RAPID QUANTIFICATION OF IRINOTECAN IN RAT PLASMA BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY-APPLICATION TO PRECLINICAL PHARMACOKINETICS

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Summary

A simple rapid and robust method for quantification of irinotecan in rat plasma has been established using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS). Imipramine was used as an internal standard. Irinotecan and internal standard in plasma sample was extracted using simple liquid-liquid extraction. The samples were injected in to a C_{18} reversed-phase column and mobile phase used was acetonitrile – 0.1% formic acid in water (90:10, %v/v) at a flow rate of 0.5 ml/min. using MS/MS in the selected reaction-monitoring (SRM) mode, Irinotecan and Imipramine were detected without any interference from rat plasma matrix. Irinotecan produced a precursor ion ($[M+H]^+$) at m/z 587.20 and a corresponding product ion at m/z 167.01 The internal standard produced a precursor ion ($[M+H]^+$) at m/z 281.20 and a corresponding product ion at m/z 86.19. Detection of Irinotecan in rat plasma by the LC-ESI/MS/MS method was accurate and precise with a quantitation limit of 10ng/ml. The proposed method was validated with linear range of 10 - 4000 ng/ml. Reproducibility, recovery and stability of the method were evaluated. The method has been successfully applied to preclinical pharmacokinetic study of Irinotecan liposome

Keywords: Irinotecan, liquid chromatography, mass spectrometry, rat plasma.

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Introduction

The antineoplastic agent irinotecan hydrochloride (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy-camptothecin (CPT-11) is a semisynthetic derivative of the natural product camptothecin[1,2]. This drug has demonstrated good antitumor activity both in vitro and in vivo against various experimental tumor models[1,3], including multidrug-resistant lines[4]. CPT-11 has shown anticancer activity against a variety of solid tumors in clinical trials, including colorectal cancer, gynecologic cancers, non-small cell and small cell lung cancers, and refractory cervical cancer[5-11]. Several HPLC methods have been developed to quantify irinotecan and its major metabolites in human plasma[12-21]. These methods involve the simultaneous quantification of the lactone and carboxylate forms or quantification of the total forms, i.e., lactone plus carboxylate. In most of these methods, both CPT-11 and SN-38 were quantified and the related compound camptothecin was used as the internal standard. In some of these published methods, chromatography was carried out with fluorescence detection. Most reported approaches involve long run time and tedious sample preparation procedures, Liquid chromatography–mass spectrometry methods have also been described[22-24] to support preclinical and clinical studies of CPT-11. Most of these HPLC and LCMS methods have been reported in human, monkey and mouse plasma and also in urine, tissue homogenates and feces.

The preclinical studies are the preliminary studies for novel drug delivery systems like liposomes, microspheres and nanoparticles. Although several published methods are available for clinical pharmacokinetic evaluation, a small number of reports are available for IRI in rat models. A sensitive, simple and reliable method with wide calibration range is a prerequisite for pharmacokinetic and toxicokinetic evaluation of IRI novel drug delivery systems. Here we describe a sensitive LC/MS/MS method to quantify irinotecan in rat plasma. The sample preparation is simple liquid-liquid extraction. In the present study, between-run and within-run precision and accuracy of the assay were evaluated, as were the effects of plasma freeze-thaw cycles and dilution. Short-term room temperature stability and long-term storage stability were also studied. The advantages of this method are less sample volume and shorter run time with wide calibration range. The method was validated as per the US-FDA guidelines[25]. This method was used for pharmacokinetic evaluation of IRI liposomes in rat.

Materials and Methods

Materials and reagents

Irinotecan hydrochloride trihydrate was procured from Varda Biotech Lab (Mumbai, India) and Imipramine hydrochloride was obtained from Sigma Aldrich (USA). Methanol (super gradient grade), tertiary butyl methyl ether and dichloromethane (HPLC grade) were purchased from Labscan (Thailand). Formic acid was purchased from Merck (Germany). Disodium ethylenediamine tetra-acetic acid (EDTA) vacutainers (USA) were used for collecting blood samples from animals. Milli-Q water purification system supplied by Millipore (USA) was used for the preparation of the aqueous mobile phase. Drug free rat plasma samples were obtained from central animal house, Manipal University, Manipal and stored at –70° C prior to use.

Equipment
LC-MS/MS analysis was performed using Surveyor HPLC system coupled with TSQ Quantum discovery max, a triple stage quadrupole LC-MS/MS instrument equipped with an electrospray ionization (Thermofinnigan Ltd., USA). LC Quan software version 2.5.6 was used for quantitation. The HPLC column used was Genesis C_{18} (100×4.6 mm, 5µ particle size) purchased from Grave Vydac (Runcom, UK).

Chromatographic conditions
The analytes were chromatographically separated using reverse-phase high-performance liquid chromatography (HPLC) with isocratic elution. The mobile phase consisted of methanol – formic acid in water (0.1%) (90:10%, v/v) at a flow rate of 0.5 mL min-1. The separation was carried out at room temperature on Genesis C18 (100×4.6 mm, 5µ particle size) analytical column and eluent was monitored at selected reaction monitoring mode.

Preparation of calibration standard and quality control (QC) samples
The Main stock solution of IRI and IS were prepared in methanol at free base concentration of 1000.0 µg mL^{-1}. Intermediate stock solution was prepared from main stock solutions by dilution with water: methanol (50:50, v/v). This intermediate stock solution was used to prepare the spiking stock solutions for calibration curve and quality control samples. Blank rat plasma was screened prior to spiking to ensure it was free of endogenous interference at retention times of IRI and IS. A nine point standard curve and quality control samples of IRI was prepared by spiking the blank plasma with 5 % v/v of IRI. The calibration curve ranged from 10.0 to 4000.0 ng mL^{-1} and quality control samples were prepared at three concentration levels of 40.0, 2000.0 and 3500.0 ng mL^{-1}.

Sample preparation
A 95.0 µL aliquot of plasma containing IRI was pipetted into centrifuge tube to which 5.0 µL of IS working solution (10.0 µg mL^{-1} Imipramine) was added and vortexed to mix. 3.0 mL of tertiary butyl methyl ether **:** dichloromethane (70:30%, v/v) was added and vortexed for five minutes using multipulse vortexer. The mixture was centrifuged at 3500 rpm at 4°C for 5 min. The supernatant was separated and evoported to dryness under stream of nitrogen at 40°C. Analyte residues were reconstituted with 1.0 mL of mobile phase and transferred to polypropylene auto sampler vials. 10.0 µL was injected to LC-MS/MS system.

Validation
This method was validated for selectivity, sensitivity, recovery, linearity, precision and accuracy, matrix effect, stability and dilution integrity.
Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other expected components in the sample. This test was performed by analyzing the blank plasma samples from different sources for interference at the retention time of IRI and IS.
Sensitivity was determined by analyzing blank rat plasma (n=6) spiked with the analyte at lowest level of the calibration curve, 10.0 ng mL^{-1} in replicates.

Linearity of the method was evaluated over the concentration range of 10.0-4000.0 ng mL^{-1}. Comparison of the slope, intercept and correlation coefficient was made for three days validation process.
The intra and inter-day accuracy and precision of the assay were tested by analysis of six QC samples at each level and at least five precision and accuracy batches on three separate days. Accuracy and precision of the quality control samples were calculated against the calibration curve.
The recovery of the IRI and IS was calculated by comparing the peak area response of extracted analytes with post spiked analytes that represent 100% recovery. Recovery was carried out at three concentrations (low, medium and high quality control sample).
The matrix effect was performed at two concentration level (low and high quality control samples) and calculated by comparing the aqueous equivalent sample peak area with post spiked sample peak area.
As a part of method validation, stability was evaluated. The stock solution stability was evaluated at room temperature and at 2-8°C by comparing with freshly prepared stock solution. All stability experiments were performed with freshly prepared calibration standards and quality control samples.
The in-injector stability was evaluated after keeping processed samples in auto sampler at 5 °C for 28 h. The bench top stability was evaluated for 6 h at room temperature. The freeze-thaw stability was performed by comparing the stability samples that had been frozen and thawed thrice with freshly prepared calibration standards and quality control samples.
The long-term stability was performed by analyzing low and high quality control samples stored at -70°C for 74 days with freshly prepared calibration standards and quality control samples. All stability evaluations were based on back-calculated concentrations.
Dilution integrity was performed to extend the upper concentration limits with acceptable precision and accuracy. 2xULOQ concentration was prepared and diluted to 2 and 4 fold with blank plasma. These samples were processed and analyzed. The results were calculated after applying appropriate dilution factor.

Pharmacokinetic evaluation of Irinotecan liposomes
Twelve young, male Wistar rats (divided into two groups I and II) (body weight 220–250 g) were obtained from the central animal house, Manipal University, Manipal, Karnataka, India. The experimental protocol for the animal studies was approved by the Institutional Animal Ethical Committee (No.IAEC/KMC/07/2008-2009). Animals were maintained under controlled temperature (25±2°C) and humidity (50±5% RH) condition in polypropylene cages filled with sterile paddy husk. Animals were fasted 12 h before dosing.
The present method was applied to evaluate the pharmacokinetic behavior of IRI liposome in rats. The effect of liposome on the plasma levels of IRI following intravenous administration was determined. Group I and II received 15 mg/kg body weight of IRI standard drug and IRI liposome formulation respectively. Plasma samples were periodically collected upto 120 h at specified time intervals of 0.083, 0.5, 1.0, 2.0, 6.0, 12.0, 24.0, 48.0, 96.0 and 120.0 h.

Results and Discussion

Method development

The objective of this work was to develop and validate a simple and sensitive assay method for the quantification of IRI in rat plasma, suitable for pharmacokinetic evaluation in preclinical studies. To achieve the same, during method development, different options were evaluated to optimize chromatography and sample extraction procedure. A mobile phase containing formic acid in water (0.1%) in combination with methanol resulted in improved response in positive ionization mode due to acidic mobile phase which donate the proton to irinotecan. Use of Genesis C_{18} (100 × 4.6 mm id, 5 µ) column resulted in maximum response with symmetric peak shape. The optimized liquid-liquid extraction technique enabled the elimination of matrix ions which are commonly interfering with ionization process.

Selectivity

Chromatographic selectivity of the method was demonstrated by the absence of interfering peaks at the retention times of IRI and IS. Six different lots of EDTA blank plasma were screened. No endogenous interference was observed at the retention time of IRI and IS. Representative chromatogram of extracted blank plasma, extracted plasma samples containing 10.0 ng mL^{-1} of IRI (LLOQ) and one unknown animal study sample containing IRI is presented in figure 1, 2 and 3 respectively.

Fig. 1. Representative chromatogram of extracted blank plasma

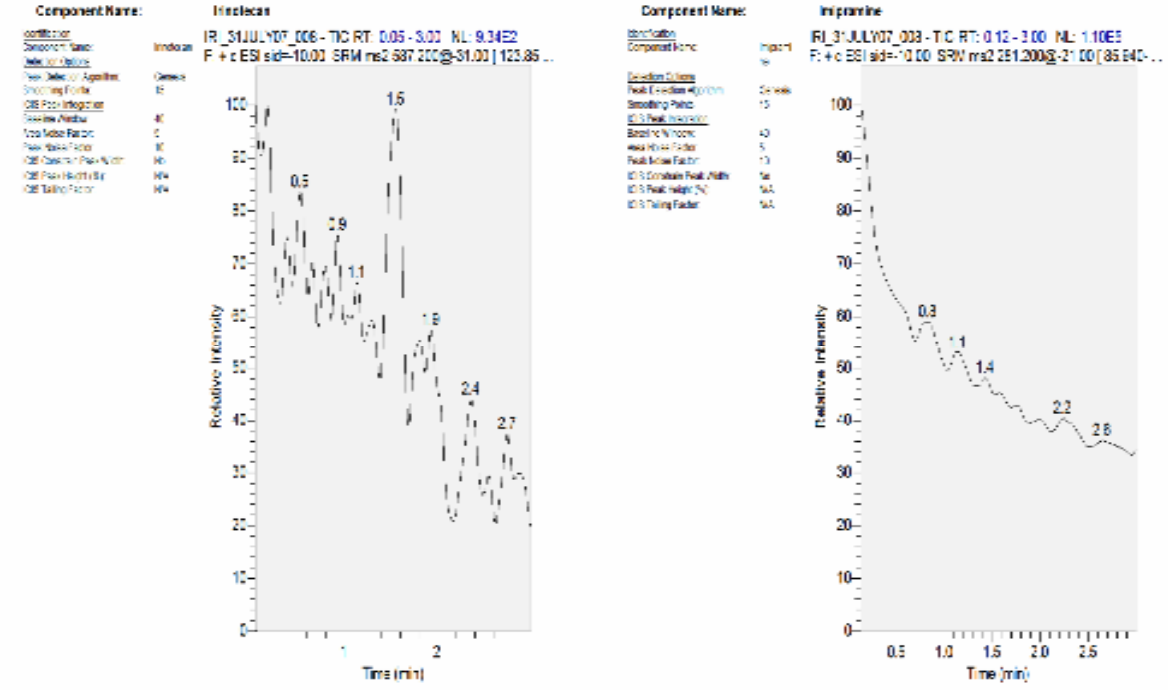


Fig. 2. Representative chromatogram of extracted plasma containing 10.0 ng/mL^{-1} of irinotecan.

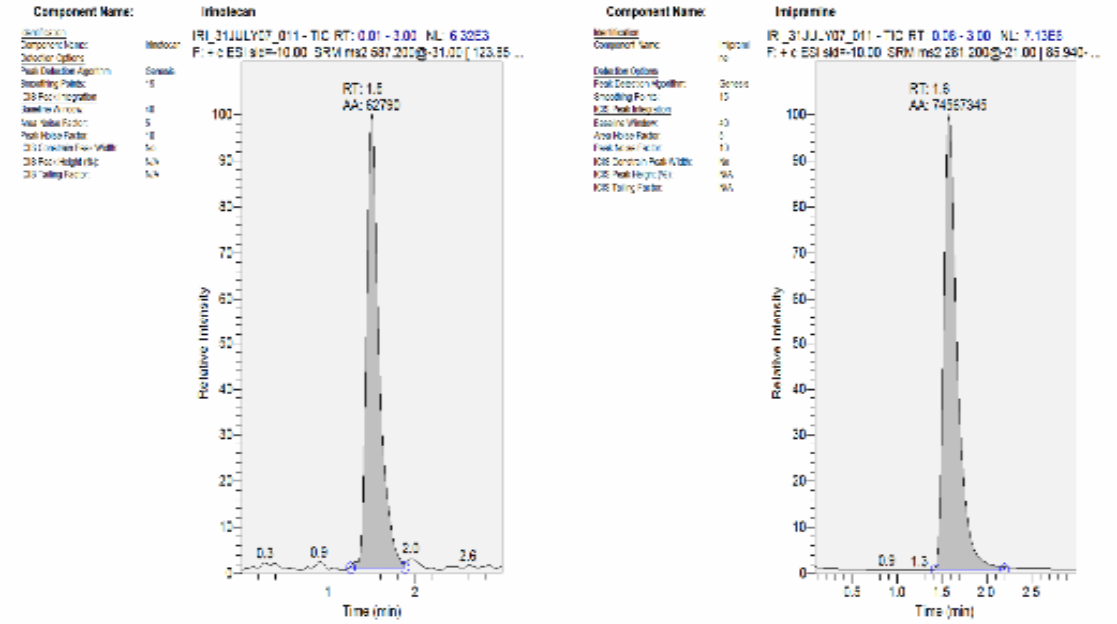
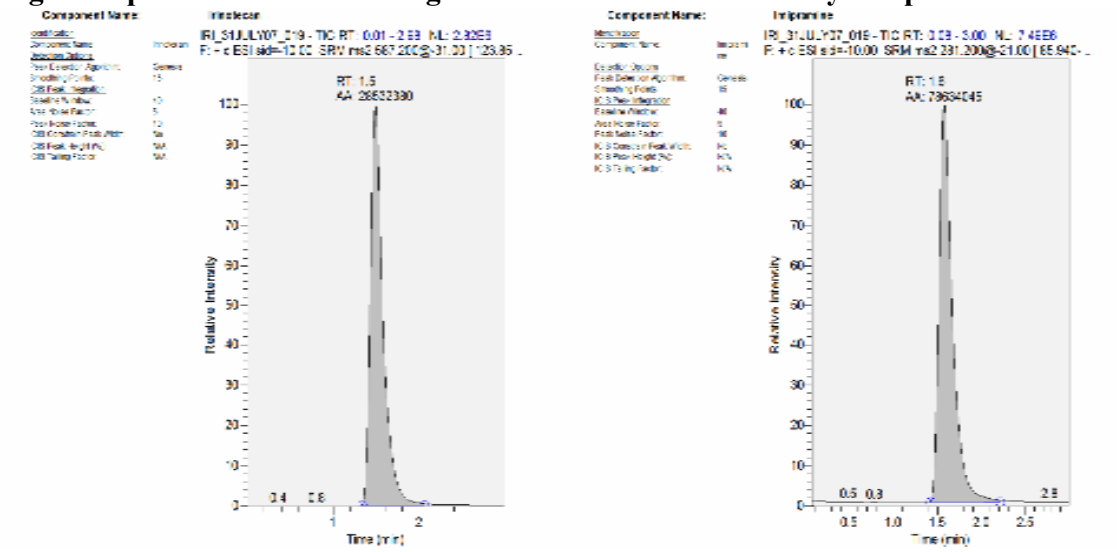


Fig. 3. Representative chromatogram of extracted animal study sample



Sensitivity (Lower limit of quantification)

The sensitivity experiment was carried out at LLOQ level. The mean percentage deviation from the nominal concentration was ≤ 6.70 % and percentage coefficient of variation was within 6.64 % and interference of the plasma endogenous constituents were found to be insignificant. Signal to noise ratio was found to be ≥ 240

Recovery

Peak areas from unextracted analyte with those of extracted analyte determined recovery. The mean absolute recovery of IRI at LQC, MQC and HQC was 81.95 % and variability across the QC level was ≤ 2.04 %. The recovery of internal standard was found to be 86.01 %.

Linearity

Representative calibration curve data of IRI are listed in table 1 and 2. The calibration curves were linear over the range of 10.0-4000.0 ng mL^{-1}. The correlation coefficient (n=5) was ≥ 0.9983 with acceptable accuracy and precision.

Table No.1. Summary of calibration curve parameters for irinotecan

Calibration curve	Slope	Intercept	r^2
1	0.0008636	-0.0003107	0.9990
2	0.0007896	-0.000191	0.9983
3	0.0008359	-0.000672	0.9989
4	0.0007609	0.001035	0.9991
5	0.0008098	-0.000426	0.9985

Table No. 2. Inter-run accuracy and precision of plasma calibration standard of irinotecan

Standard concentration (ng/mL^{-1})	Mean calculated concentration (ng/mL^{-1})	% RSD[a]	% Bias
10.000	10.109	6.48	1.09
20.000	21.079	4.09	5.40
80.000	76.398	3.83	-4.50
400.000	424.023	5.12	6.01
800.000	824.230	1.50	3.03
1600.000	1539.093	5.87	-3.81
2400.000	2421.902	0.85	0.91
3200.000	3302.324	1.54	3.20
4000.000	4107.329	2.75	2.68

[a] Relative standard deviation

Precision and accuracy

Both the intra-day and inter-day accuracy and precision of the method were determined by analysis of the blank rat plasma spiked with IRI at LLOQ, LQC, MQC and HQC levels and calculated against the calibration curve. The accuracy and precision of the method were described as percentage bias of theoretical versus measured concentrations and the percentage of the relative standard deviation, respectively. The intra-day deviation from the nominal concentration was between -2.76 to 6.94% and the intra-day precision was ≤ 6.48 % across QC levels. The inter-day deviation from the nominal concentration was between -2.25 to 5.21 % and the inter-day precision was ≤ 6.06 % across QC level. The results of accuracy and precision are enumerated in Table 3.

Table No.3. Intra-day and inter-day accuracy and precision of irinotecan in rat plasma

Spiked conc. (ng/mL^{-1})	Mean calculated conc. (ng/mL^{-1})	%RSD[a]	% Bias
Intra-day (n =6)			
10.000	10.674	6.48	6.74
40.000	42.783	3.05	6.96
2000.000	2129.034	4.50	6.45
3500.000	3403.340	3.82	-2.76
Inter-day (n =24)			
10.000	10.504	6.06	5.04
40.000	42.085	2.27	5.21
2000.000	2090.704	1.08	4.54
3500.000	3421.404	2.89	-2.25

[a] Relative standard deviation

Matrix effect

Matrix effect experiment was performed by comparing the peak area of aqueous equivalent response with six different plasma lots of post spiked sample area at LQC and HQC level. There was no significant matrix effect observed.

Stability

Stock solution stability experiment was performed at 4000.0 ng mL^{-1}. After storage for 22 days at 2-8°C and at room temperature for 7 h, more than 98.52 % of IRI remained unchanged, based on their peak areas in comparison with freshly prepared solution of IRI (10000.0 ng mL^{-1}). This suggests that the IRI stock solution was stable for at least 22 days when stored at 2-8°C and 7 h at room temperature.

Bench top stability of IRI in plasma and in-injector (5°C) stability after processing of IRI was performed at LQC and HQC levels. IRI in plasma was stable for at least 6 h at room temperature and 28 h at auto sampler maintained at 5°C with mean percentage change of ≤ 4.74 % and ≤ 4.44 % respectively. It was confirmed that repeated freeze and thawing (three cycles) of plasma samples spiked with IRI at LQC and HQC level did not affect the stability of IRI with mean percentage change of ≤ 1.27 %. Long-term stability of the IRI in plasma at -70°C was also performed after 74 days at LQC and HQC level with mean percentage change of ≤ 4.01 %. The results of the stability studies are presented in Table 4. The above results indicate that IRI is stable enough to be analyzed using this assay method.

Table No. 4. Stability sample results of irinotecan (n=6)

Stability	Specified conc. (ng/mL^{-1})	Mean comparison sample conc. (ng/mL^{-1})	Mean stability sample conc. (ng/mL^{-1})	Mean % change
In-injector [a]	40.000	41.768	40.431	-3.20
	3500.000	3573.309	3414.763	-4.44
Bench top[b]	40.000	41.768	39.789	-4.74
	3500.000	3573.309	3495.348	-2.18
Freeze and thaw[c]	40.000	41.768	42.298	1.27
	3500.000	3573.309	3609.323	1.01
Long-term[d]	40.000	39.340	37.803	-3.91
	3500.000	3479.987	3340.329	-4.01

[a] After 28 h in autosampler at 5°C.
[b] After 6 h at room temperature.
[c] After 3 freeze and thaw cycles at -70°C.
[d] At -70°C for 74 days.

Dilution integrity

The upper concentration limit of IRI can be extended to 8000.0 ng mL^{-1} with acceptable precision and accuracy of 15 % by 2-fold or 4-fold dilution with blank rat plasma. The precision was found to be ≤ 4.87 % and the mean percentage deviation from the nominal concentration was ≤ 2.25 %.

Pharmacokinetic evaluation

The mean maximum plasma concentration obtained for IRI standard and liposome formulation was 3450.521 and 951.190 ng mL^{-1} respectively. The AUC measured from 0 h to the last sampling point was higher than 90% of the value of AUC extrapolated from zero time to infinity, which indicates a suitability of the analytical method for pharmacokinetic studies.

Conclusion

A selective, sensitive and rapid LC-ESI/MS/MS method for the determination of IRI in rat plasma has been developed and validated, with a lower quantification limit of 10.0 ng mL^{-1}. Validation experiments have shown that the assay has good precision and accuracy over a wide range of 10.0 – 4000.0 ng mL^{-1}. This wide calibration range has an added advantage in pharmacokinetic evaluation of IRI by intravenous administration compared to reported method and no interference by the endogenous compounds was observed. This method was successfully applied for the pharmacokinetic evaluation of IRI liposomes in rat plasma.

Acknowledgements

The authors are grateful to Manipal University for providing animal house facility to conduct of animal experiment

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