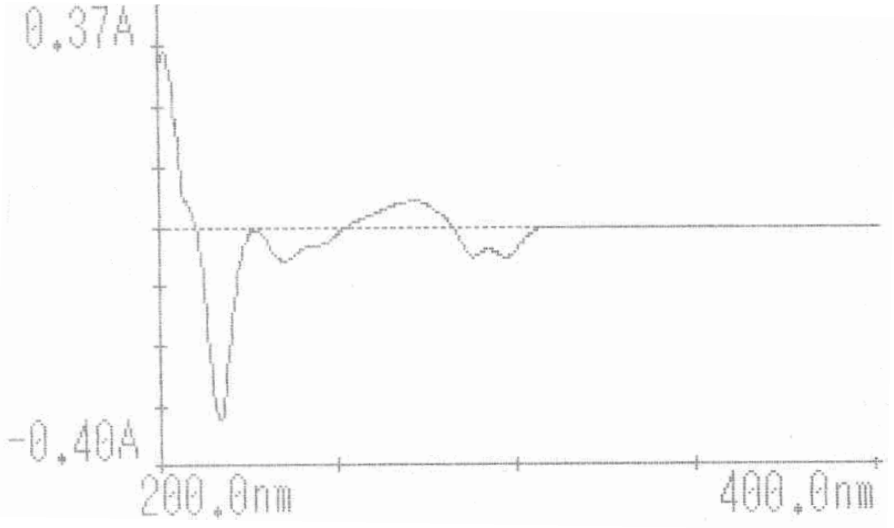

Fig. 2 First order derivative spectra of chlordiazepoxide

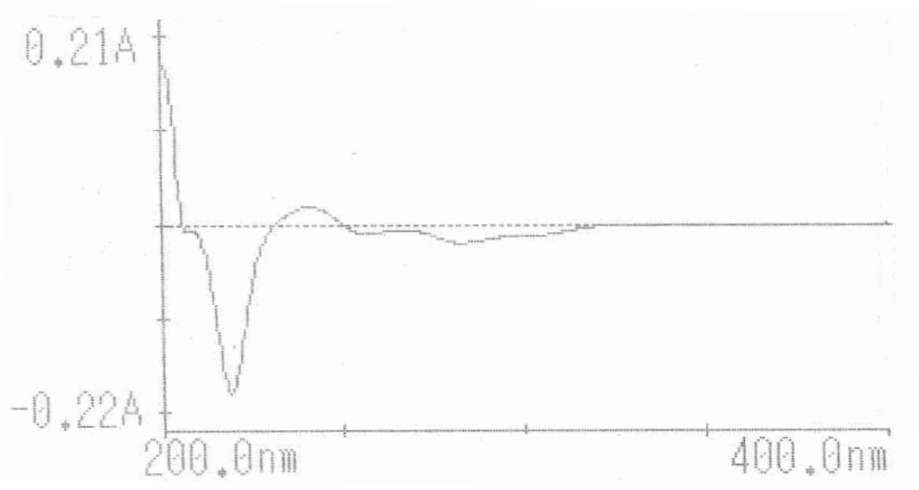

Fig. 3 First order derivative spectra of imipramine

$A = 0.0005C + 0.0008$ …… (3) **(**At λ_{283}**)**
$A = 0.0005C - 0.0002$ …….. (4) **(**At λ_{251}**)**

Where C is the concentration in µgml^{-1}, A is the peak amplitude of the first-derivative curves at 283 and 251 nm for imipramine and chlordiazepoxide respectively.

Method III Graphical absorbance ratio method:
This method is based on the method used by Ghanem *et al* which makes use of the iso-absorptive point of the two drugs i.e. the wavelength of equal absorptivity of the two components of the mixture. The iso-absorptive point was 237.5 nm in this case. The other wavelength selected is the absorption maximum of one of the components. In this case it was 284 nm, the absorption maximum of chlordiazepoxide. The concentrations of the two components are related to the ratio of the absorbances at these two wavelengths. The absorbance of the mixture was noted at 237.5 and 284 nm. Calibration curves of chlordiazepoxide and imipramine were plotted in the concentration range 8-22 µgml^{-1} (range for which Beer-Lambert's law followed). The absorptivity coefficients were determined for both the drugs and the average value was taken. These values and the absorbance ratio were used to develop equations as given below.

$$A_1 = 166.12 (Cx + Cy) = 166.12 Cx (0.7991/Qm – 1.5593) \text{ ……..(5)}$$

Where $Qm = A_2 / A_1$ and A_1, A2 are the absorbances at 237.5 and 284 nm respectively. Cx and Cy are concentrations of chlordiazepoxide and imipramine respectively.

Method IV: Multiwavelength method

The utility of multiwavelength data processing programme is to calculate the unknown concentration of component of interest present in a mixture containing both a component of interest and an unwanted interfering component by the mechanism of the absorbance difference between two points on the mixture spectra is directly proportional to the concentration of the components of interest, independent of the interfering components. It involves the estimation of two components, each time considering the second one to be an interfering one. An important criterion for this method is the presence of some absorbance in the spectrum of interfering component. The measuring wavelength can be spaced either equally of arbitrarily, depending on the measuring purpose.

Two wavelengths were selected 288.5 nm and 275.5 nm for imipramine at which chlordiazepoxide shows the equal absorbance and 244.5 nm and 224.5 nm for chlordiazepoxide at which imipramine shows equal absorbance.

Two equations were formed

$$A_1 = 0.0076C_{Imipramine} + 0.0012 \ldots\ldots\ldots \quad (6)$$

$$A_2 = 0.0199C_{Chlordiazepoxide} + 0.0215 \ldots\ldots\ldots \quad (7)$$

Where A_1 is absorbance difference between the 288.5nm and 275.5 nm and A_2 is the absorbance difference between the 244.5 nm and 224.5 nm for imipramine and chlordiazepoxide respectively.

HPTLC method

The developed HPTLC method was applied to the determination of chlordiazepoxide and imipramine in combined dosage form. The content of twenty tablets was ground to fine powder. An accurately weighed quantity equivalent to about 10 mg of chlordiazepoxide and 25 mg imipramine was transferred to a 50 ml volumetric flask, dissolved in 25 ml methanol, shaken for 15 min and volume was made up to mark with methanol. The solution was filtered through Whatman filter paper no. 41. A fixed volume of the working standard solution and sample solution were spotted as sharp bands on TLC plate. After development, the bands of the drugs were scanned at 288 nm (Fig. 4). The amount of the chlordiazepoxide and imipramine was calculated by applying suitable dilution factor and comparing peak height and peak area of the standard and the sample solution.

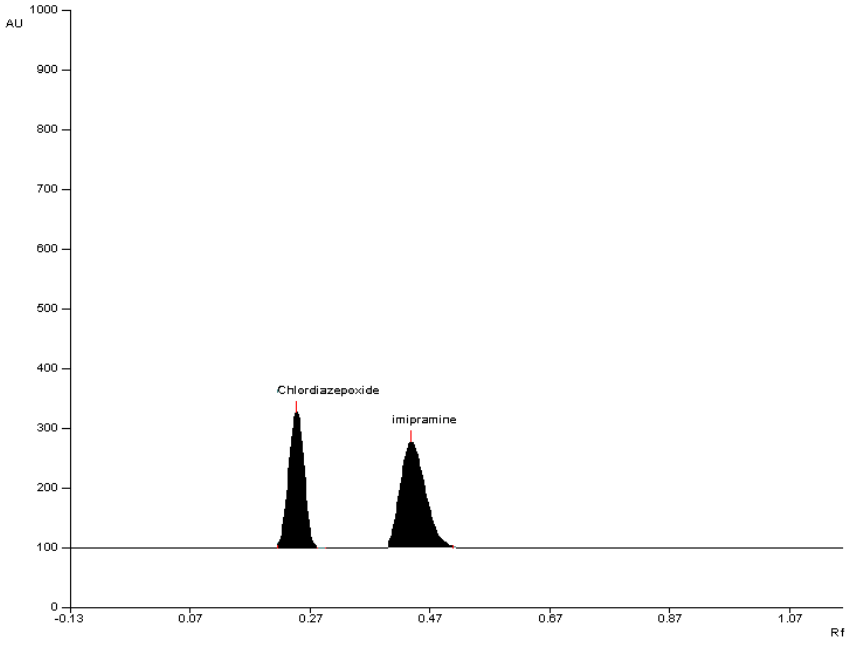

Fig.4 HPTLC chromatogram of chlordiazepoxide and imipramine

HPLC Method

The developed HPLC method was applied to the determination of chlordiazepoxide and imipramine in combined dosage form. To optimize HPLC assay parameters, the mobile phase composition and pH were studied. A satisfactory separation was obtained with a mobile phase of methanol using C18 column at ambient temperature.

The analysis was carried out by isocratic elution with flow rate 0.6 ml min^{-1} and detection at 275 nm (Fig. 5). A linear range of 1-10 µgml^{-1} was obtained as show in Table 1.The system suitability tests of HPLC method were evaluated Table 3.

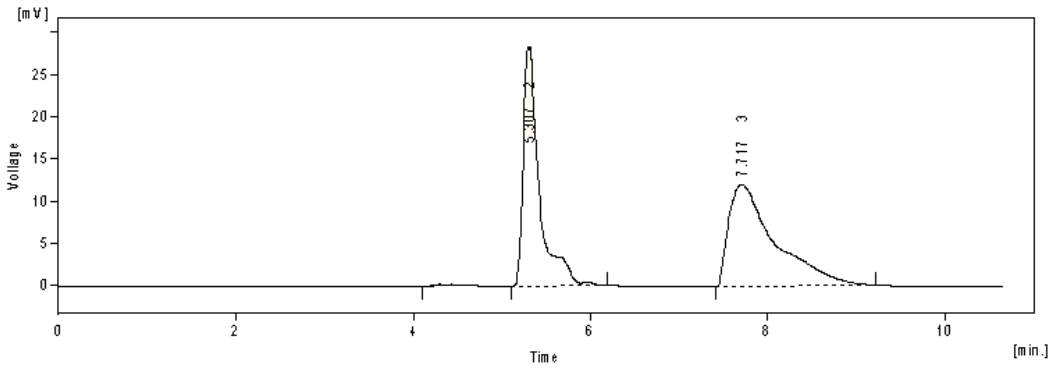

Fig. 5 HPLC chromatogram of chlordiazepoxide and imipramine

Table 1. Validation Report of Spectrophotometric, HPTLC and HPLC Methods for the Determination of imipramine

Parameters	Spectrophotometric method				HPTLC method	HPLC method
	Simultaneous equation method	Derivative spectrophotometric method	Graphical absorbance ratio method	Multiwavelength method		
Linearity range	4-18 µgml^{-1}	2-14 µgml^{-1}	8-22 µgml^{-1}	4-20 µgml^{-1}	5-9 µg	1-10 µgml^{-1}
Regression equation						
Slope	0.0058	0.0008	0.0073	0.0027	1469.8	236.33
S.D. of slope	0.0084	0.0001	0.0062	0.0024	10.23	6.86
Intercept	0.0246	0.0005	0.0182	0.0074	482.62	49.254
S.D. of intercept	0.0052	0.0012	0.0038	0.0015	14.28	15.29
Correlation coefficient	0.9984	1	0.9951	0.9989	0.9963	0.9990
S.D. of correlation coff.	0.0024	0.0004	0.0058	0.0097	0.0011	0.0026
Precision±RSD%						
Intra day[a]	99.98±0.21	99.12±0.44	98.91±0.87	99.31±0.39	100.1±0.09	99.86±0.54
Inter day[a]	99.11±0.89	99.59±0.57	100.08±0.74	99.46±0.98	100.98±0.2	99.99±0.09
Accuracy (mean)[b]±S.E.	99.79±0.72	100.12±0.29	99.47±0.24	99.12±0.19	99.99±1.02	99.14±0.80

a) Average of n=9. b) Average of n=6.

Table 2. Validation Report of Spectrophotometric, HPTLC and HPLC Methods for the Determination of chlordiazepoxide

Parameters	Spectrophotometric method				HPTLC method	HPLC method
	Simultaneous equation method	Derivative spectrophotometric method	Graphical absorbance ratio method	Multiwavelength method		
Linearity range	4-18 µgml^{-1}	4-14 µgml^{-1}	8-22 µgml^{-1}	4-20 µgml^{-1}	4-10 µg	1-10 µgml^{-1}
Regression equation						
Slope	0.0128	0.0002	0.0073	0.0253	1172.8	118.87
S.D. of slope	0.0014	0.0001	0.0062	0.0071	11.12	5.59
Intercept	0.0277	0.0005	0.0182	0.0194	527.6	47.82
S.D. of intercept	0.0004	0.0009	0.0021	0.0014	15.94	16.01
Correlation coefficient	0.9987	1	0.9951	0.9959	0.9918	0.9999
S.D. of correlation coff.	0.0002	0.0001	0.0019	0.0011	0.0026	0.0031
Precision±RSD%						
Intra day[a]	99.14±0.68	99.41±0.71	98.89±1.04	99.01±1.23	99.36±0.21	99.59±0.26
Inter day[a]	99.59±0.24	99.12±0.89	99.87±0.32	100.22±0.14	100.12±0.45	99.74±0.12
Accuracy (mean)[b]±S.E.	99.92±0.51	99.23±0.65	100.56±0.39	99.36±0.97	99.78±0.59	100.21±0.3

a) Average of n=9. b) Average of n=6.

Table 3. Results of system suitability tests of HPLC method

Parameters	Value		Comments
	Chlordiazepoxide	Imipramine	
Retention time	5.30±0.034	7.71±0.089	±standard deviation
Injection repeatability	0.53%	0.59%	RSD of 6 injections
K	3.29	4.12	Capacity factor
Theoretical plate	6182	5129	Column efficiency plate/column
HETP	0.016	0.019	Height equivalent theoretical plate

RESULTS AND DISCUSSION

For spectrophotometric methods validation parameters were studied for all the methods. Accuracy was determined by calculating the recovery and the mean was determined. Precision was calculated as repeatability and inter and intra day variation for both the drugs. All the methods were successfully used to estimate the amounts of chlordiazepoxide and imipramine in bulk as well as pharmaceutical preparations.

Table 4. Determination of chlordiazepoxide and imipramine in marketed formulations

Sample no.	Spectrophotometric method								HPTLC method		HPLC method	
	Simultaneous equation method		Derivative spectrophotometric method		Graphical absorbance ratio method		Multiwavelength method					
	Chl	Imp	Chl	Imp	Chl	Imp	Chl	Imp	Chl	Imp	Chl	Imp
1	99.33	99.33	100	100	99.66	99.86	100	100.35	100.55	100.08	99.83	100.16
2	98.83	99.46	103.33	94.66	100.16	100.06	99.74	100	103.77	98.48	100.5	99.40
3	99.83	99.13	93.33	97.33	102	94.73	99.74	99.82	101.55	99.61	100.0	100.0
4	97.16	100.8	100	96	97.83	99.26	100.25	99.91	100.77	100.26	100.0	100.3
5	100.16	99.4	96.66	97.33	98.83	100.06	99.83	100	100.55	100.08	99.33	100.06
6	98.0	100.1	93.33	101.33	99.66	99.86	100	99.91	101.11	99.64	98.08	100.3
Mean	98.88	99.70	97.778	97.775	99.69	98.97	99.92	99.99	101.38	99.69	99.62	100.03
RSD	1.151	0.635	4.128	2.54	1.369	2.100	0.197	0.185	1.213	0.650	0.847	0.334
S.D.	1.138	0.633	4.036	2.483	0.996	0.989	0.189	0.179	1.229	0.648	0.844	0.329

Choice of an analytical method depends on factors such as the nature of the drug, the complexity of the sample, and the intended use. In this study, the chromatographic conditions were affected by the physicochemical properties of imipramine and chlordiazepoxide, for example solubility, polarity, and UV absorption. The objective of the study was to develop an HPLC assay for analysis of imipramine and chlordiazepoxide as the drug substance and as the combined dosage form. Mobile phase selection was based on peak properties (symmetry, number of theoretical plates, capacity factor), run time, ease of preparation, and cost. This method uses a simple mobile phase, which can be regarded as more useful in routine analysis. Retention time repeatability during the precision studies was found to be excellent. The retention time of imipramine (7.71 min) and chlordiazepoxide (5.30 min), was satisfactory. Both the drugs gave a sharp and symmetrical peak when chromatographed under the conditions described above.

The attempt was made to develop HPTLC method for estimation of chlordiazepoxide and imipramine in combined dosage form. Literature survey revealed that there is no HPTLC method has been reported for simultaneous estimation of imipramine and chlordiazepoxide in pharmaceutical preparation. Before proceeding to the experiment the available both the drugs were standardized by the official methods. Various pure solvent and mixtures in different proportion were tried as mobile phase. However mobile phase comprising of toluene: ethyl acetate: ethanol: diethanolamine (70: 15: 4: 1 v/v/v/v) were found more suitable for quantitative separation of chlordiazepoxide and imipramine with R_f value 0.26 and 0.45 respectively with saturation period of 20 min at 288 nm. The selection of wavelength was based on nearly equal absorbance by both the component of mixture for optimum sensitivity. The calibration curves were drawn with peak height and peak area for each concentration of the drugs. This study was validated by considering various validation parameters. Validation was performed according to the guideline of ICH.

Method validation

Linearity/Range

Aliquots of different dilutions were prepared for the linearity test. Each solution was measured (or injected) three times and linear regression analysis of chlordiazepoxide and imipramine was driven (Table 1).

Precision
The precision of the methods were assessed by determining RSD values of intra-day and inter-day analysis (n=9) of chlordiazepoxide and imipramine standard solutions over 3 days (Table 1).

Accuracy
The calculated t-test and F-test are not exceeding their theoretical values at p=0.05, indicating that there is no significant difference between each method.

Standard Addition Technique
The proposed methods were applied for the analysis of the drug in pharmaceutical dosage form (Table 4). The validity of the methods was assessed by applying the standard addition technique. The results in indicate no interference from tablets excipients such as calcium carbonate, hydroxypropyl cellulose, aluminium magnesium silicate, povidone, sodium starch glycolate, saccharin sodium and magnesium stearate.

CONCLUSION

The presented work describes validated spectrophotometric; HPTLC and HPLC methods for the assay of chlordiazepoxide and imipramine in pharmaceutical dosage form and bulk drug (Table 2). The three suggested methods are simple, selective, and accurate can be used for the routine quality control analysis of the cited drug either in bulk or in dosage form without any interference from common excipients. The spectrophotometric method is rapid with low cost for both identification and quantification.

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