Imipramine hydrochloride (IPM) is a tricyclic antidepressant. Its chemical name is 10,11-dihydro-5H-dibenzo[b,f]azepine-5-(dimethylaminopropyl) hydrochloride. The molecular formula is C19H24N2 and the molecular mass is 316.9 g/mol. It is officially recognized in Indian Pharmacopoeia (IP)\(^1\), British Pharmacopoeia (BP)\(^2\) and the United States Pharmacopoeia (USP)\(^3\). Diazepam (DZM) an antianxiety, antiepileptic drug with the IUPAC name, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one. The molecular formula of diazepam is C16H13ClN2O and the molecular weight is 284.7 g/mol. It is also officially recognized in IP\(^1\), BP\(^2\) and USP\(^3\).

Imipramine hydrochloride (IPM) was studied as a single drug and in combination with other drugs\(^4-6\). Different methods like second-order derivative spectrophotometry\(^5\), chemometric method\(^6\), spectrophotometry\(^7,8\), high performance liquid chromatography (HPLC)\(^4,9,10\), fluorescence polarization immunoassay\(^10\), HPLC-diode array detection (HPLC-DAD)\(^11\), surface ionization organic mass spectrometry\(^12\) and HPLC tandem mass spectrometry (HPLC-MS/MS)\(^13\) have been employed to measure IPM. It was also determined in human serum\(^4,9,13\) and urine\(^4,8\).

Diazepam has been studied separately, in combination with other benzodiazepines and other drugs\(^14-20\) and its metabolites\(^21\). It was studied in whole blood\(^16\), plasma\(^17,18\), urine, hair and oral fluids\(^19,22\) and in pharmaceutical form\(^23-26\). These studies were carried out using analytical methods such as second-derivative spectrophotometry\(^5\), chromatography\(^\text{[15]}\), HPLC\(^\text{[16-18,26]}\), LC-MS/MS\(^\text{[18,19]}\), HPLC electrospray tandem mass spectrometry\(^20\), capillary electrophoresis...
and HPLC-electrospray ionization mass spectrometry (HPLC-ESI-MS/MS)[21], gas chromatography-negative ion chemical ionization mass spectrometry[22], spectrophotometry[23] fluorimetry[24], differential pulse polarographic determination[25] and capillary electrophoresis[26].

IPM and DZM combination is available as Imipam (La Pharma), Imidep (Sunrise Remedies), Imilor (Intra Doxis) and Imizonic plus (Acme) and many others. The motivation for the present work is that benzodiazepines and tricyclic antidepressants are often administered to patients in combination for the treatment of anxiety and depression related disorders. This clinical use necessitates quantitative estimation of these drugs in combination using a simple and rapid method.

In this paper, the development of a method for quantitative and simultaneous estimation of imipramine hydrochloride and diazepam in combination is reported. The developed method is validated by measuring linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, robustness, recovery, ruggedness and specificity based on ICH guidelines[27]. To the best of our knowledge, this is the first work reporting simultaneous determination of IPM and DZM drug combination by RP-HPLC method using ultraviolet (UV) detection.

The chemicals methanol and water (pH between 5 and 8) were of HPLC grade and procured from Merck Limited, Mumbai. Analytical grade sodium acetate and acetic acid were used (Merck Limited, Mumbai). Formulations of the drug were purchased from a local pharmacy. For filtering the prepared solutions 0.45 µm nylon membrane filter paper (Ultipore, Mumbai) was used.

A Peak HPLC system operated in isocratic mode was utilized for the present investigations. A Chromosil C18 column (250×4.6 mm, 5 µm particles) was used as a stationary phase. The HPLC system was equipped with a LC 20AT pump and a variable wavelength programmable UV/Vis detector (Shimadzu, SPD-10Avp). The samples were injected with a 20 µl Hamilton syringe. The degassing of the mobile phase was done with an ultrasonic bath sonicator and a digital electronic balance was used for weighing the materials. Chromatograms were recorded and integrated by Peak LC 7000 software (MSP Lab instruments, India). Analysis of the data was done with Microsoft Excel software. The detection wavelength of IPM and DZM was determined with an ultraviolet UV/Vis spectrophotometer (Techcomp, UV 2301) provided with Hitachi software.

To prepare the stock solution, 10 mg of IPM and DZM was weighed and dissolved in 10 ml of methanol separately in a 10 ml volumetric flask. Then the drug was sonicated for 2 min to dissolve completely. After cooling, the drug was filtered through 0.45 µm nylon membrane filter paper. A 1000 µg/ml solution was prepared, from this 2 ml was further diluted to 20 ml to get a stock concentration of 100 µg/ml solution. Required concentrations were prepared by selective dilution from the standard solution.

The RP-HPLC method with UV detection was started with the development of the mobile phase composition, flow rate, wavelength and pH. These were optimized for sharper peaks with less asymmetry and for good resolution of IPM and DZM drugs. Initially, mobile phase volume ratio was developed. Methanol and water were tested separately and in combination as a mobile phase. Further, sodium acetate was added to methanol and water, which increased the pH of the mobile phase. pH was decreased to 3.8 by using dilute acetic acid. After several chromatographic runs, methanol:water:0.1 M sodium acetate (30:50:20 v/v/v) showed better peak symmetry, good signal to noise (S/N) ratio and well separated chromatographic peaks. Similarly, different pump pressures were tried and the pressure was set to 12.5 MPa. Optimum flow rate was determined by running the solutions at different flow rates and was set to 1.0 ml/min. The elution runtime was limited to ten minutes after checking for the interference and influence of the excipients.

The optimum chromatographic conditions obtained during the method development were: mobile phase-methanol:water:0.1M sodium acetate (30:50:20 v/v/v); detection wavelength-243 nm; stationary phase-Chromosil C18 column (250×4.6 mm, 5µm); pH of the mobile phase-3.8 adjusted with diluted acetic acid; active pharmaceutical ingredient...
concentration-75 µg/ml (IPM) and 15 µg/ml (DZM); flow rate-1.0 ml/min; pump pressure-12.5±0.5 MPa and runtime-10 min.

The spectra of diluted solutions of IPM and DZM in methanol were recorded on an UV spectrophotometer by scanning the wavelength from 200 to 400 nm. The peak of maximum absorbance for both IPM and DZM showed a wavelength of 243 nm (fig. 1). For simultaneous analysis of the two drugs; first the single drugs were scanned individually and then maximum absorption wavelength was chosen from the overlay spectra. By complete separation of IPM and DZM the specificity of the developed RP-HPLC method was validated with retention time, tailing factor and resolution. The measured peaks for both the drugs were sharper and were well separated. The chromatogram of IPM and DZM from standard solution was shown in fig. 2. The retention time was 3.33±0.02 for IPM and 4.64±0.02 min for DZM for 10 min runtime, respectively. The tailing factors of IPM and DZM were 0.79 and 1.03 and these values fall in the range of recommended values. Furthermore, the theoretical plates of IPM (12184) and DZM (2862) were well above the recommended values.

The linearity measurements were carried out for six concentrations in the range 25-150 µg/ml in 25 µg/ml steps for IPM and in the range 5-30 µg/ml in 5 µg/ml steps for DZM, respectively. These measurements were fitted with a linear regression of the form $y=ax+b$ and the values of regression parameters for the curves were $r^2=0.999$ for IPM and $r^2=0.9994$ for DZM. All the linear regression parameters were statistically significant.

The precision studies of the two drugs IPM (75 µg/ml) and DZM (15 µg/ml) were done by intraday and interday precision (for three successive days) measurements. The data obtained from intraday precision and interday precision was given in Table 1. The peak areas were expressed in term of mean normalized area of six samples. Table 1 gives standard deviation (SD) and relative standard deviation (RSD) along with the recommended values of these parameters. The RSD of intraday analysis of all the samples was 0.31 and 0.61, and interday analysis was 0.85 and 0.99 for IPM and DZM, respectively. This was less than 1% indicating the method was precise. Ruggedness of IPM (75 µg/ml) and DZM (15 µg/ml) was checked by two different analysts. In total six samples were analyzed and the results were given in Table 1. The RSD was less than 2% indicating the ruggedness of the method.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the calibration curves of IPM and DZM drugs. These were determined from the sensitivity during linearity measurements. For IPM, LOD was 0.03 µg/ml and LOQ was 0.1 µg/ml. For DZM, LOD was 0.02 µg/ml and LOQ was 0.07 µg/ml.

The developed method was validated for robustness by changing the mobile phase volume ratio, flow rate and wavelength from the optimal chromatographic parameters. The percentage change of peak areas was calculated for the above three parameters. The observed change was less than 2% from the standard value (Table 2). The results indicate that the developed method was robust.

Fig. 1: Absorption spectrum of IPM and DZM. Absorption spectrum of imipramine hydrochloride (IPM) and diazepam (DZM). Absorbance as a function of wavelength. The maximum absorption was observed at 243 nm.

Fig. 2: Chromatogram of IPM and DZM. Chromatogram of imipramine hydrochloride (IPM) and diazepam (DZM), whose retention times were 3.33 and 4.64 min, respectively.
The accuracy of the proposed method was verified using standard addition technique by adding known amount of standard to samples (spiked samples). Three different percentage determinations (50, 100 and 150%) were used for the recovery studies. For each percentage level the analysis was repeated three times \((n=3)\) for IPM and DZM. The recovery percentage was compared with the actual amount. The results were presented in Table 3 as the mean concentrations and their standard deviation (SD) for each percentage level. The relative standard deviation (RSD) or the coefficient of variation (CV) and calculated percentage error was also given (Table 3). The good recovery of the two drugs IPM and DZM in the range of 100.59-101.52% and 99.47-100.33% satisfy the ICH guidelines\(^{[27]}\).

Formulation assay was performed on commercial Imipam (La Pharma) tablets. The procedure was repeated two times, separately. Twenty tablets were powdered and weighed. The powdered drug equal to 1 mg was taken and dissolved in 10 ml of methanol. From a concentration of 100 \(\mu\)g/ml solution, 75 and 15 \(\mu\)g/ml were prepared. From formulation assay studies 74.6 and 14.9 \(\mu\)g/ml were found, respectively. The assay results of the two drugs were expressed as percentage of label claims. The recovery percentages were 99.4% for IPM and 99.9% for DZM that were in good agreement within the 90 to 100% of the label claim. The chromatogram peaks of the IPM and DZM drugs were predominant in the drug sample with negligible interference from excipients, normally present in the tablets.

In summary, the described RP-HPLC method in this paper was simple and sensitive, fast and specific to carry out. The reported method facilitates for simultaneous determination of imipramine hydrochloride and diazepam with good resolution and sharper chromatographic peaks within a run time of

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**TABLE 1: VALIDATION PARAMETERS**

| Parameter          | IPM Mean±SD (n=6) | % RSD | DZM Mean±SD (n=6) | % RSD | Recommended RSD |
|--------------------|-------------------|-------|-------------------|-------|-----------------|
| Intraday precision | 1.01±0.02         | 1.79  | 0.97±0.02         | 1.62  | <2 %            |
| Intraday precision | 1.01±0.01         | 1.1   | 1.014±0.01        | 1.23  | <2 %            |
| Ruggedness         | 0.98±0.01         | 1.22  | 0.99±0.01         | 1.24  | <2 %            |

Results of validation parameters such as intraday, interday precision and ruggedness for IPM (imipramine hydrochloride 75 \(\mu\)g/ml) and DZM (diazepam, 15 \(\mu\)g/ml). RSD is relative standard deviation. RSD: relative standard deviation, SD: standard deviation.

**TABLE 2: ROBUSTNESS STUDIES OF IPM AND DZM**

| Parameter                              | Value                         |
|----------------------------------------|-------------------------------|
| Standard                               | 535134                        |
| Mobile phase                           | 535841                        |
| Methanol:water:0.1M sodium acetate     | 532425                        |
| pH (adjusted with dil. acetic acid)    | 525043                        |
| Wavelength (nm)                        | 542416                        |
|                                       | 538835                        |

Results of robustness studies of imipramine hydrochloride (IPM) and diazepam (DZM) with variation of parameters such as, mobile phase, volume ratio, pH and detection wavelength. *At 75 \(\mu\)g/ml and *at 15 \(\mu\)g/ml. IPM: imipramine hydrochloride, DZM: diazepam

**TABLE 3: RECOVERY STUDIES OF SPIKED SAMPLES OF IPM AND DZM**

| Analyte | Recovery (%) | Target Conc. \((\mu\)g/ml) | Spiked Conc. \((\mu\)g/ml) | Final Conc. \((\mu\)g/ml) | Conc. obtained \((\mu\)g/ml) Mean±SD | % Recovery Mean±SD | % Error\(^{a}\) |
|---------|--------------|-----------------------------|-----------------------------|-----------------------------|-------------------------------------|-------------------|----------------|
| IPM     | 50           | 50                          | 25                          | 75                          | 76.14±0.51                          | 101.52±0.8       | 0.38           |
|         | 100          | 50                          | 50                          | 100                         | 100.59±0.9                          | 100.59±0.9       | 0.52           |
|         | 150          | 50                          | 75                          | 125                         | 126.19±1.2                          | 100.95±0.99      | 0.57           |
| DZM     | 50           | 10                          | 5                           | 15                          | 14.94±0.25                          | 99.63±1.67       | 0.98           |
|         | 100          | 10                          | 10                          | 20                          | 19.89±0.20                          | 99.47±1.03       | 0.58           |
|         | 150          | 10                          | 15                          | 25                          | 25.17±0.31                          | 100.33±1.23      | 0.69           |

Results of recovery studies with spiked samples of imipramine hydrochloride (IPM) and diazepam (DZM) using standard addition method. % error is RSD/\(\sqrt{n}\), no of trials \(n=3\). RSD: relative standard deviation, SD: standard deviation
10 min. The presented results of validation parameters intraday and interday were precise and recovery results were accurate. They were established by statistical parameters and satisfy the ICH guidelines. Hence, this method can be used for routine quantitative analysis of these drugs in combination and in pharmaceutical preparations in industry and laboratories.

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There are no conflicts of interest.

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