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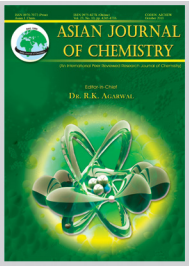

# Method Development and Validation of Topotecan Hydrochloride in $K_2$ EDTA Human Plasma by Using HPLC Coupled with Tandem Mass Spectrometry

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A rapid, sensitive and specific method based on high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) detection with electro spray ionization in positive ionization mode has been developed for the determination of topotecan hydrochloride in $K_2$ EDTA Human plasma. Sample preparation involved protein precipitation extraction with 0.1 % acetic acid in cold acetonitrile. chromatographic separation was performed on Agilent Eclipse XDB column ($C_{18}$ 150 × 4.6 mm 5µ), with a mobile phase consisting of 0.1 % acetic acid in acetonitrile and 0.5 % acetic acid in water at a flow rate of 0.7 mL/min. The analytes were then detected by monitoring the transitions m/z 422.2-219.4, 377.0 for topotecan and m/z 587.6-124.2 for irinotecan using API-4000 LC-MS/MS system (AB Sciex). Calibration curve were linear within the range of 0.5 to 50.00 ng/mL. The lower limit of quantitation was 0.5 ng/mL. The method has been fully validated in $K_2$EDTA Human plasma. This method can be successfully applied to the pharmacokinetic study sample analysis.

**Key Words: Topotecan hydrochloride, LC-MS/MS, $K_2$EDTA Human Plasma.**

## INTRODUCTION

Topotecan hydrochloride is designated chemically as (S)-10-[(dimethylamino)methyl]-4-ethyl-4,9-dihydroxy-1*H*-pyrano[3',4':6,7]indolizino[1,2-b]quinoline-3,14(4*H*,12*H*)-dione mono hydrochloride. Topotecan hydrochloride salt of a semi synthetic derivative of camptothecin with antineoplastic activity. During the S phase of the cell cycle, topotecan selectively stabilizes topoisomerase I-DNA covalent complexes, inhibiting religation of topoisomerase I-mediated single-strand DNA breaks and producing potentially lethal double-strand DNA breaks when complexes are encountered by the DNA replication machinery. Camptothecin is a cytotoxic quinoline-based alkaloid extracted from the Asian tree *Camptotheca acuminata*[1].

Few methods have been developed and validated for detection and estimation of Topotecan hydrochloride in biological media using LC-MS/MS[2] and a sensitive fluorescent HPLC method in whole blood[3]. Presently available high performance liquid chromatography (HPLC), LC-MS/MS methods lack sensitivity, more plasma volume and required more time for analysis. The current methodology describes sensitive LC-MS/MS method with an LLOQ of 0.5 ng/mL in 100 µL of plasma volume for measuring topotecan hydrochloride and irinotecan hydrochloride is used as an internal standard. At the same time, method is efficient in analyzing large number of plasma samples obtained for pharmacokinetic studies after therapeutic doses of topotecan hydrochloride.

## EXPERIMENTAL

A reference standard topotecan hydrochloride hydrate, irinotecan hydrochloride internal standard was purchased from Sigma Aldrich India. HPLC grade deionized water, acetonitrile was obtained from J.T. Baker. Formic acid, ammonium acetate was obtained from Sigma.

**Instrumentation and chromatographic conditions:** High performance liquid chromatography coupled with Tandem mass spectrometry was used for the method development and validation. Mass spectrometry model API-4000, HPLC model Agilent 1200 series equipped with binary pump, auto sampler was used to keep temperature 5 ºC, column pven temperature 40 ºC detection was made at 219.4, 377.0 m/z for topotecan hydrochloride and 124.2 for irinotecan hydrochloride using ESI positive mode ion spray ionization, Analyst 1.4 software was used for quantification. The stationary phase was Agilent eclipse XDB column ($C_{18}$ 150 × 4.6 mm 5µ).

**Optimization of LC MS method:** The LCMS method was optimized with a view to develop and estimate topotecan hydrochloride in plasma. Which took into account the complex chemical behaviour of topotecan related to the lactone opening and the keto-enol tautomerism[4-7]. The standard stock solution was diluted to a concentration of 10 ng/mL, and tested at different pH starting from 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 in 2 mM ammonium acetate Buffer pH adjusted by acetic acid by injecting into RP $C_{18}$ column. Different ratio of 0.1 % acetic

acid acetonitrile and water with 0.5 % acetic acid. The optimal ratio of mobile phase was found to be a gradient ratio 0.5 % water and 0.1 % acetonitrile (20 to 85 % v/v organic) with a total run time of 5 min. The separation was carried out at 40 ºC column temperature with a flow rate of 0.7 mL. The injection volume was 5 µL. The run time of analyte and internal standard was 1.50 and 2.58 min.

**Preparation of standard and quality control samples:** Stock solutions of topotecan hydrochloride were made up in 2 mM ammonium acetate buffer pH 4.5 adjusted by acetic acid (dilution solution) at approximately 1 mg/mL concentration under safety enclosure and these solutions were refrigerated and protected from light. Working solutions of varying concentrations of topotecan hydrochloride were prepared on the day of analysis by diluting the stocks with dilution solution. Every day before extraction the calibration curve in stabilized human plasma was prepared by spiking known amounts of topotecan hydrochloride into $K_2$ EDTA human plasma (100 µL) and internal standard (25 µL).

Calibration curve standards consisting of a set of eight non-zero concentrations ranging from 0.5 ng/mL to 50.0 ng/mL for topotecan hydrochloride were prepared. Prepared quality control samples consisted of topotecan hydrochloride concentrations of 0.5 ng/mL (LLOQ), 0.15 ng/mL (LQC), 20.0 ng/mL (MQC), 35 ng/mL (HQC) and 50.0 ng/mL (ULOQ) these samples were stored below -20 ºC. Twelve sets of LQC and HQC were stored to below -20 ºC freezer to generate stability.

**Preparation of internal standard stock solution:** Stock solutions of irinotecan hydrochloride were made up in 2 mM ammonium acetate buffer pH 4.5 adjusted by acetic acid (dilution solution) at approximately 1 mg/mL concentration under safety enclosure and these solutions were refrigerated and protected from light. Appropriate dilutions were made with dilution solution to make a IS working dilution of 30 ng/mL.

**Preparation of mobile phase, dilution and washing solution:** The mobile phase aqueous (water with 0.5 % acetic acid) and organic (0.1 % acetic acid acetonitrile) were prepared. The dilution solution was prepared with 2 mM ammonium acetate Buffer pH 4.5 adjusted by acetic acid. The washing solution was prepared by mixing in the ratio of (20:30:50 v/v) isoproponol:methanol:water.

**Sample extraction:** Before extraction bulk spiked CC and quality control samples, were removed from the deep freezer and thawed in ice bath. Calibration and quality control samples were then made ready for extraction in 2 mL polypropylene tubes. Exactly 100 µL of spiked plasma was pipette out into prelabelled polypropylene tubes, to this 25 µL of internal standard dilution (30 ng/mL) was added and vortexed for 10 secs. The spiked plasma samples were protein crashed with1.0 mL of 0.1 % acetic acid in cold acetonitrile and these samples were centrifuged at 14000 rpm and 1 mL of supernatant was transferred by pipette to another 2 mL polypropylene prelabeled tube and these samples were dried under nitrogen evaporator at 40 ºC till dryness. These dried samples were reconstituted with recon solution (90:10 water: acetonitrile 0.1 % acetic acid) of 100 µL. And these samples were transferred to auto sampler vial and 5 µL was injected into the chromatographic system.

**Method validation parameters:** The validation is performed as per US FDA and ANVISA guidelines[8,9]. The optimized LCMS method was validated with respect to the following parameters:

**System suitability:** System suitability was performed before starting each day's activity

**Specificity/selectivity:** Specificity and selectivity was performed in six different lots of plasma having $K_2$ EDTA as anticoagulant.

**Matrix effect:** Blank plasma from six independent sources of matrix were processed and then spiked with an analyte at LQC level and an internal standard at the concentration used in the method being validated just before injection in to the LC-MS/MS. An aqueous solution of analyte was prepared at LQC level with internal standard in Recon solution.

**Carry over:** Processed and injected two blank, two LLOQ, two ULOQ samples followed by re-injection of first two blank samples.

**Linearity:** A regression equation with a weighting factor of $1/X^2$ of analyte to internal standard concentration was judged to produce the best fit for the concentration -detector response relationship for topotecan hydrochloride in human plasma.

**Precision and accuracy:** The precision of the assay was measured by the percent coefficient of variation over the concentration range of LLOQ, LQC, MQC and HQC samples respectively during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ, low, middle and high quality control samples to their respective nominal values, expressed in percentage.

**Recovery:** Six sets of aqueous recovery comparison samples were prepared and injected. The recovery comparison samples of topotecan were compared against extracted samples of LQC, MQC and HQC samples.

**Dilution integrity:** Six sets of dilution integrity samples (approximately 3 times of highest standard concentration) were processed by diluting them twice and another six sets by diluting them four times using pooled plasma. These quality control samples were analyzed along with a freshly spiked and processed calibration curve standards. The quality control sample concentrations were calculated using appropriate dilution factor.

## RESULTS AND DISCUSSION

The method was validated in terms of limit of quantification, recovery, selectivity, precision, accuracy and stability.

**Specificity/selectivity:** No significant interference was observed at the RT and m/z of analyte and internal standard in all the batches screened.

**Carry over:** The % carry over was found to be 0.00 for analyte and 0.00 for internal standard.

**Linearity:** The method was validated over the range of 0.50 to 50.0 ng/mL. The correlation coefficient found to be greater than 0.9996.

**Recovery:** The recovery was determined by comparing the aqueous solution and the spiked analyte. The percentage recovery of the drug and the internal standard was calculated and it was 49.50 % and 72.0 % respectively.

**Precision and accuracy:** For the results of within-batch precision and accuracy and between batch precision and accuracy. Refer Table-1. Represents the chromatogram of LLOQ

and internal standard (Figs.1-4) and a typical chromatogram of plasma blank (Fig. 2). (Fig. 3) represents the chromatogram of upper limit of quantization.

TABLE-1
PRECISION AND ACCURACY

| Experiment | | QC level |
|---|---|---|
| Within batch precision | PA1 | 1.6 % - 3.6 % (LQC-HQC) |
| | PA2 | 2.1 % - 3.2 % (LQC-HQC) |
| | PA3 | 1.8 % - 4.0 % (LQC-HQC) |
| Within batch accuracy | PA1 | 100.9 % - 105.3 % (LQC-HQC) |
| | PA2 | 101.1 % - 106.8 % (LQC-HQC)) |
| | PA3 | 101.5 % - 106.5 % (LQC-HQC) |
| Between batch precision | - | 2.4 % - 3.8 % (LQC-HQC) |
| Between batch accuracy | - | 101.2 % - 104.7 % (LQC-HQC) |

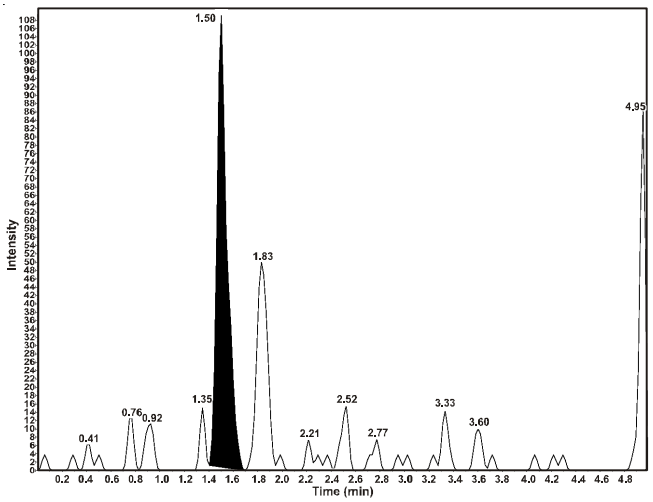


Fig. 1. Representative chromatogram of lower limit of quantization -LLOQ (0.5 ng/mL)

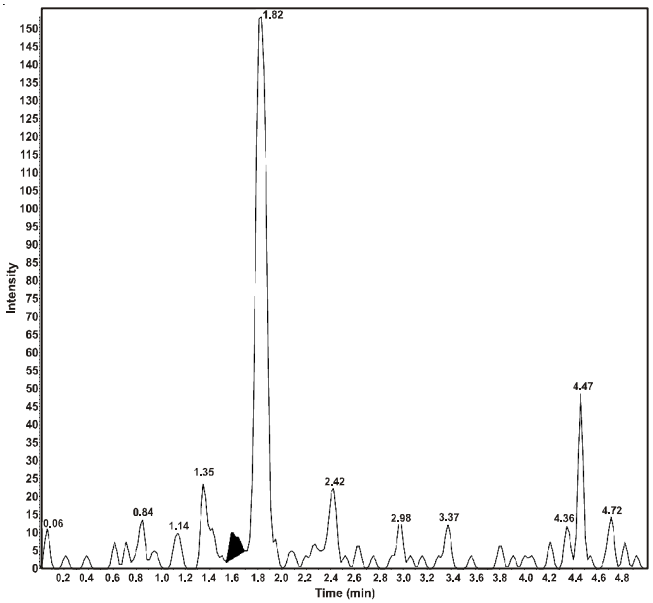


Fig. 2. Representative chromatogram of plasma blank

## Stabilities

**Bench top stability:**Bench top stability was determined for 4 hours, using six sets each of LQC and HQC. The quality control samples were quantified against the freshly spiked calibration curve standards (Table-2).

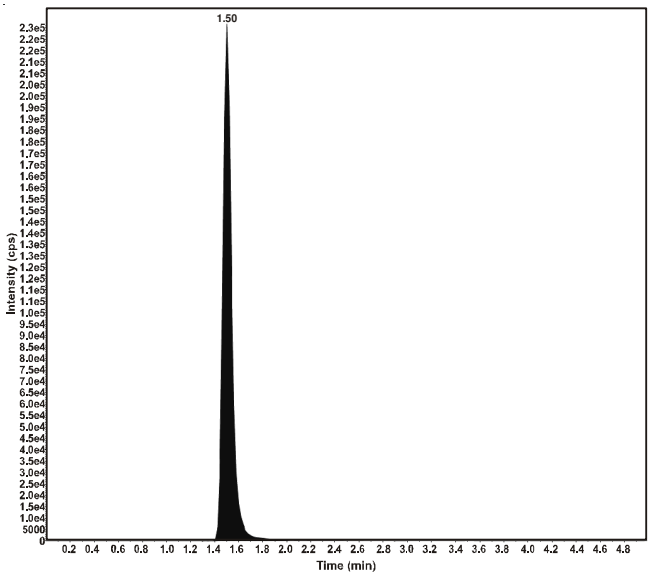


Fig. 3. Representative chromatogram of upper lower limit of quantization -ULOQ (50 ng/mL)

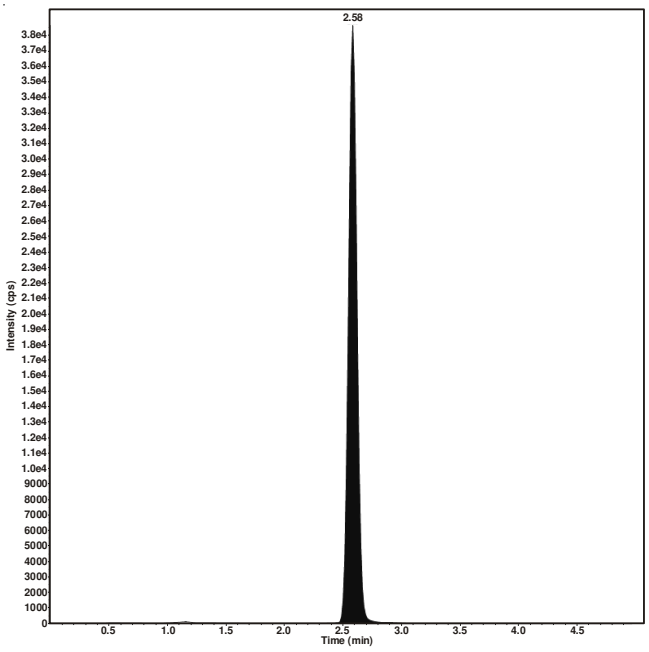


Fig. 4. Representative chromatogram of internal standard-IS

TABLE-2
STABILITY DATA

| Experiment | QC level | Change (%) | Stability duration |
|---|---|---|---|
| Bench top | LQC | -5.5 | 4 h |
| | HQC | -7.0 | |
| Freeze thaw | LQC | -5.3 | 3 Cycles |
| | HQC | -9.7 | |
| Long term stability-20°C | LQC | -10.9 | 15 Days |
| | HQC | -9.5 | |
| Auto sample stability | LQC | -4.0 | 24 h |
| | HQC | -1.5 | |
| Re-injection reproducibility | LQC | -1.8 | 24 h |
| | HQC | 0.9 | |

**Freeze-thaw stability three cycles:** The stability in human plasma was determined for three freeze-thaw cycles. Six replicates of LQC and HQC were analyzed after undergoing

three freeze-thaw cycles. The freeze-thaw quality control samples were quantified against the freshly spiked calibration curve standards (Table-2).

**Long term stability at below -20 ºC:** Six replicates of LQC and HQC were stored below -20 ºC in the freezer and deep freezer respectively for 15 days. These samples were quantified against the freshly spiked calibration curve standards. The stability of the analytes was evaluated by comparing each of the back calculated concentrations of stability QCs with the mean concentrations of the respective QCs analyzed in the first accepted precision and accuracy batch (PA-1) (Table-2).

**Auto sample stability:** Six replicates of LQC and HQC were analyzed and stored in auto sampler to prove stability. These samples were injected after a period of 24 h and were quantified against freshly spiked calibration curve standards (Table-2).

**Re-injection reproducibility:** Six replicates of LQC and HQC of the precision and accuracy batch PA-3 were retained in the auto sampler at 5 ºC for 24 h to test the re-injection reproducibility of the method. Reinjection reproducibility concentrations against the PA-3 batch concentrations (Table-2).

## Conclusion

The result showed that the analytical method was accurate, as the accuracy of quality controls were within the acceptance limits of 100 ± 15 % at their respective concentration levels. The precision around the mean value was never greater than 15 % at any of the concentrations studied. The stability studies demonstrated that Topotecan was stable during normal assay procedures and in long-term frozen storage conditions (below -20 ºC). This should allow clinical samples to be stored and analyzed efficiently. The main advantage is improved sensitivity that allows the quantification of 0.50 ng/mL of Topotecan in plasma samples.

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