CHAPTER III

SECTION I

NEW SPECTROPHOTOMETRIC METHODS FOR THE ASSAY OF PHYSIOLOGICALLY ACTIVE CATECHOLAMINES IN PHARMACEUTICAL PREPARATIONS
Catecholamines are vicinal dihydroxybenzene derivatives in which either the position 3- or 4- is unsubstituted and these positions are not sterically blocked. Dopamine (DPH) is a naturally occurring organic amine and its hydrochloride salt is being used in the treatment of shock and in acute congestive failure. It also increases the flow of blood to the kidney, which ultimately results in increased urinary output. Methyldopa (MD) is employed as an antihypertensive drug in the treatment of moderate to severe hypertension while levodopa (LD) is used in Parkinson’s disease.

In the present work, a few of catecholamines viz., DPH, MD and LD were selected for the study.

In view of their wide applications, convenient and reliable analytical methods are required for their assay. Many analytical techniques viz., HPLC\textsuperscript{1,2}, voltammetric\textsuperscript{3} and liquid chromatographic\textsuperscript{4} have been reported for their assay. But these techniques are of high cost and are not available at most of the pharmaceutical laboratories. However, photometric methods are particularly attractive because of their speed and simplicity. Consequently, a large number of spectrophotometric methods have been developed for the determination of these drugs.

Helaleh et al\textsuperscript{5} have reported a spectrophotometric method for the determination of LD and MD in pure and in pharmaceutical preparations based on the reaction with cerium (IV) in H\textsubscript{2}SO\textsubscript{4} medium. The method involves heating of the reaction mixture at 80°C for 20–25 min for colour development. This method exhibited maximum absorbances at 510 nm and at 550 nm.
obeying Beer's law in the concentration range of 200–550 ppm and 125–250 ppm with the molar absorptivity values of $1.08 \times 10^3$ and $1.15 \times 10^3$ $\text{mol}^{-1} \text{cm}^{-1}$ for LD and MD respectively.

Murad and Eyad\textsuperscript{6} have described spectrophotometric determination of MD and LD based on the formation of nitroso derivatives with 3.0 % NaN\textsubscript{2}O\textsubscript{2} and 1.0 M HCl. The nitroso derivatives exhibited absorption maxima at 470 nm and at 500 nm obeying Beer's law in the range of 4-36 ppm and 4-28 ppm for LD and MD respectively.

Salem\textsuperscript{7} has discussed the oxidation of catecholamines by periodic acid in acetate buffer of pH 4.7. The wavelength of maximum absorption was found to be 485 nm. The method involves Beer's law in the range of 2.4–28 ppm.

Recently we have reported\textsuperscript{8} a spectrophotometric method for the assay of certain vicinal dihydroxybenzene derivatives in pharmaceutical formulations based on oxidative coupling. The system obeyed Beer's law over the ranges of 1-23 ppm, 1.5-40 ppm and 2-11 ppm with molar absorptivity values of $1.8 \times 10^3$, $1.5 \times 10^3$ and $1.8 \times 10^3$ $\text{mol}^{-1} \text{cm}^{-1}$ for LD, MD and DPH respectively.

Sastry et al\textsuperscript{9} have established a method for the spectrophotometric determination of DPH and pyrocatechol (PC) using p-amino acetophenone in presence of sodium metaperiodate. The systems obeyed Beer's law in the range of 2.0–24 ppm and 0.8–12 ppm at the maximum absorption of 440 nm and 510 nm for DPH and PC respectively. Their data on Sandell's sensitivities
and molar absorptivities are: 0.066 and 0.020 µg cm\(^{-2}\) and 2.85 \(\times\) 10\(^3\) and 5.5 \(\times\) 10\(^3\) \(\text{mol}^{-1}\) cm\(^{-1}\) respectively.

Sane et al\(^{10}\) have discussed spectrophotometric methods for the determination of DPH based on its reaction with the Folin and Ciocalteu’s phenol reagent, p-phenylenediamine dihydrochloride, resorcinol, 4-aminophenazone and with potassium periodate. These methods exhibited maximum absorbances in the range of 405 nm to 600 nm obeying Beer’s law in the concentration range of 2.0 to 40 ppm with molar absorptivity values of 3.54 \(\times\) 10\(^3\) to 2.54 \(\times\) 10\(^4\) \(\text{mol}^{-1}\) cm\(^{-1}\). In these methods, they have indicated that the colour development and measurement of absorbances should be over within half an hour.

El-Kommos et al\(^{11}\) have reported the oxidation of catecholamines by sodium metaperiodate in aqueous alcoholic medium forming o-benzoquinone. The wavelength of maximum absorption ranges from 465 to 520 nm. It has the molar absorptivity value ranging from 3.9 \(\times\) 10\(^3\) - 6.25 \(\times\) 10\(^3\) \(\text{mol}^{-1}\) cm\(^{-1}\) and the Beer’s law ranging from 5.0 to 50 ppm. This method required heating at 60 ± 5°C with constant stirring for 3.0- 7.0 min.

Walash et al\(^{12}\) have used p-dimethylamino cinnamaldehyde (PDAC) and p-dimethylamino benzaldehyde (PDAB) as analytical reagents for the determination of MD and noradrenaline (ND) in pharmaceutical formulations. The method was based on the measurement of orange or purple coloured species developed when the alkaline solution of MD or ND was allowed to
react with PDAC or PDAB. The coloured products obeyed Beer's law in the concentration range 0.1-1.5 ml of $2 \times 10^{-3}$ M (or 47-714 ppm) of MD and ND.

The literature surveys of the catecholamines are presented in the Table 3.1. Most of the methods reported for the assay required prolonged treatment or heating. Moreover these methods have less sensitivity.

In the present investigation, the author has developed two simple, rapid and adequately sensitive spectrophotometric methods for the determination of dopamine hydrochloride, levodopa and methyldopa.

**EXPERIMENTAL**

**Equipments:**

As described in Chapter II.

**Preparation of reagents and chemicals:**

All chemicals used were of analytical reagent or pharmaceutical grade and quartz processed high-purity water was used throughout.

*Method A:*

A 10% solution of each of sodium nitrite and sulphamic acid and 20% NaOH were prepared in distilled water. A 1.0 M HCl was used for the study.

*Method B:*

A 0.5% aqueous solution of each of isoniazid, INH (M/s Cadila Pharmaceuticals Ltd., India) and p-dimethylaminobenzaldehyde, PDAB was prepared in distilled water. A 0.5 M NaOH was used for the study.
Preparation of standard drug solutions:

The catecholamines DPH (TTK Pharma Ltd., India), LD (Sun Pharmaceuticals Ltd., India) and MD (Indian Drugs and Pharmaceuticals Ltd., India) were prepared in distilled water and stored in amber coloured bottles in a refrigerator. These solutions were standardised by the reported method27.

Different dosage forms of catecholamines were obtained commercially from different firms.

Recommended procedures

After a systematic and detailed study of the various parameters involved as described in this chapter, the following procedures [Methods: A: Sodium nitrite and sulphamic acid and B: PDAB and INH] were recommended for the assay of catecholamines in pure and pharmaceutical formulations.

Analysis of pure drugs: $\text{Method A:}$

 Aliquots of the standard drug solution containing 15–240 µg of LD or 20–250 µg of MD were taken in a series of 10 ml calibrated flasks. A 1.0 ml of 1.0 M HCl and 2.0 ml (for MD) or 1.0 ml (for LD) of 10 % sodium nitrite were added, mixed well and kept for 5 min. After this, a 1.0 ml of 10 % sulphamic acid was added to each of the flask, mixed well and kept for another 2 min. The whole reaction was carried out in an ice cold water bath. Then 1.0 ml (for MD) or 1.5 ml (for LD) of 20 % NaOH solution was added at room temperature and the volume of the resulting red coloured solution was made upto the mark with distilled water. The absorbance of the solution was
measured after 20 min against the corresponding reagent blank at 502 nm for MD or at 497 nm for LD. Calibration curves were also constructed (Fig. 3.1).

Method B:

Various amounts of aliquots of the drug solutions (25–200 μg of MD, 10–150 μg of LD and 20–175 μg of DPH) were transferred into a series of 10 ml volumetric flasks. To these were added 3.0 ml (for DPH and LD) or 2.0 ml (for MD) of 0.5 % PDAB, 1 ml (for DPH and LD) or 2 ml (for MD) of NaOH (0.5 M) and 1.0 ml (for DPH and LD) or 2.0 ml (for MD) of INH (0.5 %). The contents of the flasks were diluted to the mark with distilled water. After mixing the contents thoroughly, the absorbances were measured after 5-7 min at 478 nm for DPH, 494 nm for MD and at 488 nm for LD. Calibration graphs were plotted (Fig. 3.2).

The calibration graphs were subsequently used for computing the amounts of catecholamines in pharmaceutical formulations.

Analysis of pharmaceutical formulations:

Tablets:

Twenty five tablets of LD or MD were weighed and finely powdered. An amount equivalent to 25 mg of the drug was weighed accurately and transferred into a 100 ml beaker. Using a mechanical stirrer, the powder was completely disintegrated and the solution was filtered. The filtrate was made upto 100 ml with distilled water and an aliquot of the drug solution was analysed using the Methods A and B as described earlier for pure drugs.
Injections:

After DPH injection solutions were appropriately diluted with distilled water to obtain the required concentrations of the drug, an aliquot of the solution within Beer's law limit was analysed by Methods A and B.

RESULTS AND DISCUSSION

Method A:

Murad and Eyad have reported a spectrophotometric method for the assay of LD and MD based on the formation of nitroso derivatives. These methods were found to be less sensitive compared to the proposed method. In the present method, the investigator observed that the coupling of nitroso derivative with sulphanic acid enhanced the sensitivity. The LD and MD when reacted with 1.0 M HCl and NaN02 at 0–5°C formed corresponding nitroso derivatives, which on treatment with sulphanic acid in presence of sodium hydroxide formed respective red coloured products (azo compounds). The probable reaction mechanism for the formation of azo compounds of LD and MD may be represented as shown in Scheme 3.1.

Method B:

Catecholamines undergo oxidation to form the corresponding aminochromes in NaOH medium which further undergo condensation with PDAB. These products on further treatment with INH formed red coloured coupling products having absorption maximum at 478 nm for DPH,
at 488 nm for LD and at 494 nm for MD. The tentative reaction mechanism for MD, a representative member may be shown as presented in Scheme 3.2.

Spectral characteristics:

The absorption spectra of azo compounds (developed by following the recommended procedures) were scanned on a spectrophotometer in the wavelength region of 360 - 800 nm (figures 3.3 and 3.4). The absorption maxima of these compounds are given in Table. 3.2.

Standardisation of experimental parameters:

The standardization of experimental parameters incorporated in the procedures of two methods was ascertained by measuring the absorbances at appropriate $\lambda_{\text{max}}$ of a series of solutions by varying one and fixing the other parameters.

Method A:

The optimum conditions in this method were fixed based on the study of the effects of various parameters viz. strength and volume of acid, sodium nitrite sulphamic acid and NaOH on colour formation and stability.

The maximum colour intensity of the respective azo compound was obtained with a volume of 1.0 ml of 1.0 M HCl, 2.0 ml (for MD) or 1.0 ml (for LD) of 10 % sodium nitrite, 1.0 ml of 10 % sulphamic acid and 1.0 ml (for MD) or 1.5 ml (for LD) of 20 % NaOH (Fig. 3.5-3.8).
Method B:

**Effect of PDAB:**

Figure 3.9 indicates that a volume of 2.0 ml (for DPH and LD) and 1.5 ml (for MD) of 0.5 % PDAB was necessary for full colour development. Below these volumes less intense coloured species were observed. It was also noticed that the excess of PDAB neither affected the stability nor the absorbance of the species. Hence a volume of 3.0 ml for DPH and LD, and 2.0 ml for MD were used for subsequent studies.

**Effect of INH:**

The effect of INH concentration was studied by recording the absorbance of the coloured species at a fixed amount of catecholamines and varying the volume of INH. It was found that a 0.75 ml (for DPH and LD) and 1.5 ml (for MD) of 0.5 % INH gave maximum stable and more sensitive coloured products and the addition of excess of INH produced no adverse effect (Fig. 3.10). Hence a volume of 1.0 ml (for DPH and LD) or 2.0 ml (for MD) of INH was maintained throughout the study.

**Effect of NaOH:**

The reaction was observed only in alkaline medium. Hence bases like sodium carbonate, ammonia buffers of different pH, ammonium hydroxide and sodium hydroxide were tried in the study. The formation or intensity of the coloured products was slow or less in sodium carbonate, ammonia buffers of different pH and ammonium hydroxide. Hence various volumes of 0.5 M NaOH were tried and good results were obtained with 1.0 ml
(for DPH and LD) and 2.0 ml (for MD) (Fig. 3.11). Hence these volumes of NaOH having the cited concentration were used for further work.

**Sequence of addition of reagents in Methods A and B:**

The order of addition of reagents in both methods is critical and hence the order suggested in the procedures is followed.

**Precision and accuracy:**

The precision and accuracy of the proposed methods were judged using known amounts of each of the catecholamine (within their respective Beer's law ranges). The low percentage relative standard deviation and percentage error values (Table 3.2) indicated good precision and accuracy of the proposed methods.

**Optical characteristics of the coloured products:**

In order to verify the ranges of Beer's law, absorbances were measured for varying amounts of each of the drug solutions with established concentration of the reagents. The Beer's law limits (Fig. 3.1 and 3.2), optimum photometric range, molar absorptivity and Sandell's sensitivity values have been evaluated. Regression analyses of Beer's law plots at their respective $\lambda_{max}$ values revealed a good correlation. Graphs of absorbances versus concentration showed zero intercept and are described by the regression equation $Y = a + bX$ (where $Y$ is the absorbance of a 1 cm layer, $b$ is the slope, $a$ is the intercept and $X$ is the concentration of catecholamine in ppm) obtained by least-squares method.

The results are given in Table 3.2.
Stability of the complexes:

The coloured species formed were stable for 80 min in Method A and for more than 5 h in Method B which make the methods more practicable for quality control.

Studies on the interference of excipients/additives:

It was studied by adding different amounts of excipients or additives generally present in the formulations of the catecholamines and assaying the catecholamines taken after developing the colour by following the procedure described in each of the method. It was found that the talc, glucose, starch, lactose, dextrose and magnesium stearate did not interfere in the determination at the levels that are usually found in dosage forms. It was also found that an antioxidant, sodium metabisulphite and sodium chloride that are usually present in DPH injections did not interfere.

Recovery studies:

The recovery technique was applied to judge the suitability of the proposed methods. For this, known quantities of pure drug were mixed with definite amounts of preanalysed formulations and the mixtures were analysed as before. The total amount of the drug was then determined using the proposed methods separately and the amount of the added drug was calculated by difference. The results were observed to be in good agreement.
Applicability of the proposed methods:

In order to justify the reliability and suitability of the proposed methods, dosage forms marketed under different trade names were analysed. The results of analysis were found to be satisfactory (Table 3.3).

Statistical analysis of the results in comparison with the official\textsuperscript{27} method:

The performance of the proposed methods was judged by calculating the Student t- and by the variance ratio F-tests. The results of analysis of various pharmaceutical formulations of catecholamines are given in Table 3.3. At 95 % confidence level, the calculated t- and F-values do not exceed the theoretical values. Thus, the results obtained reveal that a similar degree of accuracy is afforded between the proposed methods and the official method.

CONCLUSIONS

The salient features of the proposed methods are their simplicity, rapidity, reasonable sensitivity with a wide range of determination without the need for extracting or heating and involve the use of a spectrophotometer which costs much less as compared to chromatographic methods. The Method B was found to be more sensitive compared to Method A. The reagents utilised are cheaper and readily available. The absorbance values were adequately stable for a sufficient interval of time, which makes the methods more practicable. Additives and excipients used in the formulations do not interfere with the proposed methods. The data obtained by the statistical comparison (with the official method) and the recovery studies clearly indicated the good reproducibility and accuracy of the proposed methods. With these, the methods could be applied for routine quality work in pharmaceutical industries.
REFERENCES


Scheme 3.1: Reaction mechanism (Method A)

MD; $R = -(\text{CH}_2)\text{C(NH}_2\text{(CH}_3\text{)COOH)}$

LD; $R = -(\text{CH}_2)\text{CH(NH}_2\text{)COOH}$
Scheme 3.2: Reaction mechanism for MD (Method B)
Fig. 3.1 Beer's law plots of A LD and ■ MD

Fig. 3.2 Beer's law plots of ■ LD ▲ DPH and • MD (Method B)

(Method A)
Fig. 3.3: Absorption spectra of coloured products of MD and LD (Method A).

1. LD (22 ppm) + HCl + sodium nitrite + sulphamic acid + NaOH
2. MD (18 ppm) + HCl + sodium nitrite + sulphamic acid + NaOH
3. HCl + sodium nitrite + sulphamic acid + NaOH reagent blank
Fig. 3.4: Absorption spectra of coloured products of LD, MD and DPI (Method B).

1. LD (14 ppm) + PDAB + INH + NaOH
2. MD (15 ppm) + PDAB + INH + NaOH
3. DPH(11 ppm) + PDAB + INH + NaOH
4. PDAB + INH + NaOH reagent blank

Fig. 3.4: Absorption spectra of coloured products of LD, MD and DPH (Method B).
Effect of volume of sodium nitrite (10%) on the absorbance of the coloured products of • LD (16 ppm) and ● MD (20 ppm).

Effect of volume of HCl (1 M) on the absorbance of the coloured products of ▲ LD (15 ppm) and ● MD (14 ppm).
Fig. 3.7 Effect of volume of sulphamic acid (10 %) on the absorbance of coloured products of

- LD (13 ppm) and ● MD (14 ppm)

Fig. 3.8 Effect of volume of NaOH (20 %) on the absorbance of coloured products of

■ LD (14 ppm) and ● MD (17 ppm)
Fig. 3.9 Effect of volume of PDAB (0.5 %) on the absorbance of the coloured products of
- LD (9 ppm) ▲ MD (12 ppm)
and ■ DPH (13 ppm)

Fig. 3.10 Effect of volume of INH (0.5 %) on the absorbance of coloured products of
- LD (10 ppm) ▲ MD (13 ppm) and
■ DPH (13 ppm)
Effect of volume of NaOH (0.5 M) on the absorbance of the coloured products of □ LD (9 ppm) • DPH (14 ppm) and ▲ MD (13 ppm)

Fig. 3.11
Table 3.1 Literature survey of the reagents used for the determination of catecholamine derivatives

<table>
<thead>
<tr>
<th>Reagent</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>Beer’s law range, ppm</th>
<th>Remarks</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphamic acid in alkali</td>
<td>540</td>
<td>10-50</td>
<td>Require long standing for colour development</td>
<td>13</td>
</tr>
<tr>
<td>Chloranilic acid</td>
<td>325</td>
<td>2-30</td>
<td>Involves extraction into chloroform-isopropyl alcohol mixture; has less sensitivity</td>
<td>14</td>
</tr>
<tr>
<td>Sodium tungstate</td>
<td>299-300</td>
<td>0.53 - 87.5</td>
<td>The chromogen is stable for 40 min.</td>
<td>15</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>698</td>
<td>1-50</td>
<td>Heating at 40-45 °C for 10 min; chromogen is stable for 1 h.</td>
<td>16</td>
</tr>
<tr>
<td>Tetrazolium violet chloride</td>
<td>512</td>
<td>0.2-8.0</td>
<td>Heating at 50 ± 0.5°C for 30 min and cooling to 20-25 °C</td>
<td>17</td>
</tr>
<tr>
<td>MnSO$_4$</td>
<td>--</td>
<td>50-500</td>
<td>Works out for higher concentration of MD.</td>
<td>18</td>
</tr>
<tr>
<td>Compound</td>
<td>Extraction Time</td>
<td>Absorbance Range</td>
<td>Comments</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>------------------</td>
<td>--------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Trinitrobenzene sulphonic acid</td>
<td>20 min</td>
<td>--</td>
<td>This method required extraction and also 20 min. to complete the reaction</td>
<td></td>
</tr>
<tr>
<td>Thiosemicarbazide</td>
<td>45 min</td>
<td>1.0–8.0</td>
<td>This method required 45 min for completion of the reaction</td>
<td></td>
</tr>
<tr>
<td>3-methylbenzothiazolin-2-one hydrazone hydrochloride and Ce(IV)</td>
<td></td>
<td>2.0–8.0</td>
<td>Measured the absorbances after 30 min and before 120 min.</td>
<td></td>
</tr>
<tr>
<td>Ninhydrin</td>
<td></td>
<td>9.0–21</td>
<td>This method required heating and DMF is used as a solvent.</td>
<td></td>
</tr>
<tr>
<td>Ammonium molybdate</td>
<td>25–250</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>0.8–4.0</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KBrO₃</td>
<td>40–650</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium vanadate</td>
<td>100–500</td>
<td>--</td>
<td>Has very low sensitivity</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2 Optical characteristics, precision and accuracy data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values of</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MD</td>
<td>LD</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td></td>
<td>502</td>
<td>497</td>
</tr>
<tr>
<td>Beer's law limits (ppm)</td>
<td></td>
<td>2–25</td>
<td>1.5–24</td>
</tr>
<tr>
<td>Optimum photometric range (ppm)</td>
<td></td>
<td>4–22.5</td>
<td>3–22.2</td>
</tr>
<tr>
<td>Molar absorptivity (X $10^3$ mol$^{-1}$ cm$^{-1}$)</td>
<td></td>
<td>5.38</td>
<td>5.50</td>
</tr>
<tr>
<td>Sandell's sensitivity (ng cm$^{-2}$)</td>
<td></td>
<td>39.23</td>
<td>35.83</td>
</tr>
<tr>
<td>Regression equation (Y)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope, b</td>
<td>0.0249</td>
<td>0.0289</td>
<td>0.0389</td>
</tr>
<tr>
<td>Intercept, a</td>
<td>0.0082</td>
<td>-0.0154</td>
<td>-0.0031</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9998</td>
<td>0.9995</td>
<td>0.9996</td>
</tr>
<tr>
<td>Relative standard deviation (%)</td>
<td>0.98</td>
<td>0.81</td>
<td>0.89</td>
</tr>
<tr>
<td>% Range of error c</td>
<td>0.78</td>
<td>0.96</td>
<td>0.61</td>
</tr>
</tbody>
</table>

*a $Y=a+bX$ where X is the concentration of the drug in ppm.

c For six replicate analysis within Beer's law limits.
Table 3.3 Determination of catecholamines in pharmaceutical preparations by the proposed methods and their comparison with the official method\textsuperscript{27}

<table>
<thead>
<tr>
<th>Drug\textsuperscript{*}</th>
<th>Label claim (mg)</th>
<th>Official method</th>
<th>Recovery **± SD, % and their comparison with the official method</th>
<th>Proposed methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Method A</td>
</tr>
<tr>
<td>Tablets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MD\textsuperscript{a,b}</td>
<td>250</td>
<td>98.94 ± 1.01</td>
<td>98.95 ± 1.01</td>
<td>99.53 ± 0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F = 1.00; t = 1.61</td>
<td>F = 1.23; t = 1.25</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>100.02 ± 0.46</td>
<td>99.11 ± 0.55</td>
<td>99.50 ± 0.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F = 1.42; t = 1.53</td>
<td>F = 1.32; t = 1.76</td>
</tr>
<tr>
<td>LD\textsuperscript{c,d}</td>
<td>250</td>
<td>99.23 ± 1.12</td>
<td>99.12 ± 1.03</td>
<td>99.15 ± 1.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F = 1.18; t = 1.21</td>
<td>F = 1.09; t = 1.62</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>99.78 ± 0.91</td>
<td>99.04 ± 0.88</td>
<td>99.11 ± 0.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F = 1.06; t = 1.44</td>
<td>F = 1.17; t = 1.23</td>
</tr>
<tr>
<td>DPH\textsuperscript{e,f}</td>
<td>Injection 200/5 ml</td>
<td>98.88 ± 0.96</td>
<td>--</td>
<td>99.05 ± 1.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F = 1.12; t = 1.11</td>
<td></td>
</tr>
<tr>
<td>Injection 200/5 ml</td>
<td>99.55 ± 0.76</td>
<td>--</td>
<td>98.96 ± 0.87</td>
<td>F = 1.31; t = 1.14</td>
</tr>
</tbody>
</table>

\textsuperscript{*} Manufactured by \textsuperscript{a}Merind, \textsuperscript{b}IDPL, \textsuperscript{c}Sun Pharmaceuticals Ltd, \textsuperscript{d}Wallace, \textsuperscript{e}TTK Pharma and \textsuperscript{f}VHB Pharmaceuticals Pvt. Ltd.

** Average of five determinations.
CHAPTER III
SECTION II

SPECTROFLUORIMETRIC ASSAY OF DOPAMINE HYDROCHLORIDE AND LEVODOPA IN PHARMACEUTICAL FORMULATIONS AND SPIKED BIOLOGICAL SAMPLES
Owing to the growing concern with the applications of dopamine hydrochloride (DPH) and levodopa (LD) in pharmacology, increasing efforts are being directed towards the development of rapid, sensitive and selective analytical techniques as mentioned in the previous section. In addition, some spectrofluorimetric methods\textsuperscript{1-8} have also been reported for the assay of these drugs. All these methods are either time bound or lengthy or require heating or necessarily involve an antioxidant to stabilize the fluorophore.

In view of this, the investigator has attempted to develop a simple, reasonably sensitive and selective spectrofluorimetric method for the determination of DPH and LD in bulk sample, pharmaceutical preparations and spiked biological samples.

**EXPERIMENTAL**

**Equipments:**

Absorption and fluorescence spectra were obtained using a Hitachi UV-visible spectrophotometer Model U 2001 and a Hitachi spectrofluorimeter Model F 2000 respectively.

**Preparation of reagents and chemicals:**

All chemicals used were of analytical reagent or pharmaceutical grade and quartz-processed high-purity water was used throughout.

Aqueous solutions of 0.1 % resorcinol (ROL) and 0.2 % NaOH were prepared separately and used in the study.
Preparation of standard drug solutions:

Aqueous solutions of DPH and LD were prepared, standardised and stored as discussed in the first section. The stock solutions were diluted as and when required.

Different dosage forms of DPH and LD were obtained commercially from different firms.

Recommended procedures

The following procedures were suggested after a systematic and detailed study of various parameters involved in the formation of fluorescent species (as described under results and discussion) for the determination of DPH and LD in pure, pharmaceutical preparations and spiked biological samples.

Analysis of bulk samples:

Suitable amounts of each of the drug solution (2-80 µg for DPH; 1-40 µg for LD) were transferred into a series of 10 ml volumetric flasks. To each of these were added 2.0 ml of ROL and 2.0 ml (for DPH) or 5.0 ml (for LD) of NaOH. The contents of the flasks were diluted up to the mark with distilled water, mixed well and the fluorescence intensities were measured at 463 nm and at 460 nm after exciting at 374 nm and at 437 nm for DPH and LD respectively.
Analysis of pharmaceutical formulations:

*Tablets:*

The LD tablets were brought into solution as described in the previous section. A suitable aliquot of the solution was taken and analysed using the procedure described for pure drug.

*Injection:*

The DPH injection solution was suitably diluted with distilled water. An appropriate amount of the aliquot was taken and analysed using the procedure described for pure drug.

Analysis of spiked biological samples:

*Blood:*

The blood sample was treated as described in chapter II. The working solutions were prepared after diluting the solution suitably with distilled water. An aliquot of the solution was analysed as described for pure drug.

*Urine:*

The urine sample was treated as discussed in chapter II. The urine solution was diluted as required. An aliquot of this solution was analysed using the procedure described for pure drug.

**RESULTS AND DISCUSSION**

The DPH or LD, when reacted with ROL in NaOH medium yielded fluorophores instantaneously at room temperature. The attempts to characterize the fluorophores were not successful as these fluorophores could not be extracted into any organic solvent or isolated in solid form. However,
the investigator has successfully utilised the formation of fluorophores for the quantitative assay of DPH and LD in pure, dosage forms and spiked biological samples.

The absorption spectra of the fluorophores of DPH and LD were recorded in the wavelength range of 250-600 nm. The DPH and LD exhibited maximum absorption at 374 nm and at 437 nm respectively (Fig. 3.1a). The corresponding $\lambda_{\text{max}}$ were used to excite the respective fluorophore.

The fluorophores showed maximum fluorescence intensities at 463 nm and at 460 nm after excitation at 374 nm and at 437 nm for DPH and LD respectively (Fig. 3.2a). Methyldopa gave non-reproducible results and hence it could not be assayed by the proposed method.

**Optimum reaction conditions:**

The optimum reaction conditions were ascertained by measuring the emission intensity at wavelength of maximum emission after exciting the fluorophore at corresponding $\lambda_{\text{ex}}$ (also equal to $\lambda_{\text{max}}$) of a series of solutions by varying one and keeping other parameters fixed.

**Effect of reagent:**

By experiments, it was noticed that an optimum volume of 1.75-2.25 ml of ROL was necessary for complete formation of fluorophore. Less intense fluorophore was formed when the volume of ROL added was less than 1.75 ml in a total volume of 10 ml, while the fluorescence intensity decreased rapidly when its volume was more than 2.25 ml.
Effect of alkali:

The fluorescent products were formed in alkaline medium only. Several bases, \textit{viz.}, NaOH, KOH, NH$_3$, ammonia buffers of different pH and Na$_2$CO$_3$ were used to achieve higher sensitivity and longer stability of the fluorophores. The fluorescence intensity was observed to be low in KOH, NH$_3$, ammonia buffer and Na$_2$CO$_3$ when compared to that in NaOH. Further it was found that the optimum fluorescence intensity was observed with 2 ml and 5 ml of NaOH for DPH and LD respectively.

Sequence of addition of reagents:

The order of addition of reagents was found to be critical and NaOH should be added after the addition of ROL to the drug solution.

Precision and accuracy:

The precision and accuracy of the proposed methods were judged by analysing five replicates of 5 ppm of DPH and 3 ppm of LD. The precision and accuracy of the proposed method were found to be excellent as indicated from their low percentage relative standard deviation (0.58-0.7) and percentage error at 95 % confidence limit (0.57-0.69).

Analytical features:

The fluorescence intensities of the corresponding fluorophores were recorded under optimum reaction conditions. A linear relationship ($r = 0.9992$ and 0.9995) was observed between the fluorescence intensities and the concentration of the fluorophores of DPH and LD over the concentration ranges of 0.2-8 ppm and 0.1-4.0 ppm respectively. The detection limit values
calculated based on the reported method\textsuperscript{9} were found to be 0.0757 and 0.0912 \( \mu \text{g ml}^{-1} \) for DPH and LD respectively.

Stability of the fluorophores:

The fluorophores of DPH and LD were found to be stable for more than 60 min.

Interference studies:

In order to assess the possible analytical applications of the proposed method, the effects of some substances that often accompany DPH or LD in various pharmaceutical products were studied by adding different amounts of these substances to known amounts (within working ranges) of DPH and LD. The substance was considered to interfere with the determination, if the obtained fluorescence intensity values differed by more than \( \pm 2.0 \% \) from that for pure drug alone. It was found that the method did not suffer from any interference by the usual tablet diluents and excipients \textit{viz.,} talc, sucrose, starch, gelatin, lactose, dextrose, magnesium stearate and glucose in amounts far in excess of their normal occurrence in various pharmaceutical preparations of DPH and LD.

Recovery studies:

The recovery technique was applied to judge the suitability of the proposed method. For this, known quantities of pure DPH or LD were mixed with definite amounts of preanalysed formulations and the mixtures were analysed as before. The total amount of the drug was then determined using the proposed method and the amount of the added drug was calculated by
difference. The percentage recovery of DPH and LD was observed to be in the range of 99.03-101.08.

Applicability of the proposed method:

In order to justify the reliability and suitability of the proposed method, various dosage forms marketed under different trade names and spiked biological samples were analysed. The results of analysis of pharmaceutical formulations of DPH and LD obtained by the proposed method were in good agreement with those of the reported method (Table 3.1a). The data of the results of analysis of spiked biological samples (Table 3.2a) revealed a good precision with low percentage relative standard deviation values (less than 0.7).

Statistical analysis of the results of pharmaceutical formulations in comparison with the reported method:

The performance of the proposed method was judged by calculating the Student t- and by the variance ratio F-tests. The results of analysis of pharmaceutical formulations of DPH and LD are presented in Table 3.1a. At 95% confidence level, the calculated t-and F-values did not exceed the theoretical values. The results thus obtained revealed that a similar degree of accuracy was obtained between the proposed and the reported method.

CONCLUSIONS

The salient features of the proposed method are its sensitivity and selectivity along with a wide concentration range of determination. The proposed method has advantages over the reported methods1-8 which are either time bound or lengthy or require heating or necessarily involve an antioxidant
to stabilize the fluorophore. However, the reagents utilised in the spectrofluorimetric assay of DPH and LD are cheaper and readily available. The fluorophores formed are adequately stable for a sufficient interval of time which make the method more practicable. An added advantage of the proposed method is the non-interference of additives and excipients commonly present in the formulations of DPH and LD. Statistical analysis of the results also showed that the proposed method has good precision and accuracy. Hence, the proposed method could be used for routine quality and in clinical laboratories.
REFERENCES


Fig: 3.1 a Absorption spectra of DPH and LD
Fig: 3.2a Emission spectra of DPH and LD

1. 4 ppm DPH + ROL + NaOH
2. 5 ppm LD + ROL + NaOH
Table 3.1a. Determination of DPH and LD in pharmaceutical preparations and its comparison with the reported\(^8\) method

<table>
<thead>
<tr>
<th>Drug*</th>
<th>Label</th>
<th>Reported method</th>
<th>% Recovery** ± SD, % and its comparison with the reported method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LD(^{a,b})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tablet 1</td>
<td>250</td>
<td>99.17 ± 0.64</td>
<td>99.69 ± 0.70, (F = 1.19; t = 1.78)</td>
</tr>
<tr>
<td>Tablet 2</td>
<td>500</td>
<td>98.67 ± 0.91</td>
<td>98.94 ± 0.65, (F = 1.96; t = 1.91)</td>
</tr>
<tr>
<td>DPH(^{c,d})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection 1</td>
<td>200/5ml</td>
<td>98.89 ± 0.75</td>
<td>99.11 ± 0.49, (F = 2.34; t = 1.59)</td>
</tr>
<tr>
<td>Injection 2</td>
<td>200/5ml</td>
<td>99.53 ± 0.82</td>
<td>99.12 ± 0.58, (F = 1.99; t = 1.71)</td>
</tr>
</tbody>
</table>

* Manufactured by \(^{a}\)Sun Pharmaceuticals Ltd, \(^{b}\)Wallace, \(^{c}\)TTK Pharma and \(^{d}\)VHB Pharmaceuticals Pvt. Ltd.

** Average of six determinations
Table 3.2a Analysis of DPH and LD in spiked blood and urine samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPH/LD present, ppm</th>
<th>DPH/LD found* ppm ± SD, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood 1</td>
<td>4.0</td>
<td>3.99 ± 0.57</td>
</tr>
<tr>
<td>Blood 2</td>
<td>6.0</td>
<td>5.96 ± 0.47</td>
</tr>
<tr>
<td>Urine 1</td>
<td>4.0</td>
<td>3.98 ± 0.70</td>
</tr>
<tr>
<td>Urine 2</td>
<td>6.0</td>
<td>5.98 ± 0.58</td>
</tr>
<tr>
<td>LD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood 1</td>
<td>2.0</td>
<td>1.99 ± 0.51</td>
</tr>
<tr>
<td>Blood 2</td>
<td>3.0</td>
<td>2.98 ± 0.61</td>
</tr>
<tr>
<td>Urine 1</td>
<td>2.0</td>
<td>1.99 ± 0.66</td>
</tr>
<tr>
<td>Urine 2</td>
<td>3.0</td>
<td>2.99 ± 0.53</td>
</tr>
</tbody>
</table>

* Average of five determinations