Development and validation of a stability indicating high performance liquid chromatography method for trimethobenzamide

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A simple, accurate, precise and robust stability indicating RP-HPLC assay method has been developed for the estimation of trimethobenzamide in stress sample. An isocratic separation of trimethobenzamide was achieved on Kromasil 100 C-18 column (250 X 4.6mm, 5μ) with a flow rate of 1.0 ml/min and by using a photodiode array detector to detect the analyte at 213nm. The optimized mobile phase consisted of methanol: ammonium formate (44:56, v/v). The drug was subjected to different forced degradation conditions according to ICH guidelines including acid, base, neutral hydrolysis, oxidation, photolysis and thermal degradation. Degradation products were found only in basic and oxidative degradation conditions. All the degradation products got eluted in an overall analytical run time of 12min. The developed analytical method has been validated according to the ICH guidelines. Response of trimethobenzamide was linear over the concentration range of 0.5-50μg/mL ($r^2$ = 0.999). Accuracy was found to be in between 94.03% to 100.39%. Degradation products resulting from the stress studies did not interfere with the detection of the analyte.

Keywords: Trimethobenzamide. Stability indicating assay method. Method development and validation. RP-HPLC.

INTRODUCTION

Trimethobenzamide hydrochloride chemically $N\{p\{[2-(dimethylamino) ethoxy] benzyl\}\}-3, 4, 5$-trimethoxybenzamide monohydrochloride is a potent antiemetic molecule used to treat nausea and vomiting. It is prescribed to patients with gastroenteritis, medication-induced nausea and another type of similar conditions. Trimethobenzamide is an antagonist of the dopamine $D_2$ receptor. It exerts its effect on the chemoreceptor trigger zone of the medulla oblongata to suppress nausea and vomiting (DeCamp et al., 2008; Smith, Cox, Smith, 2012). It is marketed under the trade name Tebamide and Tigan, by King Pharmaceuticals and GlaxoSmithKline in the formulation of oral capsules and parenterals (Gregory et al., 2004).

Forced degradation study is helpful for the development of stability indicating method, determination of intrinsic stability of drug molecule, establish the recommended storage conditions and finally establish the quality, safety and efficacy of the drug substance and drug product (Baertschi, 2011; Blessy et al., 2014; Gregory et al., 2004; Singh et al., 2013). Literature survey reveals that till date, there is no stability indicating assay method exist for quantitation of trimethobenzamide in presence of their degradation products. In this study, a simple robust and sensitive reverse phase-high performance liquid chromatography (RP-HPLC) method has been developed for the quantitative determination of trimethobenzamide in forced degradation sample. Forced degradation studies are carried out according to ICH Q1 R2 guidelines under different stress conditions like acid, base, neutral hydrolysis, oxidative, thermal and photodegradation...
MATERIAL AND METHODS

Chemicals and reagents

Pure trimethobenzamide hydrochloride (>98%) was procured from Sterling Biological Limited (Ahmedabad). Hydrochloric acid AR grade (HCl) and sodium hydroxide AR grade (NaOH) were obtained from Merck India Pvt Ltd. Hydrogen Peroxide (H$_2$O$_2$) was purchased from Qauligens. Acetic acid AR grade was purchased from Fisher Scientific, India and S.D. Fine Chemicals Ltd. Ammonium acetate and ammonium formate were obtained from S.D. Fine Chemicals Ltd and Merck India Pvt Ltd., respectively. HPLC grade methanol (MeOH) was purchased from Fischer Scientific. HPLC grade water used throughout the analysis was obtained from the Merck Milli-Q water purification unit.

Instrument and apparatus

HPLC studies were carried out on Agilent series 1260 infinity HPLC system (Open lab software) with a diode array detector (DAD) set at 213nm for UV detection. The HPLC column Kromasil C$_18$ (250×4.6 mm, 5 μm) was used in the study. The Design of Expert (DOE) (7.0.0) modeling software (Stat-Ease Inc., Minneapolis, MN, USA) was employed for method optimization. Entire stress studies were carried out on Radley apparatus (Veego) having continuous stirring and temperature adjustable knob facility. pH meter used was Eutech instruments pH tutor, India. Other equipment required for this study were sonicator (Epei ultrasonic generator), analytical balance (Mettler Toledo), vortex meter (IKA Vortex) and a hot air oven (Yorco scientific).

Method development

Preparation of drug solution

The stock solution was prepared by dissolved the drug equivalent to 1mg of trimethobenzamide in 1mL of methanol and final volume was made up to 10mL in a volumetric flask with methanol to achieve a concentration of 100 μg/mL.

Preparation of buffer

Ammonium formate buffer (1M) was prepared by taking 6.3gm of ammonium formate in 100mL of HPLC grade water in a volumetric flask. The solution was diluted 100 times to produce a 10mM strength. The buffer was filtered using 0.2 μm The buffer was filtered using a 0.2 μm Millipore filter, pH was measured and adjusted to the desired pH using formic acid.

Ammonium acetate buffer (1M) was prepared by taking 7.7 gm of ammonium acetate in 100 mL of water (HPLC Grade water) in a volumetric flask. The solution was diluted 100 times to produce a 10 mM solution. The buffer was filtered using a 0.2 μm Millipore filter, pH was measured and adjusted to the desired pH using acetic acid.

Optimization of the method

Initial HPLC runs with trimethobenzamide at a 100 μg/mL concentration were performed using different buffers namely ammonium acetate and ammonium formate, different ratios of organic solvents (40%, 47% and 54%) and different buffer strength of 10, 15 and 20mM.

The initial trials were needed to optimize the final method. Total four factors including organic concentration, type of buffer, buffer strength and pH of the buffer were needed to optimize. Optimization of these parameters was varied over three level (high, mid and low) for four parameters including organic ratio from 40-54%, ammonium formate and ammonium acetate buffer system, 10-20 mM buffer strength, and buffer pH 5.8 and 6.8, respectively.

Generation of stress samples Acid hydrolysis

The analyte sample equivalent to 10 mg of trimethobenzamide was weighed, added into 3 mL of methanol and volume was made up to 10 mL with 5 N HCl solution. The degradation samples were kept in Radley apparatus with continuous stirring at 70 °C for 8h. After incubation, 1 mL of aliquots were withdrawn at 0, 1, 2, 4, 6 and 8 h. The samples were neutralized to pH 7 with NaOH solution, diluted and analyzed by the HPLC system.
Base hydrolysis

The analyte sample equivalent to 10mg of trimethobenzamide was weighed, added into 3 mL of methanol and volume was made up to 10 mL with 5N NaOH solution. The degradation samples were kept in Radley apparatus with continuous stirring at 70 ºC for 8 h. 1 mL of aliquots were withdrawn at 0, 1, 2, 4, 6 and 8 h. The samples were neutralized to pH 7 with HCl solution, diluted and analyzed by the HPLC system.

Neutral hydrolysis

The analyte sample equivalent to 10mg of trimethobenzamide was weighed and dissolved in 10mL of water. These degradation samples were kept in Radley apparatus with continuous stirring at 70 ºC for 24 h. 1 mL of aliquots was withdrawn at 0, 6, 12, and 24 h. The samples were diluted with water and analyzed by the HPLC system.

Oxidative degradation

The analyte sample equivalent to 10mg of trimethobenzamide was weighed and dissolved in 10mL of 10% H₂O₂ solution. These degradation samples were kept in dark area without disturbance at room temperature for 24 h. 1mL aliquots were withdrawn at 0, 6, 12 and 24 h. These samples were diluted and analyzed by the HPLC system.

Thermal degradation

The analyte sample equivalent to 10 mg of trimethobenzamide was weighed on a petri dish and kept in a hot air oven at 70 ºC for 3 days. Sampling was done at multiple time points. Samples were dissolved in methanol, diluted 10 times and analyzed by the HPLC system.

Photodegradation

An amount of 10mg trimethobenzamide was uniformly spread in a Petri dish and was exposed to direct sunlight for 24h. Sampling was done at multiple time points and analyzed by the HPLC system.

Method validation

The final optimized chromatographic analytical method was validated as per the International Conference on Harmonization (ICH) Q2(R1) guidelines for system suitability, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness (Araujo, 2009; Bhaumik et al., 2010; Bliesner, 2006; Gousuddin et al., 2017; ICH, 2005). The standard stock solution was prepared by dissolving the drug equivalent to 1mg of trimethobenzamide in 10 mL of methanol to a final concentration of 100 μg/mL.

Linearity

Standard calibration curves were generated with seven different concentrations including the lower limit of quantification by making serial volume to volume dilution of stock solution over the range of 0.5-50 μg/mL. Linear calibration curves were generated between peak area and drug concentration. The linearity was examined using linear regression, which was calculated by the least square regression method.

Accuracy

The accuracy of the developed analytical method was analyzed by injecting the analyte solution at three different level of concentrations (1.25 μg/mL, 25 μg/mL and 50 μg/mL) in triplicate. The percentage accuracy of trimethobenzamide at each level and each triplicate were calculated and the mean of percentage accuracy (n=3) and the relative standard deviation (RSD) was determined.

Precision

The precision of the developed analytical method was determined by repeatability (intraday) and intermediate precision (interday) at three different levels (1.25 μg/mL, 25 μg/mL and 50 μg/mL) in triplicate. Repeatability defines the use of the analytical procedure within a laboratory over a short period of time that was examined by assaying the samples during the same day. Intermediate precision was evaluated by comparing the assays on different days.

Limits of detection and quantitation

Limits of detection (LOD) and limit of quantitation (LOQ) were determined from the signal-to-noise ratio. The detection limit was referring to as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantitation limit was referring
to as the lowest concentration level that provided a peak area with a signal-to-noise ratio higher than ten.

**System suitability**

The system suitability was determined by taking six replicates of the drug at the same concentration of 30 μg/mL. The acceptance criteria were ±2% for the percent coefficient of variation (% CV) for the peak area, retention time of drug, theoretical plate count, and asymmetry.

**Robustness**

The robustness is one of the validation parameters that measures method capacity to remain unaffected by small, deliberate changes in chromatographic conditions. The robustness of the developed method was studied by testing the influence of small changes in the organic phase ratio (40-48%), flow rate (0.9 -1.1 mL/min) and pH (5.3-6.3) of the mobile phase.

**RESULT AND DISCUSSION**

**Optimization of the method**

The percent organic concentration, pH and buffer strength was optimized considering the retention time, asymmetry, theoretical plates and peak area of the analyte. The experimental data for optimization of the method has been shown in Table I. In order to understand the effect of different factors on the chromatographic profile of trimethobenzamide, 3D plots were generated after processing all the data using the Design Expert software and the surface response graph has been shown in Figure 1. The figure shows the 3D plot as a function of percent organic concentration, pH and buffer strength. Considering the entire data set, the final optimized mobile phase which gave consistent and robust output consisted of a mixture of methanol and 14mM of ammonium formate buffer of pH 5.8 in a ratio of 44:56 (% v/v). The flow rate of the mobile phase was set at 1.0 mL/min. The detection of the analyte was carried out at 213 nm by PDA detector. The injection volume was 10 μL. Total chromatographic run time was 12 min. To confirm these optimum set of conditions, three replicate injections at 100 μg/mL of the analyte was analyzed to determine whether their observed retention time, area, asymmetry and theoretical plates were within the desired ranges. Representative chromatograms of blank and sample containing trimethobenzamide have been shown in Figure 2.

| Run | Buffer Type | Buffer Strength (mM) | Buffer pH | Organic phase (%) | RT (Min) | Area | Asymmetry | Theoretical plates |
|-----|-------------|----------------------|-----------|-------------------|----------|------|-----------|-------------------|
| 1   | Formate     | 10                   | 5.8       | 47                | 6.44     | 7510059 | 1.07      | 5815              |
| 2   | Formate     | 20                   | 5.8       | 47                | 5.93     | 6157731 | 1.08      | 5750              |
| 3   | Formate     | 10                   | 6.8       | 47                | 8.62     | 8322752 | 1.02      | 6845              |
| 4   | Formate     | 20                   | 6.8       | 47                | 6.30     | 5397765 | 1.11      | 5450              |
| 5   | Formate     | 10                   | 6.3       | 40                | 12.34    | 7587703 | 1.10      | 5553              |
| 6   | Formate     | 20                   | 6.3       | 40                | 10.13    | 5595228 | 1.08      | 5149              |
| 7   | Formate     | 10                   | 6.3       | 54                | 5.30     | 7665004 | 1.05      | 6059              |

(continuing)
TABLE I - Experimental data for optimization of the method

| Run | Buffer Type | Buffer Strength (mM) | Buffer pH | Organic phase (%) | RT (Min) | Area | Asymmetry | Theoretical plates |
|-----|-------------|----------------------|-----------|------------------|----------|------|-----------|-------------------|
| 8   | Formate     | 20                   | 6.3       | 54               | 4.00     | 5734408 | 1.10      | 5414              |
| 9   | Formate     | 15                   | 5.8       | 40               | 11.88    | 7268780 | 1.09      | 5577              |
| 10  | Formate     | 15                   | 6.8       | 40               | 13.77    | 7323710 | 1.08      | 5310              |
| 11  | Formate     | 15                   | 5.8       | 54               | 5.08     | 7290879 | 1.13      | 6279              |
| 12  | Formate     | 15                   | 6.8       | 54               | 5.45     | 7365179 | 1.02      | 6085              |
| 13  | Acetate     | 10                   | 5.8       | 47               | 5.97     | 5249576 | 1.10      | 5116              |
| 14  | Acetate     | 20                   | 5.8       | 47               | 6.77     | 5742867 | 1.10      | 5732              |
| 15  | Acetate     | 10                   | 6.8       | 47               | 7.63     | 5167161 | 1.13      | 4941              |
| 16  | Acetate     | 20                   | 6.8       | 47               | 8.15     | 5903827 | 1.13      | 5951              |
| 17  | Acetate     | 10                   | 6.3       | 40               | 12.09    | 5229590 | 1.06      | 5041              |
| 18  | Acetate     | 20                   | 6.3       | 40               | 13.50    | 5560517 | 1.10      | 6368              |
| 19  | Acetate     | 10                   | 6.3       | 54               | 4.71     | 5456938 | 1.2       | 4959              |
| 20  | Acetate     | 20                   | 6.3       | 54               | 5.27     | 6159278 | 1.11      | 5444              |
| 21  | Acetate     | 15                   | 5.8       | 40               | 10.09    | 5325027 | 1.10      | 5557              |
| 22  | Acetate     | 15                   | 6.8       | 40               | 13.60    | 5340407 | 1.10      | 5517              |
| 23  | Acetate     | 15                   | 5.8       | 54               | 4.22     | 5443829 | 1.10      | 4919              |
| 24  | Acetate     | 15                   | 6.8       | 54               | 4.95     | 5572739 | 1.12      | 5076              |
FIGURE 1 - Surface response graphs of A) Retention time as a function of pH, buffer strength and organic ratio B) Area as a function of pH, buffer strength and organic ratio C) Asymmetry as a function of pH, buffer strength and organic ratio D) Theoretical plates as a function of pH, buffer strength and organic ratio.
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Stress degradation study

Acid hydrolysis

The drug substance was exposed to 5N HCl and kept on Radley apparatus at 70 °C temperature for 8 h. It did not show any degradation in acid hydrolysis (Figure 3A). The blank solution was also subjected to stress study in the same fashion as the drug solution which did not show any peak at the retention time of the drug.

Base hydrolysis

The drug substance was exposed to 5 N NaOH and kept on Radley apparatus at 70 °C temperature for 8h. It showed 53% degradation in base hydrolysis with two degradation products which were labeled as DP-1 and DP-2. The retention time of two degradation products was about 3.01 and 8.20 min, respectively as shown in Figure 3B. The blank solution was also subjected to stress study in the same fashion as the drug solution which did not show any peak at the retention time of the drug or degradation products.

Neutral hydrolysis

The drug substance exposed to water keeping on Radley apparatus at 70 °C temperature for 24 h. It showed no degradation in neutral hydrolysis as shown in Figure 3C. The blank solution was also subjected to stress study in the same fashion as the drug solution which did not show any peak at the retention time of the drug.

Oxidative degradation

The drug was exposed to 10% H₂O₂ at room temperature for 24 h. Samples were withdrawn at different time intervals and injected into the HPLC system and chromatogram was recorded. There was...
47% degradation with one degradation product having retention time 7.49 min as shown in Figure 4A. The blank solution was also subjected to stress study in the same fashion as the drug solution which did not show any peak at the retention time of the drug or degradation product.

**Photodegradation**

The drug sample was exposed to direct sunlight for 24h and sample were withdrawn at different time intervals from 6 to 24h. Samples were injected into the HPLC system and chromatogram was recorded. No degradation was found at the end of 24h of exposure as shown in Figure 4C. Hence the drug can be considered as photostable. The blank solution was also subjected to stress study in the same fashion as the drug solution which did not show any peak at the retention time of the drug. Results of all the degradation studies have been summarized in Table II.

**TABLE II - Summary of degradation study**

| Condition of degradation study | % of drug degraded | Retention time of degradant (min) |
|-------------------------------|--------------------|-----------------------------------|
| 5N HCl, 8h                    | No degradation     | -                                 |
| 5N NaOH, 8h                   | 53%                | 3.01 and 8.20 (DP-1) (DP-2)       |
| Neutral hydrolysis, 24h       | No degradation     | -                                 |
| Oxidative degradation, 24h    | 47%                | 7.49 (P DP-1)                     |
| Thermal degradation, 3 days   | No degradation     | -                                 |
| Photo degradation, 24h        | No degradation     | -                                 |

**Method validation**

**Linearity**

The Linearity of detector response of different concentration of drug was analyzed in the range from 0.5 to 50 μg/mL ($r^2$ =0.999). The regression equation obtained was $Y=120775x$, where $Y$ is peak area and $X$ is a concentration of the analyte (μg/mL). This equation was used to determine the amount of the analyte present in the stability samples.
**Accuracy**

The average intraday accuracy at low (1.25 μg/mL), medium (25 μg/mL) and high (50 μg/mL) level was found to be in the range of 94.22 to 101.18%. The average interday accuracy was found to be 94.03 to 100.63%. The results for intra and interday accuracy study has been summarized in Table III.

**TABLE III** - Intraday and interday accuracy and precision data of the analyte

| Level          | Runs | Mean found (µg/mL) | SD  | %CV  | Accuracy |
|----------------|------|-------------------|-----|------|----------|
| Low (1.25µg/mL)| 1    | 1.26              | 0.01| 0.65 | 101.18   |
|                | 2    | 1.25              | 0.01| 0.94 | 100.08   |
| Medium (25µg/mL)| 1   | 23.55             | 0.07| 0.29 | 94.22    |
|                | 2    | 23.46             | 0.32| 1.37 | 93.84    |
| High (50µg/mL) | 1    | 50.20             | 0.19| 0.39 | 100.39   |
|                | 2    | 50.18             | 0.22| 0.44 | 100.37   |

| Inter-day      | 1.26 | 0.01 | 0.94 | 100.63 |
|----------------|------|------|------|--------|
| Low (1.25µg/mL)| 23.51| 0.21 | 0.91 | 94.03  |
| Medium (25µg/mL)| 50.19| 0.19| 0.37 | 100.38 |

**Precision**

Precision was calculated by studying three replicates of each of the concentration of low level, mid level and high level on different intervals on the same day (intraday) and on a different day (inter-day) and their mean, SD and %RSD was calculated. The RSD was found to be in between 0.29 to 1.37% for intraday and 0.37 to 0.94% for interday precision study. The low RSD value after repeated injections (below 2%) indicated that the developed method was precise. Table III is showing the results of intraday and interday precision study.

**Limit of detection and limit of quantitation**

The limit of detection and limit of quantitation was determined by signal to noise ratio method. It was analyzed at the concentration level of 0.01 µg/mL. The LOD and LOQ obtained were 0.01 and 0.033 µg/mL, respectively.

**System Suitability**

Six replicate injections of at the concentration level of 30 µg/mL of the analyte were injected into the HPLC system and different parameters including retention time, area, theoretical plate and asymmetry were determined for each individual peak. Mean, SD and %RSD were calculated for the six peaks. The RSD value was found to be 0.10% for retention time, 0.91% for peak area, 0.91% for asymmetry of the peak and 0.48% for theoretical plate count. All the RSD values were less than 2%. The result of the system suitability study has been summarized in Table IV.

**TABLE IV** - Result of system suitability study

| Retention time | Area      | Asymmetry | Theoretical plates |
|----------------|-----------|-----------|--------------------|
| 1              | 3499964   | 0.91      | 4325               |
| 2              | 3534443   | 0.91      | 4279               |
| 3              | 3548169   | 0.92      | 4324               |
| 4              | 3588087   | 0.92      | 4293               |
| 5              | 3574268   | 0.9       | 4333               |
| 6              | 3524654   | 0.91      | 4315               |
| Mean           | 3544931.00| 0.91      | 4311.50            |
| SD             | 32486.21  | 0.008     | 21.03              |
| %RSD           | 0.10      | 0.91      | 0.48               |
Robustness

Robustness was performed by changing the conditions like a minor change in pH, flow rate and organic ratio. The %RSD of area and asymmetry after minor flow rate change were 0.43 and 0.87, respectively. After changing the pH, the %RSD value were 1.41 and 0.97 and after changing the organic ratio it was 1.01 and 0.82, respectively. The analytical output was found to be unaffected due to a minor change in the tested chromatographic parameters. Results of the robustness study have been summarized in Table V.

CONCLUSION

A simple, robust and sensitive stability-indicating HPLC assay method has been developed for quantification of trimethobenzamide in presence of their degradation products. The developed analytical method was accurate, precise and robust which is capable to separate and quantify all the degradants in stress samples. Degradation products were found only in basic hydrolysis and oxidative degradation. However, there was no degradation in acid, neutral, photo and thermal degradation. The developed method has been validated according to the ICH guideline. The developed method can be used for analysis of stability samples in routine quality control of trimethobenzamide in pharmaceutical industries. This research work thus can be considered to have a significant contribution to the field of pharmaceutical analysis.

ACKNOWLEDGMENT

The authors acknowledge the financial support of Department of Pharmaceuticals, Ministry of Chemical and Fertilizer, Government of India and NIPER-Ahmedabad. Authors also acknowledge the support of NIPER-Ahmedabad for providing necessary facilities and infrastructures.

CONFLICT OF INTEREST

We do not have any conflict of interest to declare.

| Parameter      | Area          | % RSD | Asymmetry | %RSD |
|----------------|---------------|-------|-----------|------|
|                | Trial 1       | Trial 2 | Trial 1 | Trial 2 |
| Flow rate      | 0.9           | 1218035 | 1223027 | 0.87  | 0.87 |
|                | 1.0           | 1217873 | 1210114 | 0.43  | 0.86 | 0.87 | 0.87 |
|                | 1.1           | 1209249 | 1215879 | 0.86  | 0.86 | 0.85 |
| pH             | 5.3           | 1227131 | 1229872 | 0.92  | 0.93 |
|                | 5.8           | 1217873 | 1210114 | 1.41  | 0.92 | 0.91 | 0.97 |
|                | 6.3           | 1258238 | 1242839 | 0.91  | 0.93 |
| % Organic ratio| 40            | 1211656 | 1205559 | 0.91  | 0.92 |
|                | 44            | 1230115 | 1224745 | 1.01  | 0.9  | 0.91 | 0.82 |
|                | 48            | 1229629 | 1238726 | 0.91  | 0.9  |
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Received for publication on 03rd October 2018
Accepted for publication on 23th October 2018