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Research Article

# ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF IMIPRAMINE AND DIAZEPAM IN TABLET DOSAGE FORM BY RP-HPLC

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**ABSTRACT**

Objective:To develop a new validated reverse phase high performance liquid chromatography method for the simultaneous estimation of Imipramine and Diazepam in tablet formulation.

Methods: The chromatographic analysis was carried out on X Bridge C18 column (150×4.6 mm, 5µ), phosphate buffer with pH 3.4 & Acetonitrile (55:45) as mobile phase, at a flow rate of 1.0 ml/min and detected at wave length 250 nm.

Results: The retention times for Imipramine and Diazepam were 2.2 min and 5.6 min respectively. The percentage recoveries for Imipramine and Diazepam were 99.68% and 100.31% respectively. The regression value for both the drugs was found to be 0.999, which showed the linear response from 62.5-625µg/ml for Imipramine and 12.5-125µg/ml for Diazepam.

Conclusion: This method was fast, accurate, precise and sensitive hence it can be employed for routine analysis of tablets containing both drugs.

**Keywords:** Imipramine, Diazepam, RP-HPLC, Simultaneous estimation.

## INTRODUCTION

Imipramine is chemically 3-(10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*,*N*-dimethyl-propan-1-amine (figure-1). Imipramine (IMI) is a tricyclic antidepressant used to treat mental depression. Its primary use in multiple sclerosis is to treat bladder symptoms, including urinary frequency and incontinence [1]. The molecular formula of IMI is C19H24N2, it is an acyclic drug. It is freely soluble in water, chloroform and 95%ethanol. Generally IMI is available in the market, Imipramine hydrochloride. It is a yellowishwhite crystalline powder. Diazepam is chemically 7-chloro-1, 3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (figure-2). Diazepam (DIA) is a benzodiazepine generally used as hypnotic, anxiolytic and muscle relaxant. DIA is also routinely prescribed as the standard first-line treatment for acute convulsions and prolonged status epilepticus [2]. Several high-performance liquid chromatographic (HPLC) methods have also been reported for the determination of IMI and DIA individually [3-8] and in combination with other drugs [9-12]. However, there is no simultaneous method reported for their simultaneous estimation. Hence we have planned to develop a validated reversed-phase HPLC method for the estimation of these drugs in combined dosage form as per ICH guidelines [13-18].

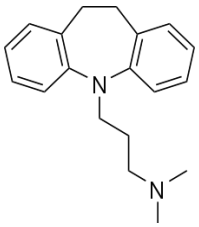


**Fig.1: Structure of Imipramine**

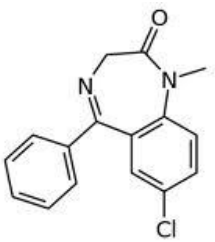


**Fig. 2: Structure of Diazepam**

## MATERIALS AND METHODS

### Instrumentation

Chromatography was performed with Water's 2695 HPLC system provided with Hamilton Syringe, auto sampler and 2996 Photodiode array detector. All HPLC systems were equipped with a column compartment with temperature control and an on-line degasser. Sample acquisition, analysis and reporting were performed by Empower 2 (waters) chromatography software.

### Reagents and chemicals

Pharmaceutically pure sample of Imipramine and Diazepam were obtained from Spectrum Pharma Research Solutions, Hyderabad as gift samples along with their analytical reports. HPLC grade Water, Acetonitrile and Methanol were obtained from Ranchem and Commercial tablets of Imipramine (25mg) and Diazepam (5mg) Depsol Forte were procured from the local market.

### Chromatographic condition

The isocratic mobile phase consisted of buffer: Acetonitrile pH 3.4 in the ratio of 55:45v/v at a flow rate of 1.0 ml/min X Bridge C18 column (150×4.6 mm, 5µ) was used as stationery phase. The detection wave length for both the drugs is 250nm.

### Preparation of standard stock solution

Accurately weigh about 25mg of Imipramine and 5mg of Diazepam drugs into clean and dry 10ml volumetric flasks individually and dissolve in 10ml of diluents to get a concentration of 2500µg/ml of Imipramine and 500µg/ml of Diazepam (stock solution).

### Preparation of working standard solutions

Aliquot of 0.25ml, 0.5ml, 1.0ml, 1.25ml and 1.5ml and 2.5ml were pipette out from stock-A into 10 ml volumetric flask separately and volume was made up to 10ml with diluent. This gives the solutions of 62.5µg/ml, 75µg/ml, 250µg/ml, 312.5µg/ml, 375µg/ml and 625µg/ml respectively for Imipramine, and 12.5µg/ml, 25µg/ml, 50µg/ml, 62.5µg/ml, 75µg/ml and 125µg/ml respectively for Diazepam.

### Method validation

### System suitability tests

To ensure the validity of the analytical procedure, a system

suitability test was established. Data from six injections of 10µL of the working standard solutions of IMI and DIA were used for the evaluation of the system suitability parameters like tailing factor, the number of theoretical plates and retention time.

**Linearity**

By appropriate aliquots of the standard IMI and DIA solutions with the mobile phase, six working solutions ranging between 62.5-625µg/ml for Imipramine and 12.5-125µg/ml for Diazepam were prepared. Each experiment was performed in triplicate according to optimized chromatographic conditions. The peak areas of the chromatograms were plotted against the concentration of IMI and DIA to obtain the calibration curve.

**Accuracy**

Recovery studies by the standard addition method were performed with a view to justify the accuracy of the proposed method. Previously analyzed samples of IMI and DIA to which known amounts of standard IMI and DIA corresponding to 50%, 100% and 150% of label claim were added. The accuracy expressed as the percentage of analyte recovered by the proposed method.

**Precision**

Precision was determined as repeatability and intermediate precision, in accordance with ICH guidelines. The intra-day and inter-day precision were determined by analyzing the samples of IMI and DIA. Determinations were performed on the same day as well as well as on consequent days.

**Limit of detection and the limit of quantification**

Limit of detection (LOD) and limit of quantification (LOD) of IMI and DIA were determined by calibration curve method. Solutions of both IMI and DIA were prepared in linearity range and injected in triplicate. Average peak area of three analyses was plotted against concentration. LOD and LOQ were calculated by using following equations.

$$LOD = (3.3 \times Syx)/b, \quad LOQ = (10.0 \times Syx)/b$$

Where Syx is residual variance due to regression; b is slope.

**Robustness**

The robustness of the method was performed by deliberately changing the chromatographic conditions. The organic strength was varied by ±5%, column temperature was varied by ±5$^0$c and the flow rate ±0.1mL.

**Sample preparation**

20 tablets were weighed, powdered and calculated the average weight of each tablet. Then the weight equivalent to 5 tablets was transferred into a 100 mL volumetric flask, 80mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 2ml was pipetted out into a 10 ml volumetric flask and made up to 10ml with diluent to get sample solution.

**RESULTS AND DISCUSSION**

**Method development:**

Initially reverse phase liquid chromatography seperation was tried to develop using various ratios of Methanol: water, Acetonitrile: water as mobile phase, in which both the drugs did not respond properly, and the resolution was also poor. The organic content of mobile phase was also investigated to optimize the seperation of both drugs. To improve the tailing factor, the P$^H$ of the mobile phase becomes important factor. At P$^H$ – 3.4 both drugs eluted with better seperation.

Thereafter, phosphate buffer (P$^H$-3.4)- Acetonitrile (55:45v/v) was selected as a mobile phase, at a flow rate of 1.0 ml/min. The stationary phase was X Bridge C18 column (150×4.6mm, 5µ). Imipramine and Diazepam shows maximum absorption at the wave length of 250nm was selected as the detection wave length. The retention times were found to be 2.2 min and 5.6 min for Imipramine and Diazepam respectively. The chromatogram obtained was shown in the figure 3.

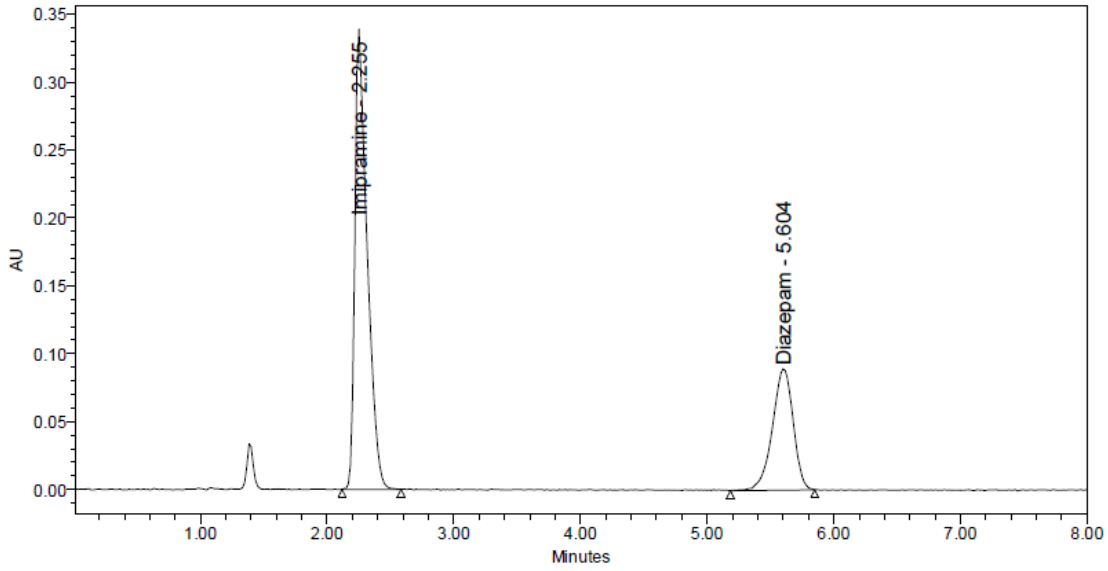


**Fig. 3: Chromatogram of Imipramine and Diazepam**

**Method Validation**

**System suitability:** System suitability parameters like number of theoretical plates, HETP and peak tailing were determined. The values for the parameters were shown in the table-1.

**Table 1: System suitability of Imipramine and Diazepam**

| Parameters | IMI | DIA |
|---|---|---|
| No. of theoretical plates | 2251 | 5599 |
| Tailing factor | 1.6 | 0.9 |
| Mean Area | 2295524 | 1027231 |

**Linearity:** The linearity response for both drugs Imipramine and Diazepam was between 62.5-625µg/ml (Figure 4 A) and 12.5-125µg/ml (Figure 4 B) and the linearity were represented by the regression equation as shown below.

y (IMI )= 9756.x + 8864 ($r^2$ = 0.999) y(DIA) = 21192.x -15163 ($r^2$ = 0.999)

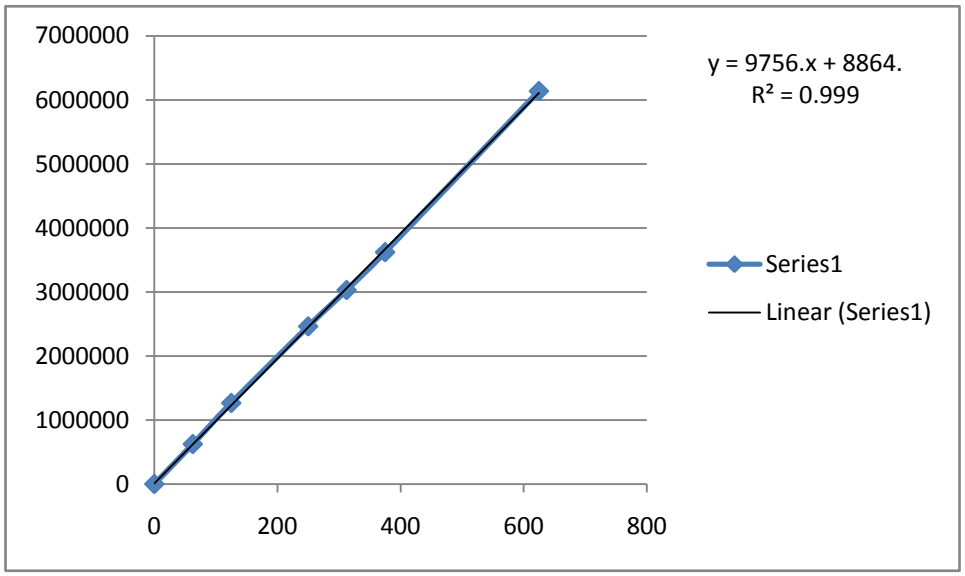


**Fig. 4 (A): Calibration curve for Imipramine**

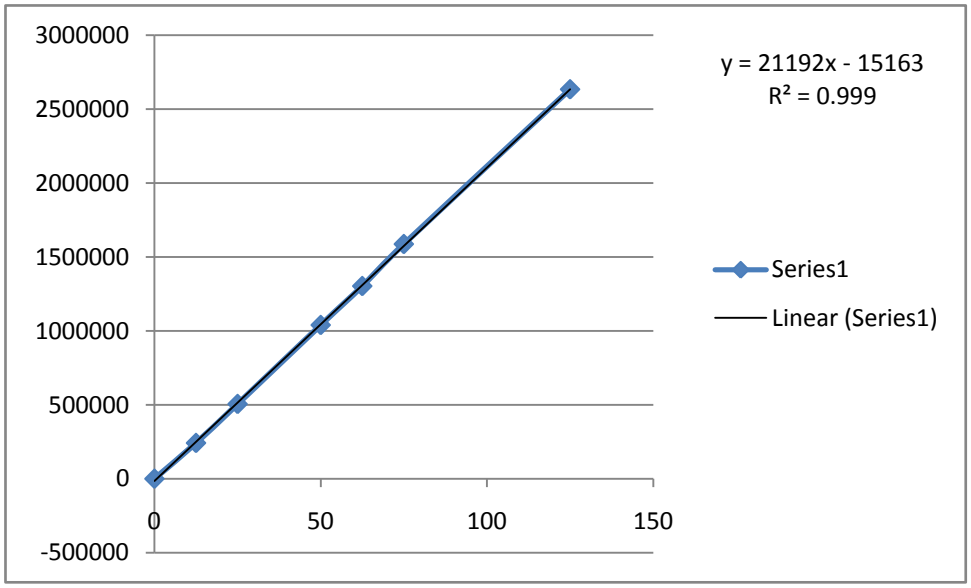


**Fig. 4(B): Calibration curve for Diazepam**

**Accuracy:** Recovery studies were performed to validate the accuracy of developed method. A define concentration of standard drug solution was added to preanalyzed sample solution and recovery was studied. The results are as shown in the table 2.

**Table 2: Results of Accuracy of IMI and DIA**

| Preanalysed sample solution conc. (ug/ml) | | Standard drug conc. (ug/ml) | | % Recovered | |
|---|---|---|---|---|---|
| **IMI** | **DIA** | **IMI** | **DIA** | **IMI** | **DIA** |
| 250 | 50 | 125 | 25 | 99.44 | 99.90 |
| 250 | 50 | 125 | 25 | 98.88 | 98.64 |
| 250 | 50 | 125 | 25 | 99.075 | 98.76 |
| 250 | 50 | 250 | 50 | 98.71 | 100.63 |
| 250 | 50 | 250 | 50 | 98.93 | 101.28 |
| 250 | 50 | 250 | 50 | 100.87 | 101.24 |
| 250 | 50 | 375 | 75 | 100.69 | 100.56 |
| 250 | 50 | 375 | 75 | 101.22 | 100.85 |
| 250 | 50 | 375 | 75 | 99.31 | 100.95 |
| | | | MEAN | 99.68 | 100.31 |
| | | | SD | 0.967 | 1.001 |
| | | | %RSD | 0.97 | 0.99 |

**Precision**

**Repeatability:** Six replicates in same concentration were analyzed in same day for repeatability and results were found to be within acceptable limits (RSD <2) as shown in table 3**.**

**Intermediate Precision:** Six replicates in same concentration were analyzed on two different days and two analysts for day to day and analyst to analyst variation and results were found to be within acceptable limits(RSD<2) as shown in table 3.

**Table 3: Results of Precision of IMI and DIA**

| | Repeatability (%Assay) | | Day to Day (%Assay) | |
|---|---|---|---|---|
| | IMI | DIA | IMI | DIA |
| Sample 1 | 99.67 | 99.31 | 99.35 | 100.07 |
| Sample 2 | 101.35 | 99.91 | 100.31 | 99.91 |
| Sample 3 | 99.35 | 101.11 | 100.07 | 101.6 |
| Sample 4 | 99.82 | 100.58 | 100.08 | 101.46 |
| Sample 5 | 100.93 | 100.82 | 100.04 | 101.73 |
| Sample 6 | 98.83 | 98.25 | 100.11 | 101.68 |
| %Mean | 99.99 | 99.99 | 99.99 | 101.07 |
| SD | 0.961 | 1.075 | 0.329 | 0.846 |
| %RSD | 0.96 | 1.07 | 0.32 | 0.83 |

**Robustness:** The robustness study was performed by slight modification in flow rate of the mobile phase, pH of the buffer and composition of the mobile phase. The samples of Imipramine and Diazepam were analyzed under these changed experimental conditions. The change was made in the ratio of mobile phase by ±5%, column temperature ±5$^0$c and the flow rate ±0.1mL. There were no significant changes in the chromatography pattern when the above modifications were made in the experimental conditions, showing that the method is robust.

**Stability of sample solution:** the sample solution injected after 24hr did not show any appreciable change. Results are shown in table 4.

**Table 4: Stability data of IMI and DIA**

| Drug | % Assay at 0 hr | % Assay at 24 hr |
|---|---|---|
| IMI | 99.31 | 99.83 |
| DIA | 101.08 | 101.64 |

**LOD and LOQ:** LOD and LOQ were determined by calibration curve method. IMI and DIA solutions were prepared in the concentration range of 62.5-625 and 12.5-125 µg/ml respectively and injected in triplicate. Average peak area of three analyses was plotted against concentration. LOD and LOQ were calculated by using following equations.

$$LOD = (3.3 \times Syx)/b,\ LOQ = (10.0 \times Syx)/b$$

Where Syx is residual variance due to regression; b is slope. LOD and LOQfor IMI were 3.202543 and 9.704677 µg/ml respectively and for DIA were 0.147769 and 0.447783 µg/ml respectively.

**Tablet analysis:** Content of IMI and DIA were found in the commercial tablets by the proposed method and results were shown in Table 5.

**Table 5: Results for HPLC Analysis of Tablets**

| Sample No | Peak area | | % Assay | |
|---|---|---|---|---|
| | IMI | DIA | IMI | DIA |
| 1 | 2276586 | 1033210 | 98.67913 | 100.07 |
| 2 | 2298471 | 1031538 | 99.62774 | 99.91 |
| 3 | 2293103 | 1048982 | 99.39506 | 101.60 |
| 4 | 2293347 | 1047563 | 99.40564 | 101.46 |
| 5 | 2292412 | 1050266 | 99.36511 | 101.73 |
| 6 | 2293949 | 1049741 | 99.43173 | 101.68 |
| | | AVG | 99.31 | 101.07 |
| | | SD | 0.326 | 0.846 |
| | | RSD | 0.32 | 0.83 |

**CONCLUSION**

Simultaneous estimation of Imipramine and Diazepam in tablet dosage form by RP-HPLC method was developed and validated. For both the drugs Imipramine and Diazepam, the regression value was found to be 0.999, which shows the linear response from 62.5-625µg/ml for Imipramine and 12.5-125µg/ml for Diazepam. Selectivity experiment showed that there is no interference or overlapping of peaks either due to excipients or diluents with the main peak of Imipramine and Diazepam. The % RSD for precision is <2 which confirms that method is sufficiently precise and the total run time required for the method is only 8 min for eluting both the drugs. So, this method is fast, accurate, precise and sensitive hence it can be employed for routine quality control of tablets containing both drugs in industries.

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