RP-HPLC ANALYTICAL METHOD FOR THE QUANTITATION OF TOLBUTAMIDE IN FORMULATION VEHICLE: APPLICATION TO HOMOGENECITY AND STABILITY EVALUATION IN PRE-CLINICAL STUDY FORMULATION SAMPLES

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ABSTRACT

A novel, simple, specific, sensitive and reproducible high-performance liquid chromatography (HPLC) assay method has been developed and validated for estimation of tolbutamide. The HPLC method includes use of Diphenhydramine Hydrochloride as an internal standard (IS). The chromatographic analysis was performed using Waters Alliance High Performance Liquid Chromatography system equipped with UV detector and PC based data system with Empower Software. Chromatographic separation was achieved on symmetry sheild, RP 18, 5 µM (Waters) column maintained at 40°C temperature using gradient mobile phase composition with (0.01 M Ammonium Acetate in Milli-Q Water, pH adjusted to 5 using Acetic Acid) and acetonirile at a flow rate of 1.00 mL/min with a total run time of 10 min. The eluate absorbance was monitored using an UV detector set at 258 nm wavelength. Method validation was performed as per ICH guidelines and the results met the acceptance criteria. The calibration curve was linear over a concentration range of 25 to 2508 µg/mL ($r^2 \geq 0.997$). Precision & accuracy batches analysed on 3 different days revealed accuracy of tolbutamide in vehicle within the range of 100 to 114%, 91.5 to 107% and 91.2 to 110%, respectively and Inter day (i.e. within batch) accuracy was 107, 98.9 and 98.7 % at Low, Middle and High QC levels, respectively. The validated HPLC method was...
successfully applied for assay, homogenity and stability determination of preclinical study formulation samples.

**KEYWORDS:** Tolbutamide; HPLC; Method validation; Homogeneity; Pre-clinical Formulation.

**INTRODUCTION**

Diabetes mellitus condition is defined as elevated glucose levels either due to deficiency of insulin in body (Diabetes mellitus type-1) or resistance to insulin by cells (Diabetes mellitus type-2). Anti-diabetic drugs that increase insulin output from pancreas are termed under 'Secretagogues' class. Based on chemical classification Secretagogues drugs can be subdivided into Sulfonylureas, Biguanides, Thiazolidinediones, Alpha-glucosidase inhibitors, Meglitinides and Combination of Sulfonylureas and metformin (biguanide). Tolbutamide is first generation sulfonylurea drug that exerts its pharmacological activity via higher release of insulin from β cells in pancreas. These drugs may induce hypoglycemia (in few cases) due to excess insulin production and release (Bertram G. Katzung (1996)). Major side effects observed with tolbutamide are Hypoglycemia, Weight gain, Hypersensitivity (cross allergicity with sulfonamides) and drug interactions (Increased hypoglycemia with cimetidine, Insulin, Salicylates and Sulfonamides). Salicylates displace tolbutamide from its binding site on plasma binding proteins which lead to increase in free tolbutamide concentrations and thus hypoglycemic shock.

John W. Ho. et.al. (1993) in their publication ‘Determination of tolbutamide hydroxylation in rat liver microsomes by high-performance liquid chromatography: effect of psychoactive drugs on in vitro activity’ discussed HPLC analysis approach for estimation of inhibition of microsomal tolbutamide hydroxylation reaction by psychoactive drugs. Group shared their observations on impact of proper wavelength selection during assay. Tolbutamide and its metabolites have close absorbance maxima and need to be separated chromatographically for proper quantisation strategy adoption during any in-vivo or in-vitro study with possibility of metabolites presence possibilities in samples. Group used liquid-liquid extraction with diethyl ether for sample extraction and UV detection of hydroxytolbutamide at 240 nm wavelength after chromatographic seperation.

Ying Liu et.al. (2009) in their publication ‘A simplified method to determine five cytochrome P450 probe drugs by HPLC in a single run’ discussed HPLC analysis approach for estimation
of five different probe substrates i.e. Caffeine, Chlorzoxazone, Tolbutamide, Metoprolol and Midazolam from rat plasma for evaluation of CYP1A2, 2C9, 2D6, 2E1 and 3A4 activities. Method involved liquid-liquid extraction of rat plasma samples using chloroform and HPLC analysis on RP-C18 column at UV detection wavelength of 230 nm. Researchers concluded limitation of single wavelength used for multiple compounds and specified a possibility on improvements in their next research work.

D. Madhu Latha et.al. (2013) in their publication ‘Development and validation of RP-HPLC method for quantitative analysis tolbutamide in pure and pharmaceutical formulations’ discussed the HPLC analysis approach for estimation of tolbutamide involving dilution of samples with mobile phase (Methanol: 0.1% Orthophosphoric acid: Acetonitrile :: 10:30:60) and chromatographic separation on Zodiac C18 (250 X 4.6 mm, 5 µ) with UV detection 231 nm wavelength.

In present research, we demonstrate a analytical strategy for quantisation of tolbutamide in formulation samples with increased sensitivity i.e. 100 ng on column load and extended linearity range. Further the method was validated as per regulatory guidelines recommendations and employed for evaluation of experiments related to stability and homogeneity assessment studies.

MATERIALS AND METHODS

Chemicals and reagents: Tolbutamide and Diphenhydramine Hydrochloride was procured from Sigma Aldrich, St. Louis, USA. HPLC grade acetonitrile were purchased from JT Baker and Ammonium Acetate from Sigma Aldrich, St. Louis, USA. Acetic Acid, AR Grade was procured from Rankem chemicals, Ranbaxy Fine Chemicals Limited, New Delhi, India. All other chemicals/reagents were of research grade and used without further purification.

HPLC operating conditions: The HPLC system used was Waters 2695 Alliance system (Waters, Milford, USA) equipped with performance PLUS inline degasser along with an auto-sampler was used to inject 20 µL aliquots of the processed samples on a HPLC Column: Symmetry shield, RP 18, 5 µM (Waters) which was maintained at controlled elevated temperature (40 ± 1°C). An gradient phase comprising Acetonitrile: Ammonium Acetate, (pH 5.0) (v/v) delivered at a flow-rate of 1 mL/min was used for attaining chromatographic resolution between Tolbutamide and IS.
Preparation of stock and CC/QC solutions

Standard blank samples were spiked 10 µL of water into 90 µL blank formulation vehicle and vortexed to mix thoroughly. Standard zero samples were prepared by spiking 10 µL of water into 90 µL blank formulation vehicle and vortexed to mix thoroughly. Calibration curve standards and Quality control samples were prepared by spiking 10 µL of working solution into 90 µL of blank formulation vehicle and vortex to mix thoroughly. To these sample, 400 µL of IS working solution was added and mixed thoroughly by vortexing. All the above samples were centrifuged at 14000 RPM for 5 minutes. Finally about 200 µL supernatant was transferred into inserts kept in pre-labeled 1 ml HPLC glass vials and caped with polyethylene plugs/caps.

Sample preparation: The test item for each dose were weighed on separate butter papers, and carefully transferred into a 10 mL measuring cylinder. Small aliquot of the vehicle was added to dissolve the test substance and final volume was made up to the mark (10 mL) with the vehicle to get the desired concentration (40 & 160 mg/mL). These dose formulations were handled at room temperature (20 - 24°C) during preparation and storage.

Validation procedures: A full validation according to the OECD Principles of Good Laboratory Practice for the testing of chemicals as specified by International [C (97) 186/Final] Legislation.ICH Guidelines: Validation of analytical procedures: Text and methodology Q2 (R1) and Australian pesticides and veterinary medicines authority guidelines for the validation of analytical methods for active constituents, agricultural and veterinary chemical products, October 2004 was performed.

System suitability

System suitability test(s) was performed on three different days prior to each day of method validation at the medium QC concentration level. Analytical batch of system suitability was consisting of six injections of medium QC level in diluent solution. Samples were prepared

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<tr>
<th>Time (min.)</th>
<th>Solvent Flow Rate (mL/min)</th>
<th>Mobile Phase A (%) Ammonium acetate pH 5</th>
<th>Mobile Phase B (%) Acetonitrile</th>
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by spiking 10 µL of MQC working solution into 490 µL IS working solution and vortex to mix thoroughly and analysed using HPLC.

**Selectivity**
Selectivity was performed with six different batches (prepared separately) of formulation vehicles and standard concentration spiked at LLOQ level in each lot separately. Samples were prepared by spiking 10 µL each of Milli-Q water (as blank) and LLOQ working solution into 90 µL of formulation vehicle. 400 µL of IS working solution was added to each sample and vortexed, centrifuged and analysed using HPLC.

**Autosampler carryover test**
Mobile phase was directly injected and samples of LLOQ and ULOQ concentration levels were prepared by spiking 10 µL each of LLOQ and ULOQ working solution into 490 µL IS working solution, respectively and vortex to mix thoroughly and analysed using HPLC.

**Linearity of calibration standards**
Linearity of calibration standards were plotted out on three different days (three times along with P&A batches on three different days). A standard curve was comprising of 8 non-zero standards including lowest and highest concentration in duplicate (excluding Standard Blank and Standard Zero). Calibration standards for analysis were prepared by spiking 10 µL of respective working solution into 90 µL of formulation vehicle, processed accordingly and analysed using HPLC conditions specified earlier.

**Precision and accuracy (P&A)**
Precision and accuracy was determined on three different days following injection of standard blank, zero standard blank and 8 non zero calibration standard with duplication of LLOQ and ULOQ injection. The precision and accuracy samples were comprising six sets each of LQC, MQC and HQC concentration level samples representing the entire standard curve range.

**Fortification Levels of Test substance**
Homogenicity, Bench-top and long term stability tests for dose formulation samples were carried out at the following concentrations of tolbutamide in the vehicle:

- 40 mg/mL (Low Dose)
- 160 mg/mL (High Dose)
Preparation of Dose Formulations
The test item for each dose were weighed on separate butter papers, and carefully transferred into mortar. Compound was wetted with vehicle to make a paste with pestle and the volume was made up with the vehicle to get the desired concentration of 40 and 160 mg/mL.

Homogenicity
A volume of 0.5 mL of dose formulation was withdrawn from top, middle and bottom level in duplicates using measuring cylinder with the help of micropipette in 1.5mL eppendorf tube and was used for the estimation of homogenicity. On each occasion of sampling, each dose formulation was mixed well before sampling. One sample from vehicle and six samples from each dose level (low and high) was drawn and analysed for the tolbutamide concentration.

Samples were prepared by spiking 100 µL of Vehicle, 2 aliquot from top layer, 2 aliquot from the middle layer and 2 aliquot from the bottom layer into 400 µL IS working solution, respectively and vortex to mix thoroughly, centrifuged and analysed using HPLC.

Stability
Stability of test substance in formulation vehicle was determined at different time intervals (short term/bench top stability at 0, 1, 2, 4 and 6 hr and long term stability at day-4 and day-8). Stability of test substance was evaluated in formulation vehicle at 40 and 160 mg/mL concentrations. The stability samples were analyzed using a freshly spiked calibration curve and quality control samples (2 replicates of LQC, MQC and HQC). Six replicates of stability samples were analyzed per concentration and mean back-calculated concentrations of stability samples was compared against mean of first day (initially observed) concentrations for stability evaluation. On each occasion of sampling, each dose formulation was mixed well before sampling.

Processing procedure: 100 µL each of six replicates of stability samples were spiked into 400 µL IS working solution respectively processed accordingly and analysed using HPLC.

Bench-top stability and Long-term stability: Stability samples was kept at ambient temperature and an aliquot was subjected for analysis after completion of stability duration i.e. 0,1, 2, 4 and 6 hrs. Samples were processed and stored in refrigerated condition till
analysis. For long term stability samples were kept at ambient temperature and an aliquot was subjected for analysis after completion of stability duration i.e. 4 and 8 days.

RESULTS AND DISCUSSION

System Suitability and Selectivity: The % CV of peak area ratio of six consecutive injections on three different days was in the range of 0.04 to 0.11, which met the acceptance criteria (i.e. % CV of Peak Area Ratio should be less than or equal to 5%). The % CV of retention time of Tolbutamide and Diphenhydramine (IS) on three different days was in the range of 0.03 to 0.07 and 0.04 to 0.11, respectively which met the acceptance criteria (i.e., % CV of retention time of analyte and internal standard should be less than or equal to 2% of the mean retention time of the analyte and internal standard). Thus, it is concluded that the system was suitable for the analysis.

There was no interference observed at the retention time of tolbutamide and internal standard in all six replicates of formulation vehicle batches (blanks) vs standards spiked at LLOQ concentration analysed. The results met the acceptance criteria (i.e. response of interfering peak(s) at the retention time of the Tolbutamide and internal standard peak should be ≤ 20% & ≤ 5 % respectively, to the corresponding LLOQ standard). These results suggest the competence of the method to differentiate and measure Tolbutamide and internal standard in presence of other constituents of matrix in the sample.

Linearity of Calibration Standards

Linearity of calibration standards analysed along with three different precision & accuracy batches revealed accuracy of all the standard curve points were in the range of 96.2 to 103 %, 96.4 to 108 % and 96.3 to 103 % respectively of three different batches which met acceptance criteria (i.e.75% of standards must have accuracy within or equal to 85 to 115% of theoretical and 80 to 120% of theoretical for the LLOQ). The correlation coefficient (r²) of calibration plots was greater than 0.997 for all batches, which met the acceptance criteria (i.e. correlation coefficient (r²) value for standard curve should be ≥ 0.98).

Within-run (Intra-Day): Precision & accuracy batches analyzed on 3 different days revealed accuracy of tolbutamide in formulation vehicle was in range of 100 to 103 %, 110 to 114 % and 103 to 114 %, respectively at LQC level, 91.5 to 96.5 %, 102 to 107% and 92.9 to 106 %, respectively at MQC level and 91.2% to 97.6%, 99.0 to 106 % and 93.5 to 110 %, respectively at HQC level, which met the acceptance criteria (i.e. at least 67% of the total QC samples and at least 50% of QC samples at each level should have back-calculated
concentration values within ±15% of the nominal concentrations and 75% of standards must have accuracy within or equal to 85 to 115% of theoretical.

Precision & accuracy batches analyzed on 3 different days have precision (%CV) of 0.99, 1.30 and 4.32 %, respectively at LQC level, 2.20, 1.38 and 5.80 %, respectively at MQC level and 2.52, 2.55 and 6.35 %, respectively at HQC level, which met the acceptance criteria (i.e., %CV for low, medium and high QC concentrations should not exceed 15%).

**Between-run (Inter-Day):** Inter day mean accuracy was 107, 98.9 and 98.7 at LQC, MQC & HQC levels, respectively. Inter day mean precision (%CV) was 4.32, 5.51 and 4.90 at LQC, MQC & HQC levels, respectively which met the acceptance criteria (i.e., mean accuracy should be within ±15% of the nominal concentration at LQC, MQC and HQC and %CV for low, medium and high QC concentrations should not exceed 15%).

**Homogeneity**

Linearity of calibration standards analyzed along with homogeneity samples revealed accuracy of all the standard curve points were in the range of 94.9 to 109 % which met acceptance criteria (i.e., 75% of standards must have accuracy within or equal to 85 to 115% of theoretical and 80 to 120% of theoretical for the LLOQ). The correlation coefficient (r²) of calibration plots was 0.994, which met the acceptance criteria (i.e., correlation coefficient (r²) value for standard curve should be ≥ 0.98). Quality control samples analyzed with homogeneity samples were having accuracy in the range of 93.5 to 106 % at LQC level, 88.2 to 101 % at MQC level and 101 to 105 % at HQC level, which met the acceptance criteria (i.e., at least 67% of the total QC samples and at least 50% of QC samples at each level should have back-calculated concentration values within ±15% of the nominal concentrations and 75% of standards must have accuracy within or equal to 85 to 115% of theoretical). Accuracy of dose formulation samples (2 replicates from top layer, 2 replicates from the middle layer and 2 replicates from the bottom layer of each dose formulation) were found to be in range of 105 to 107 % at 40 mg/mL concentration and 99.3 to 105 % at 160 mg/mL concentration which met the acceptance criteria (i.e., back-calculated concentration values should be within ±10% of the nominal concentrations). The % CV of 40 & 160 mg/mL of dose formulation was found to be 1.43 & 0.65, respectively, which met the acceptance criteria (i.e., %CV of accepted samples should be less than or equal to 10%). Dose formulation of test substance in formulation vehicle was found to be homogenous at 40 & 160 mg/mL concentration.
Stability
Linearity of calibration standards analysed along with Bench-top, Day-4 & 8 stability samples revealed accuracy of all the standard curve points were in the range of 92.9 to 105 % and 93.1 to 105 % respectively, which met acceptance criteria (i.e., 75% of standards must have accuracy within or equal to 85 to 115% of theoretical and 80 to 120% of theoretical for the LLOQ). The correlation coefficient ($r^2$) of calibration plots was greater than 0.998 in all batches.

Accuracy for stability control samples analysed with Day-4 & 8 stability samples were having accuracy in the range of 86.1 to 91.8 % (day-4) & 92.8 to 113% (day-8) at LQC level, 89.0 to 91.9 % (day-4) & 96.0 to 114 % (day-8) at MQC level and 85.3 to 85.7 % (day-4) & 107 to 109 % (day-8) at HQC level, which met the acceptance criteria.

Bench-top stability of dose formulation samples at 40 & 160 mg/mL concentration were found to be stable on bench top at ambient temperature upto 6 hr. The % change of 40 & 160 mg/mL dose formulation was found 0.12 and 0.03 at 1hr, 0.27 and 0.22 at 2hr, 0.29 and 0.25 at 4hr and 0.38 and 0.24 at 6hr, respectively.

Long-term stability of dose formulation 40 & 160 mg/mL samples was found to be stable for 8 days at refrigerated condition (6 ± 2 °C temperature). The % change of 40 & 160 mg/mL dose formulation was found -3.93 and -4.33 on day-4 and -3.68 and -4.26 on day-8 respectively.

FIGURES

Fig. 1: Blank formulation vehicle with without IS
Fig. 2: Blank formulation vehicle with IS

Fig. 3: Calibration standard level-5 spiked in formulation media with IS

Fig. 4: Calibration curve for IS response in standards
CONCLUSION

Dose formulation of tolbutamide was found to be stable at refrigerated temperature for 8 days at 40 & 160 mg/mL concentration during long-term stability evaluation. Also formulation was found to be homogeneous and stable at ambient temperature condition for 6 hours. Considering limited number of publications on analytical strategies for tolbutamide, our research can provide a firm standpoint in terms of initial development and pharmacokinetic studies for tolbutamide. Analytical method proposed showed very consistent and accurate results in studies conducted for evaluation of method performance.

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