CHAPTER 2

METHODS FOR THE ANALYSIS OF TRICYCLIC ANTI-DEPRESSANT DRUGS

2.1. Introduction

The tricyclic antidepressants (TCA’s) are one of the largest groups of drugs for the treatment of psychiatric disorders such as depression, mainly endogenous major depression. The analyses of these compounds are important for obtaining optimum therapeutic concentrations and for quality assurance in pharmaceutical preparation. Therapeutic drug management is most common for the tricyclic anti depressants (TCA’s). Therapeutic drug management (TDM) of TCA’s is important due to wide inter-individual variability in pharmacokinetic, production of active metabolites, and a high risk of drug-drug interactions. In addition, TDM of some TCAs can be used to optimize dose, wherein concentration relationships are recognized for both therapeutic response and potentially life-threatening toxicity. In many clinical scenarios, TDM of TCA’s is accomplished by currently available point of care or automated immunoassays that provide a total TCA concentration.

This chapter describes an analytical methods designed to quantify clinically and pharmaceutically significant concentrations of two TCA’s (dothiepin and doxepin) in human plasma using liquid chromatography-tandem mass spectrometry (LCMS/MS), and spectrophotometric methods in pure form and in pharmaceutical dosage forms.

2.2. Drug profile

Dothiepin hydrochloride

It is an important and commonly prescribed drug for major depressive disorder, anxiety, insomnia, neuropathic pain/chronic pain and resistant depression. It is commercially available under trade names Prothiaden75, Dothep50 etc. Dothiepin hydrochloride (DOT) is chemically known as \((E, Z)-3-(\text{dibenzo}[b,e]\text{thiepin-11}(6H)\text{-ylidene})-\text{N,\text{N-di-methylpropan-1-amine}}\). Dothiepine has the following molecular structure:
Doxepin hydrochloride is a white or faintly yellow crystalline powder. It is freely soluble in water, alcohol and methylene chloride. The molecular formula of doxepine is C\textsubscript{19}H\textsubscript{21}NS. HCl and molecular weight is 331.9 g/mol.

**Doxepin hydrochloride**

Doxepin hydrochloride (DOX) is chemically known as (3E)-3-(6H-benzo[C] [1] benzoxepin-11-ylidene)-N, dimethylpropan-1-amine. It is a psychotropic agent with tricyclic antidepressant and anxiolytic properties. It is used to treat depression, anxiety disorders, and as a second line treatment of chronic idiopathic urticaria. Known under many brand names such as Aponal, Adapine, Doxal (orion), Deptran, Sinquan and Sinequan. Doxepine is also used for the treatment of sleep maintenance, and the trade name of doxepin for this indication is Silanor. Doxepine has the following molecular structure:

\[ \text{HCl} \]

Doxepin hydrochloride is a white to off white powder. It is freely soluble in water, alcohol and methylene chloride. The molecular formula of doxepin is C\textsubscript{19}H\textsubscript{21}NO and molecular weight is 279.376 g/mol.

2.3. Literature review

The pharmacological and pharmaceutical importance of these drugs resulted in an increasing need for analytical methods for their detection and quantification. There is a wide variety of techniques used for analysis, from titrations to very advanced
techniques using highly sophisticated instrumentation. The most common techniques used include HPLC, gas chromatography, UPLC, LC-MS, GC-MS and CE-MS (capillary electrophoresis-mass spectrometry).

Among the various analytical methods, spectrophotometric methods for the determination of micro quantities of drugs have received considerable attention due to their simplicity, reliability and availability of number of chromogenic reagents.

Many scientific endeavors are dependent upon accurate quantification of drugs and endogenous substances in biological samples. The focus of bio-analysis in the clinical and pharmaceutical industry is to provide a quantitative measure of the active drug and/or its metabolites for the purpose of pharmacokinetic, toxico-kinetics, and bioequivalence studies.

**Spectrophotometry methods**

Hisham *et al.* [1] have developed two spectrophotometric and spectrofluorimetric methods for the analysis of tramadol, acebutolol and dothiepin in pharmaceutical preparations. The reagent used was 5% malonic acid prepared in acetic anhydride and the mixture was heated at 60 °C and make up with 95% ethanol. Beers law was obeyed in the range 5-25 µg/mL. The absorbance was measured at 329 nm and further continued spectrofluorimetric measurement, the fluorescence intensity was measured at 431-434 nm emission wavelengths with excitation at 389 nm against the reagent blank 95% ethanol.

Sameer and Basavaiah [2] have reported two simple spectrophotometric methods for the determination dothiepine hydrochloride using alizarin red S as an ion-pairing reagent. In the first method, the formed ion-pair complex was extracted with dichloromethane and the absorbance of the yellow colored complex was measured at 445 nm against the reagent blank. In second method, the ion pair complex of DOT–alizarin red S treated with dichloromethane and 1% alcoholic KOH, the contents were mixed well and measured the resulting violet colored species at 570 nm against the reagent blank.

El-Sayed and Hassan [3] have described three extractive colorimetric methods for the estimation of DOT in bulk sample and in dosage forms. First two methods
based on the formation of an ion pair complexes with methyl orange (A) and orange G (B). Method C depends on ternary complex formation between cobalt thiocyanate and the drug. The linear concentration range was 0.1-12 µg/mL for method A, 0.5-11 µg/mL for method B and 3.2-80 µg/mL for method C. Evaluated wave lengths were 423, 498 and 625 nm for methods A, B, and C respectively.

Two kinetic spectrophotometric methods for DOT in bulk and in drug formulation were described by Taha [4]. The first method was based on oxidation reaction of the drug with alkaline KMnO₄ at room temperature for a time of 25 min. The absorbance of the colored manganate ions was measured at 610 nm. In second method, reaction kinetics was studied between the drug and 4-chloro-7-nitrobenzofurazan (NBD-Cl) in the presence of 0.1 mol/L sodium carbonate, and the absorbance was measured at 470 nm. The linear range of these two methods was 4-24 and 50-250 µg/mL, respectively.

Revanasiddappa and Manju [5] have described spectrophotometric method for the doxepin, amitriptyline, and nortriptyline hydrochlorides in pure and in dosage forms. This method is based on the oxidative coupling of the drugs with 3-methylbenzothiazolin-2-one hydrazone in the presence of iron(III) chloride in 1 M hydrochloric acid. The method requires costly reagent.

Aliphatic nature of doxepin was spectrophotometrically determined with copper(II). Eugeniusz somogyi et al. [6] explained, the ions of copper(II) in the acetone medium create bonds with some compounds of the nature of tertiary aliphatic amines. Calibration curve for doxepine over the range of 3.16-7.90 mg/mL and the absorbance was measured at 474 nm. The method is less sensitive.

An extractive spectrophotometric determination of doxepin was reported in the literature [7]. It was developed for the assay of doxepin hydrochloride based on the formation of stable ion-pair complex between the drug and cobalt-thiocyanate in 2 N hydrochloric acid medium. The formed ion-pair complex was extracted with dichloroethane. The absorbance was measured at 625 nm against the reagent blank. Linearity range was 0.5-30 µg/mL.

One spectrofluorimetric method was reported for the determination of doxepin hydrochloride in commercial dosage forms by Nafisur Rahman et al. [8]. The method
was based on the fluorescent ion pair complex formation of the drug with eosin Y in the presence of sodium acetate-acetic acid buffer solution of pH 4.52. It was extracted with dichloromethane. This extracted complex showed fluorescence intensity at the wavelength of 567 nm after excitation at 464 nm. The linearity of the method was 0.1-0.8 µg/mL.

**Chromatographic methods**

Few chromatographic methods such as LC-MS/MS, HPLC, GC-MS and UPLC/MS/MS methods have been reported for the analysis of DOT. The author has described a brief account of these methods in the following paragraphs.

Xiang Chen *et al.* [9] have described a LC-ESI-MS method for dothiepine hydrochloride in human plasma. Samples were prepared using extraction solvents n-hexane: 2-propanol (95:5). The separation was achieved on Ultimate XB C18 column (2.1 x150mm, 5µm). The total run time per sample is 4.0 min.

Taylor *et al.* [10] have described a reverse phase HPLC method that will simultaneously measure dothiepine and its three metabolites in plasma using extraction solvent diethyl ether. Working range of this method is 50-1000 ng/mL.

Determination of antidepressant drug in oral fluid using tandem mass spectrometry was explained by Coulter *et al.* [11]. Drug was quantified using mixed-mode solid phase extraction and mass spectrometric detection was performed in positive electro spray ionization mode. The mobile phase was optimized in methanol.

Baden Horst *et al.* [12] have reported LC-MS/MS method for the determination of doxepin and its metabolite in human plasma. The sample was extracted with n-hexane: isoamyl alcohol and was separated on Phenomenex Luna C18 5µ, 150x2.1mm with a mobile phase methanol-water-formic acid.

Jiang-he Yan *et al.* [13] have described a method for stereo selective and simultaneous measurement of *cis* and *trans* isomers of doxepin and *N*-desmethyl doxepin in plasma by HPLC. Here, mobile phase consists of combination of hexane-methanol-nonylamine, and extraction solvent was a mixture of n-pentane-2-propanol (95:5). The calibration curve was linear over the range 1-200 ng/mL (plasma) and 1-400 ng/mL (urine).
Other techniques

An ion-selective electrode method [14] has been reported for quantitative analysis of dothiepin HCl. Two assay procedures have been developed using dothiepin selective electrodes. The first electrode was based on drug, galvanic acid ion pair complex, bis(2-ethylhexyl-phthalate) and poly vinyl chloride (PVC) matrix. A mixture of drug, tungstosilic acid, bis (2-ethylhexyl sebactate) as plastisizer and poly vinyl chloride matrix was used as a second electrode. The electrodes show a response in the range of $10^{-2}$ to $10^{-4}$ and $10^{-1}$ to $10^{-4}$ M dothiepine over the pH range of 5 to 8 for the two electrodes, respectively.

From the above literature survey, it is found that, only few spectrophotometric and LC-MS/MS methods are available for the determination of dothiepin and doxepin. In this chapter, two simple and reproducible spectrophotometric methods using Ce(IV)- leuco crystal violet(LCV) in [Section 2A], methyl red-bromate-bromide [Section-2B] in pure and in dosage forms are discussed.

The author has developed a simple LC-MS/MS method for the determination of dothiepin in human K$_2$EDTA plasma, and presented in Section 2C.
Section-2A

Development and validation of an indirect visible spectrophotometric method for doxepin and dothiepin in pure and the tablet dosage forms

2A.1 Introduction

Leuco crystal violet (LCV) is chemically,4,4',4''-methylidynetris(N, N-dimethyl aniline) is a crystalline white powder. It is commonly used by police agencies to develop dilute bloodstains (enhance shoe prints in blood). It is soluble in water in presence of phosphoric acid. It has the following molecular structure:

![Molecular Structure of LCV]

LCV has been used as a reagent for the spectrophotometric determination of selenium [15], arsenic [16] saccharin [17], antimony [18] and cerium [19]. In the present section the author has described his findings of spectrophotometric method for the determination of doxepin (DOX) and dothiepin (DOT) using LCV-Ce(IV) reagent system.

2A.2 Experimental

2A.2.1 Apparatus

All absorbance measurements were performed using a Systronics Model 166 digital spectrophotometer provided with 1-cm matched quartz cells. An Elico 120 digital pH meter was used for pH measurements.

2A.2.2 Reagents

Analytical reagent grade chemicals and reagents were used, and double distilled water was used throughout the experiment.
Standard solution of dothiepin and doxepin. The pure grade doxepin (DOX) and dothiepin (DOT), certified to be 99.99 % pure were received from Raja Laxmi Fine chemicals India Ltd Bangalore India, as a gift sample and were used as received. A stock standard solution equivalent to 100 μg/mL of DOX and DOT were prepared by dissolving 10 mg of the pure drug in a separate 100 mL calibrated flasks using distilled water.

Standard Ce(IV) solution (1000 μg/mL). Prepared by dissolving 0.3916 g ammonium ceric nitrate (BDH, Anal R) in 100 mL water containing 0.5 mL of conc. HNO₃. A working standard solution was prepared by a suitable dilution of a standard solution as and when required.

Leuco crystal violet (LCV, 0.025%). Prepared by dissolving 250 mg of LCV (Sigma-Aldrich, Steinhein, Germany) in 200 mL of water containing 3 mL of 85 % phosphoric acid to 1000 mL volumetric flask and shaken gently until the dye dissolved. The contents of the flask were then diluted to the mark with distilled water.

Sulfuric acid. 0.5 M was used.

Acetate buffer (pH-4.0). It was prepared by dissolving 13.6 g of sodium acetate tri hydrate in 80 mL of water. The solution pH was adjusted to 4.0 with acetic acid, and the mixture was diluted to 100 mL with water.

2A. 2.3 General procedures

Procedure for the preparation of calibration graph

Aliquots of standard DOX and DOT solutions (0.0, 0.5, 1.0, …..6.0 mL) were transferred separately into a series of 10 mL standard flasks. To this, 0.6 mL of Ce(IV) (50 μg/mL) and 0.5 mL each of 0.5 M H₂SO₄ and 0.025 % LCV were added. The reaction mixture was kept in a water bath (40 ºC) for 5 min, cooled to room temperature before being the contents were diluted to the mark with acetate buffer of pH-4.0 and mixed well. The absorbance of the formed dye was measured at 590 nm against distilled water. For each drug, a blank was prepared similarly omitting the drug and its absorbance was measured against distilled water. The decrease in absorbance corresponding to the consumed cerium(IV) and in turn, to the drug
concentration, was obtained by subtracting the absorbance of the blank solution from that of the test solution. The calibration graph was drawn by plotting the difference in absorbance (test and blank solution) of the formed dye against the concentration of the DOX and DOT. The amount of the DOX and DOT were determined from the unknown sample using the concurrent calibration curve or regression equation derived from the Beer’s law data.

2A. 2.4 Determination of DOX and DOT in pharmaceutical preparations

In order to determine the contents of DOX and DOT in commercial dosage forms, two brands of tablets containing SPECTRA Ranbaxy (Solus) 25 mg and DOTHIP (Micro Synapse) 25 mg used in the investigation were purchased from local commercial sources, and the contents of ten tablets were weighed accurately and ground into a fine powder. An amount of the powder equivalent to 25 mg each of the DOX and DOT was weighed separately and transferred into a 100 mL calibrated flasks and 50 mL water was added. The contents were shaken for about 30 min; the volume was diluted to the mark with water and mixed well. Then it was filtered using a Whatman no. 42 filter paper. The filtrate containing DOX and DOT separately was at a concentration of 100 μg/mL was subjected to analysis by the procedure described above (2A.2.3).

2A.3 Results and Discussion

The method involves the oxidation of the drugs (DOT and DOX) by the known excess of Ce(IV) in acidic medium. The unreacted Ce(IV) oxidize LCV to violet colored crystal violet (CV) in sulfuric acid medium (pH -1.0-2.3) on heating (at 40 ºC) in a water bath for 5 min. The violet color of the formed dye was developed in an acetate buffer medium (pH-3.7-4.3) showing a maximum absorption at 590 nm. The reaction pathway for the proposed method is shown in Scheme1. Based on the above observations, a simple spectrophotometric method to the determination of DOX and DOT was developed and validated as per the current ICH guidelines.
Scheme 1. Probable reaction pathway

Optimization of experimental parameters

The various experimental parameters affecting the formation of the reaction product were optimized.

Effect of time

The influence of the reaction time on the absorbance of the colored product was studied on 10 μg/mL of DOX and DOT separately with LCV as mentioned under standard procedure for the preparation of calibration graph. The optimum reaction time was found to be 5 min for this method. Then, the formed dye was stable for a period of one week.

Effects of acidity and temperature

The oxidation of LCV by Ce(IV) was studied. Of the various acid (Sulfuric, hydrochloric and phosphoric) studied, sulfuric acid was found to be the best acid for the system. Constant absorbance readings were obtained in the 0.1-1.5 mL range of 0.5 M sulfuric acid [(or) pH -1.0-2.3] at temperature 40 °C for 5 min for this method. An increase of the pH above 4.3 markedly affected the stability and sensitivity of the dye. Color development did not take place below pH -1.0. Hence, a volume of 0.5 mL of 0.5 M sulfuric acid in a total volume of 10 mL was used in all subsequent work and a temperature of 40 °C was maintained for 5 min.

Effects of reagent concentration and buffer media

The optimum concentration of LCV leading to maximum color stability was found to be 0.5 mL of LCV reagent per 10 mL of the reaction mixture. The
absorbance values were constant in the pH range of 3.7 - 4.3 of acetate buffer. Hence, the reaction mixture was diluted to the mark with acetate buffer of pH- 4.0. The formed colored dye was stable for more than a week.

2A.4 Method Validation

The proposed method has been validated for linearity, sensitivity, precision, accuracy, selectivity and recovery.

Linearity and sensitivity

The analytical parameters for the spectrophotometric determination of DOX and DOT by the proposed method are given in Table 2A.1. Under optimum reaction conditions, a linear relation was obtained between the absorbance and concentration of DOX and DOT in the range 0-60 μg/mL for both DOX and DOT [Fig.2A.1.(a), (b)]. The calibration graph is described by the equation: \( Y = a + bx \), where \( y = \) absorbance, \( a = \) intercept, \( b = \) slope and \( x = \) concentration, obtained by the method of least squares. The correlation coefficient, intercept and slope for the calibration data and sensitivity parameters such as apparent molar absorptivity and Sandell’s sensitivity values, the limits of detection and quantification calculated as per the current ICH guidelines [21] are summarized in the Table 2A.1. The limits of detection (LOD) and quantification (LOQ) were calculated according to the ICH guidelines using the formulas: \( \text{LOD} = 3.3\sigma/s \) and \( \text{LOQ} = 10\sigma/s \), where \( \sigma \) is the standard deviation of reagent blank determinations and \( s \) is the slope of concentration curve.

![Calibration curves for a) DOX and b) DOT](image-url)
**Table 2A.1.** Analytical and regression parameters of the proposed method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DOX</th>
<th>DOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}, \text{nm}$</td>
<td>590</td>
<td>590</td>
</tr>
<tr>
<td>Beer’s law range ($\mu\text{g/mL}$)</td>
<td>0 – 60</td>
<td>0 – 60</td>
</tr>
<tr>
<td>Molar absorptivity ($\epsilon$)</td>
<td>$3.05 \times 10^3$</td>
<td>$2.60 \times 10^3$</td>
</tr>
<tr>
<td>(L/mol/cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sandell’s sensitivity ($\mu\text{g/cm}^2$)</td>
<td>0.1036</td>
<td>0.1610</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>0.0109</td>
<td>0.0078</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>0.0091</td>
<td>0.0056</td>
</tr>
<tr>
<td>Regression coefficient (r)</td>
<td>0.9980</td>
<td>0.9958</td>
</tr>
<tr>
<td>$S_a$</td>
<td>0.01897</td>
<td>0.01726</td>
</tr>
<tr>
<td>$S_b$</td>
<td>0.0003</td>
<td>0.0003</td>
</tr>
<tr>
<td>LOQ ($\mu\text{g/mL}$)</td>
<td>1.4444</td>
<td>1.6990</td>
</tr>
<tr>
<td>LOD ($\mu\text{g/mL}$)</td>
<td>0.3468</td>
<td>0.5607</td>
</tr>
</tbody>
</table>

* $y = a + bx$, where $x$ is the concentration of DOX and DOT in $\mu\text{g/mL}$ and $y$ is the absorbance at the respective $\lambda_{\text{max}}$. $S_a$ is the standard deviation of the intercept, $S_b$ is the standard deviation of the slope.

**Accuracy and Precision**

The within day studies of the precision and accuracy was evaluated by performing replicate analysis of each drug samples at three different concentrations (low, medium and high) (Table 2A.2) within the working limits, each being repeated five times. The RE (%) and RSD (%) values were obtained for the developed method and was found to be satisfactory. The percentage recovery values ranged between 97.5 - 100.1 % with relative standard deviation of less than 3%. The analytical results obtained from this investigation are summarized in **Table 2A.2**.
Table 2A 2. Evaluation of accuracy and precision

<table>
<thead>
<tr>
<th>Method</th>
<th>DRUG taken</th>
<th>DRUG found*</th>
<th>RE</th>
<th>SD</th>
<th>SEM</th>
<th>RSD</th>
<th>ROE**</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX</td>
<td>10</td>
<td>9.736</td>
<td>2.64</td>
<td>0.103</td>
<td>0.039</td>
<td>1.053</td>
<td>±1.05</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>29.965</td>
<td>0.12</td>
<td>0.118</td>
<td>0.045</td>
<td>0.395</td>
<td>±0.395</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>49.766</td>
<td>0.47</td>
<td>0.429</td>
<td>0.162</td>
<td>0.861</td>
<td>±0.861</td>
</tr>
<tr>
<td>DOT</td>
<td>10</td>
<td>9.966</td>
<td>0.34</td>
<td>0.086</td>
<td>0.032</td>
<td>0.8623</td>
<td>±0.862</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>29.434</td>
<td>1.89</td>
<td>0.249</td>
<td>0.094</td>
<td>0.8462</td>
<td>±0.845</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>49.657</td>
<td>0.69</td>
<td>0.256</td>
<td>0.097</td>
<td>0.5148</td>
<td>±0.514</td>
</tr>
</tbody>
</table>

RE. Relative error; SD. Standard deviation; SEM. Standard error of mean; RSD. Relative standard deviation; ROE. Range of error

* Mean value of five determinations

**At the 95% confidence level for 6 degrees of freedom.

2A.5 Application to analysis of commercial samples

The validity of the proposed method was ascertained by the statistical comparison of the results obtained by a reference method with the proposed method by applying Student’s t-test for accuracy and F-test for precision in some commercial formulations. The results of an assay of DOX and DOT were statistically compared with the reference method [4,5] at the 95% confidence level and showed that there is no significant difference between the proposed and reference methods and the label claim (Table 2A.3).
**Table 2A.3.** Results of determination of DOX and DOT in tablets and statistical comparison with the reference method

<table>
<thead>
<tr>
<th>Tablet brand name</th>
<th>Nominal amount mg per tablet</th>
<th>Found*(% of nominal amount ± SD)</th>
<th>Reference method [4, 5]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Found*</td>
<td>DOX</td>
</tr>
<tr>
<td>SPECTRA Ranbaxy (Solus)</td>
<td>25</td>
<td>100.4±0.85</td>
<td>99.59±0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t=0.88, F=2.07</td>
</tr>
<tr>
<td>DOTHIP (Micro Synapse)</td>
<td>25</td>
<td>100.76±1.20</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t=0.64, F=1.16</td>
</tr>
</tbody>
</table>

*Mean value of five determinations.

Tabulated t and F-values at 95% confidence level are 2.77 and 6.39, respectively.

**Recovery study**

To test the applicability of the proposed method, recovery experiments were carried out by standard addition method. In this study, pre-analyzed tablet powder was spiked with pure drug at three different concentrations and the total was found by the proposed methods. Each determination was repeated three times. The recovery of the pure drug added was quantitative and revealed that co-formulated substances did not interfere in the determination. The results of recovery study are compiled in Table 2A.4.
Table 2A. 4 Results of recovery experiments via the standard addition technique

<table>
<thead>
<tr>
<th>Tablet brand name</th>
<th>Drug mg per tablet</th>
<th>Tablet solution added, μg/mL</th>
<th>Pure drug added, μg/mL</th>
<th>Total found, μg/mL</th>
<th>Pure drug recovered*, %±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPECTRA Ranbaxy (Solus) 25</td>
<td>10</td>
<td>10</td>
<td>19.95</td>
<td>99.51±0.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>29.85</td>
<td>99.26±0.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>30</td>
<td>40.01</td>
<td>100.02±0.34</td>
<td></td>
</tr>
<tr>
<td>DOTHIP (Micro Synapse) 25</td>
<td>10</td>
<td>10</td>
<td>20.04</td>
<td>100.35±0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>29.98</td>
<td>99.91±0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>30</td>
<td>39.89</td>
<td>99.64±0.17</td>
<td></td>
</tr>
</tbody>
</table>

* Mean value of three measurements
Section-2B

Sensitive visible spectrophotometric method for the determination of doxepin and dothiepin in pure and tablet dosage forms using methyl red

2B.1. Introduction

Methyl red

Methyl red is chemically known as (2-N,N-Dimethyl-4-aminophenyl) azobenzene-carboxylic acid, also called Acid red 2, is an indicator dye that turns red in acid solutions. It is an azo-dye, and is a dark red crystalline powder. Methyl red is a pH indicator; it is red in pH under 4.4, yellow in pH over 6.2, and orange in between, with a pKa of 5.1. It has the following molecular structure:

\[
\text{Methyl red} \quad \text{(2-N,N-Dimethyl-4-aminophenyl) azobenzene-carboxylic acid}
\]

Methyl red has been used as a reagent for the spectrophotometric determination of trace level of arsenic quantification through bromination of methyl red [20]. In the present section, the author has described his findings of spectrophotometric method for the determination of doxepin (DOX) and dothiepin (DOT) using methyl red-bromate bromide reagent system.

2B. 2. Experimental

2B. 2.1 Apparatus

All absorbance measurements were performed using a Systronics Model 166 digital spectrophotometer provided with 1-cm matched quartz cells. An Elico 120 digital pH meter was used for pH measurements.

2B. 2.2 Reagents

Analytical reagent grade chemicals and reagents were used, and double distilled water was used throughout the experiment to prepare all solutions.
Standard solution of dothiepin and doxepin

The preparation of pure drug solutions of DOT and DOX was described in Section 2A.2.2.

Bromate-Bromide Mixture

A stock standard solution of bromate-bromide mixture solution equivalent to 100 µg/mL KBrO₃ was prepared by dissolving accurately weighed 0.05 g of KBrO₃ (S.D. Fine Chem. Ltd., Mumbai, India) and 0.5 g of KBr (Merck, Mumbai, India) in water and diluted to the mark in a 500 mL calibrated flask. The stock solution was diluted appropriately with water and 4.25 M H₂SO₄ to get the working concentration 10 µg/mL of KBrO₃.

Methyl red (0.01 %)

It was prepared by dissolving 0.1 g of methyl red in 3 mL of 4.5 M NaOH and diluting to 100 mL with water to get 0.1% solution. Then, 10 mL of this solution diluted to 100 mL, after acidifying it by adding 3 mL of 4.25 M H₂SO₄. 4.25 M H₂SO₄ was prepared by adding 24 mL of concentrated H₂SO₄ to 50 mL of water, cooled and then diluting to 100 mL in a calibrated flask.

2B. 2.3 General procedure

Procedure for the preparation of calibration graph

Different aliquots (0.0, 0.5, and 1.0…..3.5 mL) of standard DOX and DOT solutions (10 µg/mL) were transferred separately into a series of 10 mL calibrated flasks. To each flask, 1.25 mL of bromide-bromate mixture was added, and the flasks were stoppered. Finally, 0.5 mL of 0.01% methyl red was added to each flask and mixed well before diluting to 10 mL with distilled water and the absorbance of the solution was measured at 520 nm against the corresponding reagent blank. The calibration graph was drawn by plotting the absorbance against the concentration of DOX and DOT and amount of DOX and DOT was computed from the calibration curves Figs. 2B.1 (a) and (b).
2B. 2.4 Determination of DOX and DOT in pharmaceutical preparations

Preparation of tablet solution of DOX and DOT was described in detail in Section 2A.2.4.

2B.3 Results and discussion

In the present investigation, a known excess of bromine is used to oxidize the studied drugs (DOX and DOT) in an acidic condition. The unreacted bromine bleaches the color of the azo-dye, methyl red, thereby a decrease in bromine concentration. This reaction formed the basis of DOX and DOT determination.

When methyl red is bleached (brominated) completely with bromine, the absorbance at 520 nm decreases and reaches minimum value. In the presence of drug concentration (DOX and DOT), bromine is reduced to bromide and the unreacted bromine decolorized the methyl red. Thus, with increasing concentration of drug, higher amount of bromine is reduced and this is observed by a linear increase in the absorbance due to the unbleached methyl red at 520 nm.

The difference in concentration of unbleached methyl red in the reaction mixture gives the exact concentration of the drug. The absorption spectra [Fig. 2B.2] show that, a linear increase in absorbance with increasing drug concentration. The possible reaction pathway is given in Scheme 1.
Absorption spectra

Absorption spectra of methy red, methyl red with bromine and methyl red with different concentration of DOX/DOT [0.5, 1.0 and 2.5 µg/mL] and bromine under acidic conditions were recorded against reagent blank in the region 450-600 nm [Fig. 2B.2]. Methyl red (MR), under acidic conditions has a maximum absorbance at 520 nm (curve 1). On treatment with bromine, the color of the methyl red bleaches due to bromination or cleavage of azo-bond. When methyl red is bleached completely with bromine, the absorbance at 520 nm decreases and reaches minimum value (curve 2). With an increase in drug concentration (DOX/DOT), there is a corresponding decrease in bromine concentration, and as a result less brominated/bleached methyl red is obtained (curves 3, 4 and 5). Here, DOT is used as a model compound, since DOX is also behaved similarly to it.

Fig. 2B.2. Absorption spectra for DOT
2B.3.1 Optimization of experimental variables and method development

Of all various acids (HCl, H$_2$SO$_4$, HNO$_3$) studied, sulphuric acid was found to be an ideal medium for the two steps involved in the developed method. The effect of acid concentration on the measured species was investigated and the results showed that 4.25 M H$_2$SO$_4$ was optimum for the bromination reaction of the drug as well as the dye.

Preliminary experiments were performed to fix the upper limits of the dye that could be determined spectrophotometrically, and it was found to be 0.5 mL of 0.01 % methyl red to produce maximum absorbance.

Similarly, the concentration of KBrO$_3$, in the presence of large excess of KBr to produce a convenient minimum absorbance with methyl red was found to be 1.25 mL of bromate-bromide mixture. Hence, different amount of drugs reacted with 1.25 mL of potassium bromate followed by reaction of unreacted bromine with methyl red gives increase in absorbance for both the drugs which is shown in calibration curves.

**Reaction time and colour stability**

In order to study the effect of reaction time between drugs under investigation (DOX and DOT) and the bromine generated *in situ*, the absorbance of the colored species was measured after different reaction times (5.0 - 120 min) and the results showed that the reaction was complete within 2–3 min. After completion of the reaction between the drug and the bromine, the residual bromine would brominate the dye and this bromination process was found to be complete in 10 min. The pink colour of the oxidized drug was stable for at least 90 min.

2B.4 Method validation

2B.4.1 Analytical data

A linear correlation was found between the absorbance at $\lambda_{\text{max}}$ and concentration of drug. The graphs showed negligible intercept and are described by the equation; $Y = a + bx$ (where $y =$ absorbance of 1 cm layer of solution; $a =$ intercept, $b =$ slope and $x =$ concentration in μg/mL), the regression of the Beers law data using the method of least square was made to evaluate the slope (b), intercept (a)
and correlation coefficient (r) for each system and the values are presented in Table 2B.1. The optical characteristics such as Beer’s law limits, molar absorptivity and Sandell’s sensitivity values of both drugs are also given in Table 2B.1. The limit of detection (LOD) and quantitation (LOQ) were calculated according to ICH guidelines [21].

**Table 2B.1**: Analytical and regression parameters of the proposed method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DOX</th>
<th>DOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}, \text{nm}$</td>
<td>520</td>
<td>520</td>
</tr>
<tr>
<td>Beer’s law range (μg/mL)</td>
<td>0.0-3.5</td>
<td>0.0-3.5</td>
</tr>
<tr>
<td>Molar absorptivity ($\varepsilon$, (L mol$^{-1}$ cm$^{-1}$))</td>
<td>1.94 $\times$ 10$^4$</td>
<td>5.89 $\times$ 10$^4$</td>
</tr>
<tr>
<td>Sandell sensitivity (μg cm$^{-2}$)</td>
<td>0.016</td>
<td>0.0056</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>-0.0015</td>
<td>-0.0048</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>0.0623</td>
<td>0.1842</td>
</tr>
<tr>
<td>Regression coefficient (r)</td>
<td>0.9985</td>
<td>0.9987</td>
</tr>
<tr>
<td>Standard deviation of a ($S_a$)</td>
<td>0.0063</td>
<td>0.0171</td>
</tr>
<tr>
<td>Standard deviation of b ($S_b$)</td>
<td>0.0019</td>
<td>0.0053</td>
</tr>
<tr>
<td>LOQ (μg/mL)</td>
<td>0.0326</td>
<td>0.0290</td>
</tr>
<tr>
<td>LOD (μg/mL)</td>
<td>0.0107</td>
<td>0.0095</td>
</tr>
</tbody>
</table>

* $y = bx + a$ where $x$ is the concentration of DOX and DOT in μg/mL and $y$ is the absorbance at the respective $\lambda_{\text{max}}$. $S_a$ is the standard deviation of the intercept, $S_b$ is the standard deviation of the slope.

**2B.4.2 Precision**

The precision of the method was calculated in terms of intermediate precision (intra-day and inter-day). Three different concentrations of drugs (DOX and DOT) were analyzed in replicate measurements for DOX and DOT in the same day (intra-day precision) and five consecutive days (inter-day precision). The RSD (%) values of intra-day and inter-day studies showed that the precision was good. The results are given in Table 2B.2.
2B.4.3 Accuracy

The accuracy of an analytical method was expressed as the closeness between the reference value and the found value, the accuracy was evaluated as percentage relative error between the measured concentrations and taken concentrations for DOX and DOT. The results obtained are compiled in Table 2.B.2 and show that the accuracy is good.

Table 2.B.2. Evaluation of Intra-day and inter-day accuracy and precision

<table>
<thead>
<tr>
<th>Drug studied</th>
<th>Drug taken, μg/mL</th>
<th>intra-day&lt;sup&gt;a&lt;/sup&gt;</th>
<th>inter-day&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>drug found&lt;sup&gt;c&lt;/sup&gt;, μg/mL</td>
<td>Precision&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Accuracy&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>DOX</td>
<td>1</td>
<td>1.00 ± 0.015</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.01 ± 0.008</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.01 ± 0.012</td>
<td>0.40</td>
</tr>
<tr>
<td>DOT</td>
<td>1</td>
<td>1.02 ± 0.008</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.99 ± 0.006</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.99 ± 0.01</td>
<td>0.34</td>
</tr>
</tbody>
</table>

a. Mean value of five determinations, b. Mean value of five determinations, c. Mean value of three determinations, d. Relative standard deviation (%), e. Bias %: (found-taken/taken)×100.

2B.5 Application to analysis of commercial samples

To check the validity of the proposed method, DOX and DOT were determined in some commercial formulations. The result obtained from the determination is in close agreement with the results obtained by the proposed method and the label claim. Statistical analysis of the results using Student’s t-test for accuracy and F-test for precision revealed no significant difference between the proposed method and literature methods [4, 5] at the 95 % confidence level with respect to accuracy and precision (Table 2B.3).
**Table 2B.3.** Results of determination of DOT and DOX in tablets and statistical comparison with the reference methods

<table>
<thead>
<tr>
<th>Tablet brand Name</th>
<th>Nominal amount mg per tablet</th>
<th>Found* (% of nominal amount ± SD)</th>
<th>Reference methods [4, 5]</th>
<th>DOX</th>
<th>DOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPECTRA Ranbaxy (Solus)</td>
<td>25 mg</td>
<td>99.8 ± 0.82</td>
<td>99.05 ± 0.5</td>
<td>t = 0.87, F = 2.22</td>
<td>-</td>
</tr>
<tr>
<td>DOTHIP (Micro Synapse)</td>
<td>25 mg</td>
<td>100.85 ± 1.14</td>
<td>-</td>
<td>99.80 ± 0.96</td>
<td>t = 1.54, F = 1.41</td>
</tr>
</tbody>
</table>

*Mean value of five determinations
Tabulated t and F-values at 95% confidence level are 2.77 and 6.39, respectively.

**2B.5 Recovery study**

The recovery and validity of the proposed method were further ascertained by performing recovery studies. Pre-analyzed tablet powder was spiked with pure drugs (DOX and DOT) at three different concentration levels and the total was found by the proposed method. Each determination was repeated three times. The recovery of pure drug added was quantitative and revealed that co-formulated substances did not interfere in the determination. The results of recovery study are compiled in **Table 2B.4.**
Table 2B.4: Results of recovery experiments via the standard addition technique

<table>
<thead>
<tr>
<th>Drug with brand name</th>
<th>Tablet solution µg/mL</th>
<th>Pure drug added, µg/mL</th>
<th>Total found µg/mL</th>
<th>Pure recovered* % ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPECTRA Ranbaxy (Solus), 25mg</td>
<td>1</td>
<td>0.5</td>
<td>1.49</td>
<td>98.49±0.51</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.0</td>
<td>1.98</td>
<td>98.96±0.78</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5</td>
<td>2.49</td>
<td>99.68±0.36</td>
</tr>
<tr>
<td>SPECTRA Ranbaxy (Solus), 25mg</td>
<td>1</td>
<td>0.5</td>
<td>1.48</td>
<td>97.88±0.97</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.0</td>
<td>1.99</td>
<td>99.21±0.73</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5</td>
<td>2.48</td>
<td>99.41±1.06</td>
</tr>
</tbody>
</table>

* Mean value of three measurements
Section-2C

LC-MS/MS method for the determination of dothiepin hydrochloride in human plasma

2C.1. Introduction

LC-MS/MS is a powerful technique used for many applications that has very high sensitivity and selectivity. Generally, its applications oriented towards the detection and potential identification of chemicals in the presence of other chemicals (in complex mixture) and in urine or plasma sample analysis. Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. MS works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios.

LC-MS/MS is very commonly used in pharmacokinetic studies of pharmaceuticals and thus it is most frequently used technique in the field of bioanalysis. These studies give information about how quickly a drug will be cleared from the hepatic blood flow and organs of the body. MS is used for this due to high sensitivity and exceptional specificity compared to UV, and it has short analysis time. The major advantage of MS has is the use of tandem MS/MS. The detector may be programmed to select certain ions to fragment. The process is essentially a selection technique, but is in fact more complex. The measured quantity is the sum of molecule fragments chosen by the operator. As long as there are no interferences or ion suppression, the LC separation can be quite quick. It is common now to have analysis time of 1 minute or less by MS/MS detection, compared to over 10 minutes with UV detection or HPLC analysis.

2C.2. Experimental

2C.2.1. Materials

The pharmaceutical grade antidepressant drugs doxepin and dothiepin were received from Rajalakshmi Fine Chem. Yelahanka, Bangalore and purity was 99.9%. Methanol and tertiary butyl methyl ether (TBME) were purchased from Merck specialities Private Limited, Mumbai. HPLC grade methanol was used throughout the experiment. All other reagents were analytical grade. A milli-Q water (Millipore,
Mosheium, France) water purification system was used to obtain water for LC-MS/MS analysis. Ammonium acetate (Fluka, Switzerland) and sodium hydroxide (Qualigens Fine chemicals, Mumbai) were used. Doxepin was used as an internal standard.

2C.2.2 Reagents

2 mM ammonium acetate: It was prepared by dissolving 0.1958 g of ammonium acetate in water and diluting to 1000 mL in a calibrated flask with water and sonicate it.

Mobile phase [2 mM ammonium acetate and methanol (20:80, v/v)]: Mobile phase was prepared by mixing a 200 mL of 2 mM ammonium acetate solution and 800 mL of methanol in a 1000 mL reagent bottle, shaken well and filter through 0.45 µm membrane filter, and then it was degassed by sonication before use.

0.1 M NaOH: It was freshly prepared by dissolving 0.4 g of sodium hydroxide pellets in 100 mL of distilled water.

2C.2.3. Apparatus

Mass spectrometric method detection was performed on an API 4000 triple Quadrupole instrument, using the multiple reaction-monitoring (MRM) modes. A Shimadzu LC =10AD pump was used for LC-MS/MS analysis. Turbo electro spray ionization (ESI) interface in positive mode was used. The data Acquisition was performed with Analyst 1.4.2 software.

2C.2.4. Preparation of standards and quality control samples

Stock solution of dothiepine was prepared by dissolving the accurately weighed reference standard of dothiepine in methanol to yield concentration of 1000 µg/mL. Working standard solutions of dothiepine in the concentration range of 0.025–2.52 µg/mL were prepared by dilution of the stock solution with methanol–water (50:50, v/v). The stock solution of I.S. was prepared by dissolving appropriate amounts of doxepin in methanol to give a concentration of 1000 µg/mL. An I.S. working solution of 100 ng/mL was obtained by diluting the stock solution of doxepin with methanol: water– (50:50 v/v). All the solutions were stored at 2- 8 °C and brought to room temperature before use.
Calibration standards were prepared daily by spiking 40 µL of the appropriate working standard solutions to 2.0 mL of the blank plasma giving concentrations of 0.50, 1.01, 2.42, 4.84, 12.11, 24.22, 40.37 and 50.46 ng/mL. QC samples, which were used in the validation and during the pharmacokinetic study, were prepared at the beginning of the experiment by independent dilution at three levels of plasma concentration: 1.39, 16.13, 40.32 ng/mL representing low quality control sample (LQC), medium quality control plasma (MQC), and high quality control plasma (HQC) concentration levels, respectively, and stored at −20°C after preparation. The standards and quality controls were extracted on each analysis day along with the unknown sample.

2C.2.5. Operating conditions

Chromatographic separation was achieved on a Discovery C18 column, (5 cm x 4.6 mm, 5µM, Supelco). The liquid flow rate was 1.0 mL/min. The column oven temperature was maintained at 45 °C. The HPLC system was connected to the mass spectrometer via an ESI interface. The mass spectrometer was operated in the positive ion detection mode with the spray voltage set at 4kV. CAD and curtain gas were used i.e. nitrogen and zero air gas, respectively. The collision–induced dissociation (CID), argon was used as collision gas at the pressure of approximate 1.2 torr. Quantification was performed using selected multiple reaction monitoring (MRM) mode of the transitions of m/z→Q1-296.2 >223.2amu for dothiepine and m/z→Q1-280.2 >107.3 amu for doxepin (IS) with a scan time of 5.0070 m sec. The optimized collision energy of 33 and 30 eV was used for analyte and IS, respectively.

2C.2.6. Plasma sample preparation

Frozen plasma samples were thawed to room temperature prior to extraction. A 50 µL of IS working solution concentration (100 ng/mL) and 100 µL of 0.1M sodium hydroxide were added to 500 µL of plasma sample in 5.0 mL glass tubes, and the mixture was vortexed. The sample was extracted with 2.5 mL of TBME by vibramax mixing for 10 min and centrifuged at 4500 rpm for 5 min. The upper organic layer was then transferred in to another cleaned glass tubes and evaporated to dryness under a stream of nitrogen at 40 °C, the residue was reconstituted in 0.5 mL of mobile phase followed by vortexing. The supernatant was transferred in to auto injector vials, and an aliquots of 10 µL was injected on to the LC-MS/MS system.
2C.2.7. Method validation procedure

Plasma samples were quantified using the ratio of the peak area of dothiepine to that of internal standard (IS). As the assay parameter, peak area ratios were plotted against dothiepine concentrations and the standard curve is in the form of $y = mx+c$. To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on three separate days. The calibration curves were fitted by least square regression using $1/x^2$ as weighting factor of the peak area ratio of dothiepine to IS versus dothiepine plasma concentrations.

Accuracy and precision were assessed by determining QC samples using six replicate preparations of plasma samples at four concentration levels on three separate days using three separate sources of human plasma. Precision was calculated as the relative standard deviation (R.S.D) within a single run and between different assays and the accuracy as the percentage of deviation between nominal measured concentrations.

The lower limit of quantification of dothiepine was experimentally defined as the lowest concentration in plasma samples at which within and between run precision were <20% and accuracy varying between 80 and 120%.

Three sets of six quality control (QC) samples, at low (LQC), medium (MQC) and high (HQC) concentrations of 1.39, 16.13, 40.32 ng/mL, respectively, were processed according to the method of plasma sample preparation previously described in Section 2C.2.6, but without addition of I.S. The extraction yield was calculated by dividing the mean peak area of the extracted samples by the corresponding mean peak area unextracted samples (100% aqueous). The recovery of the analyte need not be 100%, but the extent of recovery of an analyte should be consistent, precise and reproducible.

Recovery of IS was also evaluated by comparing the mean peak areas of six standards solutions to mean peak areas of six standard solution spiked in extracted plasma samples of the same concentration.

The stability procedures to evaluate the stability of the dothiepine in the human plasma under distinct timing and temperature conditions are shown below:
**Bench top stability**: Three aliquots of plasma spiked with dothiepin at 1.39 and 40.32 ng/mL (low and high quality control samples) were left at room temperature for 8 h, and then analyzed. It was investigated to ensure that dothiepin was not degraded in plasma samples at room temperature for a time period to cover the sample preparation.

**In injector or auto sampler rack or processed sample stability**: Due to the need of occasional delayed injection of extracted samples, this was carried out at two different concentrations (low and high) were extracted, loaded on to the auto sampler and kept up to 36 h before injection. There is no appreciable change occurs, ensure that the drug was not degraded in plasma samples.

**Freeze thaw stability**: This stability was carried out at two concentrations (low and high). And it was assessed by exposing the samples i.e. low and high quality control samples to three freeze thaw cycles, each cycle consists of removing the QC samples from the freezer, thawing them unassisted to room temperature, kept at room temperature up to thawing i.e. 1.0 h and re-freezing at – 20 °C.

2C.3. Results and discussion

The objective of this study was developing a sensitive and selective LC-MS/MS method for the quantitation of the dothiepin in human plasma. A method was needed a simple preparation step including liquid-liquid extraction and lesser-run time, so that the resulting dothiepin and IS could be analyzed with conventional reverse phase chromatographic conditions.

2C.3.1. Method optimization

In bioanalytical methods, three most widely employed biological sample preparation methodologies namely liquid-liquid extraction (LLE), protein precipitation (PPT), and solid phase extraction (SPE). In these three methods, LLE is cheaper than SPE and PPT. In SPE extraction, cartridges like Oasis HLB 1cc (30 mg), Phenomenex Strata-X, Alumina, etc were used. They are highly expensive. In PPT method, ion suppression from the endogeneous substances in plasma observed when treated with precipitating agent such as methanol, acetonitrile and trichloroacetic acid. This method requires long run time (>10 min). In the analysis of large batches, small
response was observed. The column pressure was also increases due to the supernatant liquid, which was very difficult to separate and evaporate. If the supernatant was injected directly on to the LC-MS/MS system for analysis, the peak shape was not good. More matrix effects were also observed.

In the present study, several extraction buffers include both acidic and basic buffers like 1% orthophosphoric acid, 0.1 M HCl, 1% formic acid and basic buffers like 0.1M Na₂CO₃, K₃PO₄, borax etc, and extraction solvents such as ethyl acetate (100%), diethyl ether: ethyl acetate (80:20, v/v). ethyl acetate:n-hexane: 70:30, v/v. and tertiary butyl methyl ether (TBME) were studied.

The combination of 0.1 M NaOH and TBME was used for the extraction of drug in plasma. Recovery of the sample and also good peaks were obtained with this combination.

2C.3.2. LC-MS/MS optimization

The LC-MS/MS method for the determination of dothiepin in human plasma was established. The signal intensity obtained in the positive mode was much higher than that in the negative ESI mode. The ESI was chosen since the sensitivity and linearity for the analyte were better. By positive ESI mode, the analyte and internal standard were formed predominantly as protonated molecules [M+H]+ in full scan mass spectra. Fig. 2.C.2 (a) - (d) displays product ion spectra of [M+H]+ ions from the analyte and IS. Dothiepine gave an intense product ion at m/z 296.2 and 223.1; doxepin gave an intense ion m/z 280.2 and 107.3. Therefore, the ions at m/z 296.2>223.1 and 280.2>107.3 were used for quantification of both dothiepin and IS in the MRM acquisition.
Fig. 2C.2 (a) parent ion mass spectra of dothiepin (Q1)
Fig. 2C.2 (b) daughter ion spectra of dothiepin (Q3)
Fig. 2C.2 (c) parent ion mass spectra of doxepin (Q1)
Fig.2C.2 (d) daughter ion spectra of doxepin (Q3)
2C.3.3. Chromatography

The ionization of the analyte especially at low concentrations was easily suppressed, which resulted in the linearity of narrow concentration range. In order to avoid the ion suppression induced by endogenous substances, mobile phase compositions and choice of buffer were evaluated during the experiment. In the early stage of development 10 mM ammonium acetate and ammonium formate, 5 mM ammonium acetate and ammonium formate were tried in the combination with acetonitrile. Mass spectrometric response was less compared to 2 mM ammonium acetate: methanol. Percentage composition of organic phase was increased, the retention time of analyte and IS become shortened, significant matrix effect (ion suppression) was observed. When little formic acid was added, to increase the efficacy of eluent, it causes high base line.

The mobile phase acetonitrile and 5mM ammonium acetate 70:30 v/v was studied. The retention time of analyte and IS were 0.4 and 1.0 minutes, respectively, but here one enhancement peak was observed at same retention time of the analyte. Hence, this composition of the mobile phase was rejected. Columns like BDS Hypersil X-terra, hypersil gold, cyano column, chromolith were investigated. But, were no good resolution and peak shapes occurred. Thus, the combination of ammonium acetate and methanol in the ratio (20:80) was used as a mobile phase in this method.

2. C.4. Method validation

2. C.4.1. Selectivity and matrix effect

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. The [Fig. 2C.3 (a-d)] shows the typical chromatograms of a blank plasma sample, blank plasma sample spiked only with IS, blank plasma sample spiked with dothiepine at the LLOQ and IS, and typical chromatogram of dothipin in higher concentration, respectively. No significant interference from endogenous substance with analyte or IS was detected.
Fig. 2C.3 (a) Blank plasma chromatogram
Fig. 2C.3 (b) blank plasma sample spiked only with IS
Fig. 2C.3(c). Blank plasma sample spiked with dothiepin at the LLOQ and IS
Fig. 2C.3(d) Typical chromatogram of dothiepin
The peak area ratios (analyte to IS) in extracted blank matrix spiked with solution of 1.39 ng/mL was in the range of 98-101% compared with those of in the same nominal solution prepared with the mobile phase, and the CV value from six different batches of plasma were less than 5%. These results indicate that ion suppression or enhancement from the plasma matrix was negligible under the experimental conditions.

2. C.4.2. Linearity and lower limit of quantification

The linear regression of the peak area ratios versus concentration was fitted over the concentration range of 0.50 to 50.46 ng/mL in human plasma. A typical equation of the calibration curves was as follows: 
\[ y = 0.0779x - 0.00751 \quad (r= 0.999) \]
where y is the peak area ratio of analyte to IS and x is the plasma concentration of dothiepin. Good linearity was observed in this concentration range and the same is presented in Fig. 2C.4.
Fig 2C.4: Calibration curve of DOT in (ng/mL)
The lower limit of quantification was 0.51 ng/mL for determination of dothiepin in plasma. At the LLOQ level, within and between–day precisions were 0.83 and 1.0 %, respectively. The accuracy was 98.41% to 99.00%.

2. C.4.3. Precision and accuracy

Table 2.C.1 summarizes the within and between–day precision and accuracy for dothiepine evaluated by assaying the QC samples. In this assay, the intra- and inter-day precision ranged from 0.76% to 1.13% and 0.83 to 7.27%, respectively. The accuracy was within 87.85 to 99 and 93.58 to 98.41%, respectively. The values were within the acceptable range and the method was accurate and precise.

Table 2.C.1 Precision and accuracy determination of dothiepin in human plasma

<table>
<thead>
<tr>
<th>Nominal conc. (ng/mL)</th>
<th>Within run (n=6), Measured conc. (ng/mL), Mean ± SD (RSD)</th>
<th>% Recovery*</th>
<th>Between run (n=24), Measured conc. (ng/mL), Mean ± SD (RSD)</th>
<th>% Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.51</td>
<td>0.50±0.005 (1.0%)</td>
<td>99.00</td>
<td>0.49±0.004 (0.83%)</td>
<td>98.41</td>
</tr>
<tr>
<td>1.39</td>
<td>1.30±0.01 (0.76%)</td>
<td>94.23</td>
<td>1.29±0.09 (6.93%)</td>
<td>93.58</td>
</tr>
<tr>
<td>16.13</td>
<td>14.50±0.12 (0.83%)</td>
<td>89.95</td>
<td>15.13±1.1 (7.27%)</td>
<td>93.84</td>
</tr>
<tr>
<td>40.32</td>
<td>35.42±0.40 (1.13%)</td>
<td>87.85</td>
<td>38.39±2.10 (5.47%)</td>
<td>95.22</td>
</tr>
</tbody>
</table>

*Average of six determinations.

2. C.4.4. Recovery and stability

Mean extraction recoveries of dothiepin at 1.39 (low-QC), 16.13 (medium-QC), and 40.32 (high-QC) ng/mL were 58.54, 60.45 and 60.00%, respectively. Mean recovery of the internal standard (100 ng/mL) was 67.8% (n=6).

Table 2C.2 lists the data for bench top, auto sampler and freeze thaw stability. The result indicates that the analyte was stable under any of the storage conditions described below and no stability related problems would be expected during the samples routine analysis.
Table 2.C.2. Stability of dothiepin under various conditions investigated in the experiment

<table>
<thead>
<tr>
<th></th>
<th>Nominal conc. (ng/mL)</th>
<th>Found conc.*, (ng/mL)</th>
<th>% CV</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bench top stability (n=6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.39</td>
<td>1.30</td>
<td>1.72</td>
<td>93.73</td>
<td></td>
</tr>
<tr>
<td>40.32</td>
<td>35.48</td>
<td>0.97</td>
<td>88.00</td>
<td></td>
</tr>
<tr>
<td><strong>Auto sampler stability (n=6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.39</td>
<td>1.29</td>
<td>0.13</td>
<td>93.22</td>
<td></td>
</tr>
<tr>
<td>40.32</td>
<td>35.30</td>
<td>0.97</td>
<td>87.55</td>
<td></td>
</tr>
<tr>
<td><strong>Freeze thaw stability (n=6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.39</td>
<td>1.28</td>
<td>1.30</td>
<td>92.43</td>
<td></td>
</tr>
<tr>
<td>40.32</td>
<td>35.55</td>
<td>5.62</td>
<td>88.17</td>
<td></td>
</tr>
</tbody>
</table>

*Average of six determinations.

2. C.5. Application in bioequivalence study

This method is to be highly selective and suitable for bioavailability and bioequivalence studies of different formulations containing dothiepin.

Conclusions

The developed spectrophotometric methods with cerium(IV)–LCV, and methyl red-bromate-bromide systems are cost effective and non toxic reagents for the DOT and DOX determination in their pure and in dosage forms. These methods are simple, rapid and not taking more than 10-15 minutes for the assay. The spectrophotometric methods are more selective than the existing UV, HPLC and other methods and are free from extraction steps. The methods were made use of simple and cheap chemicals and are based on well-characterized color reactions. The technique employed is inexpensive but was demonstrated to provide the sensitivity comparable to the expensive techniques like HPLC. This method can serves as an alternative for
the existing methods for the routine analysis of the studied analytes in pure and in pharmaceuticals.

Compared to other known LC-MS/MS and HPLC methods, this new method offers accurate, reliable, cost effective and reproducible results for dothiepin. All reported methods having run time more than 4.0 min. But, in the proposed procedure having less analysis time 2.1 min and K₂EDTA human plasma was used and the validated range was 0.5 to 50 ng/mL for dothiepin.

The proposed methods are compared with the existing methods and all these are presented in Table 2C.5.
Table 2C.5 Comparison of proposed methods with some of the reported analytical methods

<table>
<thead>
<tr>
<th>SL.No.</th>
<th>Reagents</th>
<th>Beer’s law limit (µg/mL)</th>
<th>Remarks</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Malonic acid</td>
<td>5-25</td>
<td>60 °C heating, and acetic anhydride used</td>
<td>[1]</td>
</tr>
<tr>
<td>2</td>
<td>Alizarin red S</td>
<td>-</td>
<td>Extractive method</td>
<td>[2]</td>
</tr>
<tr>
<td>3</td>
<td>Methyl orange</td>
<td>0.1-12</td>
<td>Extractive methods</td>
<td>[3]</td>
</tr>
<tr>
<td></td>
<td>orange G</td>
<td>0.5-11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cobalthiocyanate</td>
<td>3.2-80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Alkaline KMnO₄,</td>
<td>4 – 24</td>
<td>Kinetic spectrophotometry</td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td>4-chloro-7-nitrobenzofurazan</td>
<td>50-250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3-methylbenzothiazolin-2-one hydrozone</td>
<td>-</td>
<td>Costly reagent and less sensitive</td>
<td>[5]</td>
</tr>
<tr>
<td>6</td>
<td>Copper(II)</td>
<td>3.16 - 7.90 (mg/mL)</td>
<td>Less sensitive</td>
<td>[6]</td>
</tr>
<tr>
<td>7</td>
<td>Cobalt thio cyanate</td>
<td>0.5-30</td>
<td>Extraction method</td>
<td>[7]</td>
</tr>
<tr>
<td>8</td>
<td>n-hexane:2-propanol</td>
<td>-</td>
<td>LC-ESI. 4 min run time</td>
<td>[9]</td>
</tr>
<tr>
<td>9</td>
<td>Protein precipitation</td>
<td>-</td>
<td>UPLC method run time 5.0 min</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Diethyl ether (100%)</td>
<td>50-1000(ng/mL)</td>
<td>HPLC difficult to separate with Diethyl ether</td>
<td>[12]</td>
</tr>
<tr>
<td>11</td>
<td>Solid phase extraction (SPE)</td>
<td>-</td>
<td>LC-MS method, Extraction cartridge cost more.</td>
<td>[13]</td>
</tr>
<tr>
<td>12</td>
<td>n-Hexane: isoamyl alcohol</td>
<td>-</td>
<td>LC-MS/MS method</td>
<td>[14]</td>
</tr>
<tr>
<td>13</td>
<td>Hexane:methanol:nonylamine mobile phase</td>
<td>1-200 ng/mL(plasma), 1-400ng/mL(urine)</td>
<td>HPLC method</td>
<td>[16]</td>
</tr>
<tr>
<td>14</td>
<td>(a) LCV-Ce(IV)</td>
<td>(a) 0-60</td>
<td>Highly sensitive</td>
<td>Present methods</td>
</tr>
<tr>
<td></td>
<td>(b) Methyl red-bromate-bromide mixture</td>
<td>(b) 0-3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(c) LCMS/MS (dothiepin)</td>
<td>(c) 0.5-50 ng/mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
REFERENCES


[20]. Kempahanumakkagaari Suresh kumar Malingappa pandurangappa “*American journal of analytical chemistry*, 2012, **3**, 455