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RP-HPLC Method for Simultaneous Estimation of Free and Entrapped Isoniazid and Ciprofloxacin HCL in Lipid Polymer Hybrid Nanoparticles

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ABSTRACT

Bioanalytical methods of Reverse Phase-High performance liquid chromatography (HPLC) was developed and validated for simultaneous estimation of Isoniazid (INH) and Ciprofloxacin Hydrochloride (CIP HCl) encapsulated in lipid polymeric hybrid nanoparticles (LPNs) in plasma and in organ homogenates of lung, liver, spleen and kidney of mice. Chromatographic separation was done using Agilent® C18 bonded silica column of dimensions 150 × 4.6 mm, 5µmwith flow rate 1.0 ml/min, injection volume 20 µland column temperature 40°C. The mobile phase consists of a mixture of 70 volumes of 0.1 percent v/v of trifluoroacetic acid(TFA) and 30 volumes of acetonitrile (ACN). The results indicated that the developed method was linear and selective for all matrices studied. Analysis of accuracy and precision showed adequate values, with relative standard deviation values lower than 5, which are in accordance with USFDA guidelines for bioanalytical method validation. Isoniazid (INH) and Ciprofloxacin Hydrochloride (CIP HCl) were stable in plasma and tissue homogenates under different storage and processing conditions. This method was applied to study the pharmacokinetic and biodistribution profile of both drugs in free form and in bound state with lipid nanoparticles. The results showed that polymeric nanoparticles showed higher drug accumulation in the target site i.e. lung as compared to non-target organs fulfilling our aim of developing a HPLC method for the simultaneous estimation of both drugs and their application in determination of pharmacokinetic and pharmacodynamic potential of the lipid nanoparticles.

Keywords: HPLC, bioanalytical, Tuberculosis, Stability, LOD

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INTRODUCTION

Tuberculosis (TB) is one of the leading causes of morbidity and mortality worldwide. According to the World Health Organization, Mycobacterium tuberculosis (M. TB), the causative agent of tuberculosis, infects about one-third of the world's population, and causes 9 million new cases of active tuberculosis and 1.7 million deaths annually[1, 2].Although an effective therapeutic regimen is available for the treatment of TB, owing to the long duration and complexity, non-compliance of patients to prescribed regimens is high[3, 4]. Therefore, a higher efficacy of treatment and a significant reduction of treatment duration are urgently needed and derive researchers to pursue novel drug delivery systems in order to be therapeutically effective[5]. Administration of Isoniazid has been considered beneficial for the treatment of tuberculosis. Similarly, administration of Ciprofloxacin has also been considered important for the treatment of tuberculosis especially for drug resistant tuberculosis[6, 7]. Instead of using single drug, we used a newer combination, one was Isoniazid (INH) and other was Ciprofloxacin Hydrochloride (CIP HCl). Isoniazid is a well known 1stline antiTB drug. It acts on extracellular as well as on intracellular TB. On the other hand Ciprofloxacin Hydrochloride (CIP HCl) is a 2nd line anti TB drug. Many studies evidenced the usefulness of CIP in tuberculosis treatment, especially in drug resistant tuberculosis[8, 9]. Thus combination of both these drugs could be a gunshot treatment for tuberculosis, especially for drug resistant tuberculosis treatment. Numerous methods have been successfully tried for individual estimation of INH and CIP HCl by HPLC. However, there is no report of a validated HPLC method for the simultaneous determination of Isoniazid (INH) and Ciprofloxacin Hydrochloride (CIP HCl) in biological samples and in nanocarriers systems. Structure of Isoniazid (INH) and Ciprofloxacin hydrochloride (CIP HCl) is shown in figure 1.

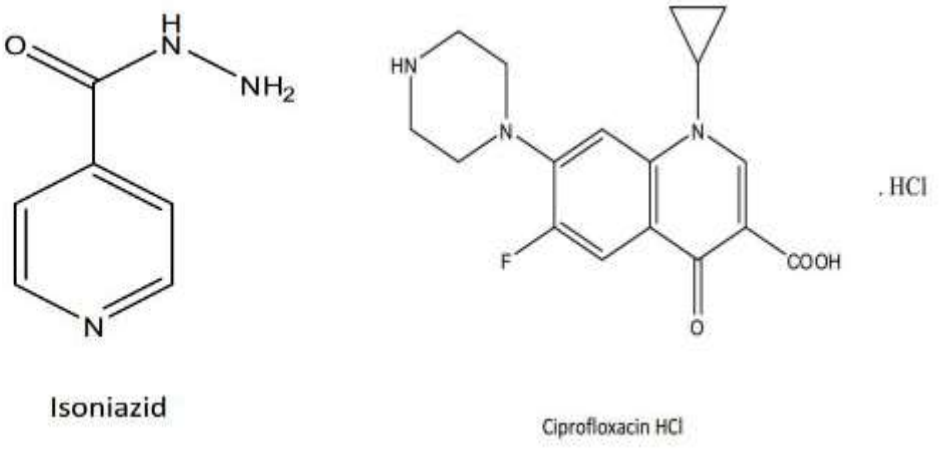

Figure 1: Structure of Isoniazid (INH) and Ciprofloxacin Hydrochloride (CIP HCl)

In the present study, a suitable, sensitive, reproducible and accurate RP-HPLC method for the simultaneous estimation of Isoniazid and Ciprofloxacin Hydrochloride in biological samples as per USFDA guidelines for bioanalytical method validation[10]and nanocarriers as per ICH guidelines for analytical method validation[11]has been developed and validated. The pharmacokinetic and pharmacodynamic profile of the drugs in free forms and in encapsulated form into the lipid polymer hybrid nanoparticles (LPN)was determined to illustrate the application of the developed method[12, 13, 14]. LPN is a new delivery system which combined the beneficial characteristics of liposomes as well as nanoparticles[15, 16], thus was successfully developed and used for the combined delivery of the drugs in lung.

MATERIALS AND METHOD

Reagents

Drugs, Ciprofloxacin Hydrochloride (CIP HCl) and Isoniazid (INH) werekind giftsfrom Lupin Pharmaceutical Pvt. Ltd., Aurangabad, India. Soy bean Lecitin (LC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy (Polyethylene-glycol)-2000] (DSPE-PEG_{2000})were gift samples from Lipoid, Germany.Poly (lactide-co-glycolide) PLGA and Dichloromethane (DCM) were purchased from Sigma Aldrich, USA andused for the preparation of lipid polymer hybrid nanoparticles (LPNs).HPLC grade acetonitrile (ACN) and trifluoroacetic acid (TFA) were used for the preparation of mobile phase and purchased from Sigma-Aldrich, USA. All other chemicals and solvents used wereof suitable analytical grade.

Lipid Polymer Hybrid Nanoparticles (LPNs) Preparation

Drug loaded lipid polymer hybrid nanoparticles (LPNs) were prepared as per method reported by Wang and group with some modification. Briefly, oil phase consists of 20 mg soya lecithin (LC), 80 mg PLGA, and 4.2 mg DSPE-PEG_{2000} were dissolved in 5 mL dichloromethane (DCM) to form the oil phase, while 10 mg drug was dissolved in 300 µL deionized water to form the internal aqueous phase. Next, the aqueous drug solution was emulsified in the PLGA organic solution by sonication for 1 min. The resultant nano-emulsion was poured into 12 mL deionized water and sonicated again for 1 min. Afterwards, the nano-emulsion was stirred overnight at room temperature to evaporate off DCM and resultant nanoparticle suspension was centrifuged twice at 11,000 rpm to remove the non-encapsulated drug and excess lipid[17]. Both INH and CIP HCl loaded LPNs were prepared by same method. Further, drug loaded LPN were converted into dry powder inhaler (DPI) using spray drying ((Labultima spray dryer, Mumbai, India) and these drug loaded spray dried LPNs (SD LPN) were used for the further study.

HPLC Analysis

HPLC analysis was performed using Waters 515 Series pumps combined with a Waters PDA 2998 series photo diode array detector. The column used was Agilent® C18 bonded silica (5 µm, 4.6 x 150 mm) with column temperature 40°C, flow rate 1.0 ml/min and injection volume was 20 µl.Analyte weighing, for preparation of calibration standards and quality controls (QC), was done on a microbalance, Mettler-Toledo AB 204-S. All mobile phase solutions were filtered by Rocker 410 vacuum filter assembly using 0.22 micron (µ) membrane filters and degassed ultrasonically by Steryl 40050 bath sonicator before use. The HPLC system was controlled by a PC workstation using Empower software installed on it.

Mobile Phase Systems

Mobile phase for INH and CIP HCl consist of a mixture of 70 volumes of 0.1 per cent v/v of trifluoroacetic acid (TFA) and 30 volumes of acetonitrile (ACN). A stainless steel C18 column of dimensions 15 cm x 4.6 mm, (5 µm) was used for the study. Flow ratewas 1.0 ml per minute, while detection was set at272 nm with injection volume of 20 µl.

Preparation of stock solutions

Accurately weighed 100 mg each of INH & CIP HCl was transferred into 100 mL volumetric flask, dissolved and diluted up to mark with water to obtain a standard stock solution of 1000 µg/ml each of INH and CIP HCl. An aliquot of the stock solution (1 mL) was transferred to 10 ml volumetric flask and diluted to the mark with water to obtain working standard solution of 100µg/ml each of INH& CIP HCl. The solution was filtered through a 0.22 µ membrane filter and 20 µL samples were injected each time.

Preparation of calibration curve (in mobile phase)

A five point calibration curve for INH and CIP HCl was prepared from the 1000 µg/ml stock solution results in the final concentration of 10 ug/ml, 50 ug/ml, 100 ug/ml, 150 ug/ml, 200 ug/ml for INH and 10 ug/ml, 50 ug/ml, 100 ug/ml, 150 ug/ml, 200 ug/ml concentration for CIP HCl respectively (working solutions). Calibration curves were constructed by plotting the values of peak area with their respective concentration. The linearity was assessed by linear regression analysis, which was calculated by least square method.

Animals

Male Mice weighing 18-30 g were used for bioanalytical study. Animals were acclimatized to laboratory conditions for a week before starting the experiment. The protocol was duly approved by Institutional Animal Ethical Committee (IAEC) of ISF College of Pharmacy, Moga, Punjab, India (ISFCP/IAEC/CPCSEA/Meeting No. 11/2014/Protocol No. 202). For validation of protocol,

blank plasma samples were used. Blood was withdrawn, from retro-orbital plexus of native mice (n=3), transferred into heparinized vials and centrifuged at 2000 RPM for 10 minutes to obtain clear plasma which was distributed in plastic tubes, sealed tightly and stored frozen at a temperature of -20^{o}C till further analysis.

Preparation of calibration curve (in Plasma)

A six point calibration curve for INH and CIP HCl was prepared from the 1000 µg/ml stock solution results in the final concentration of 100 ug/ml, 150 ug/ml, 200 ug/ml, 250 ug/ml, 300 ug/ml and 350 ug/ml for INH and 50 ug/ml, 100 ug/ml, 150 ug/ml, 200 ug/ml, 250 ug/ml and 300 ug/ml concentration for CIP HCl respectively (working solutions) in plasma. Calibration curves were constructed by plotting the values of peak area with their respective concentration. The linearity was assessed by linear regression analysis, which was calculated by least square method. All solutions were stored in refrigerator at 5.0 ± 3.0^{o}C. The bulk spiked calibration curve plasma samples were stored at -20^{o}C.

Plasma sample preparation

Blood sample was collected from retro orbital plexus of mice in heparinized tubes and plasma was separated at once with centrifugation at 5000 RPM for 10 min. About 0.1 ml of supernatant plasma was taken and 0.7 ml acetonitrile was added for deproteinization phenomena. Above prepared mixture was vortexed for 5 min and centrifuged (10,000 RPM) for 10 min. About 0.2 ml of the supernatant was taken out and diluted with 1.8 ml diluting solvent (mixture of 70 volumes of 0.1% v/v of trifluoroacetic acid (TFA) and 30 volume of acetonitrile). The prepared solution was filtered through milli pore syringe filter of pore size 0.22 µand sample solution was injected into the rheodyne injector of an HPLC system for analysis.

METHOD VALIDATION

System suitability

The system suitability test represents an integral part of method validation and used to ensure retention time, peak area, peak asymmetry, number of theoretical plates and resolution. System suitability was performed by determining the AUC for medium quality control [(MQC) without spiking into plasma] sample injected into the HPLC before the start of each analytical run and its comparison with the average AUC value obtained for the MQC, upon repetitive injections (n = 3).

Specificity

Blank plasma samples (n= 3) were prepared according to the sample preparation procedure described above and screened for the presence of any interfering peaks corresponding to the retention of either INH or CIP HCl[18].

Sensitivity

The limit of detection (LOD) of the bioanalytical methods is lowest concentration level giving a peak area of three times the noise. Limit of quantitation (LOQ) is the lowest concentration in the standard curve which provides signal to noise ratio higher than 10 with accuracy between 50-150 % and with precision of 20%[18].

Recovery

The absolute recovery was computed from the peak area of INH/CIP HCl standard solutions to those containing INH/CIP HCl LPNs at different concentrations. It was determined by comparing the peaks areas obtained after extracting plasma standards with areas obtained for corresponding stock standards. The study was accomplished at three concentration levels (50, 100, and 150 ug/ml) for both the drugs (n=3).

Inter-day and Intra-day precision and accuracy

Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) for 3 consecutive days. Three different concentrations of INH and CIP HCl were analyzed in six independent series in the same day (intra-day precision) and 3 consecutive days (inter-day precision). Every sample was injected in triplicate. The accuracy of the method, which is defined as the nearness of the true value and found value, was evaluated as % bias for INH and CIP HCl according to the following equation:

$$\%\ accuracy = \frac{observedconcentration}{nominalconcentration} \times 100$$

Stability at various storage conditions

The drug stability in biological matrices is a function of storage conditions, chemical properties of the drug and the matrix. For all the stability studies, low, medium and high quality control (QC) samples were used in triplicate. The stability at 24 hr (after plasma sample extracts had been exposed to the same temperature conditions as in autosampler) was analyzed. The freeze-thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing the QC samples unassisted at room temperature and then refreezing for 12-24 hrs followed by analysis. The bench-top stability was evaluated by keeping the QC samples at room temperature for 4 hr and then samples were processed and analyzed. Subsequently, the concentrations (50, 100 and 150 ng/mL) in plasma samples and organ homogenate samples were checked[18].

Pharmacokinetic and Organ distribution study

Pharmacokinetic and biodistribution studies for simultaneous estimation of INH and CIP HCl in mice plasma and organs of interest (lung, liver, spleen and kidney) after administration of the

developed formulations were conducted. Mice were divided into two groups, which include Group 1, to whom, oral free drugs [(combination of free INH and free CIP HCl) (INH and CIP HCl = 10mg each)] were administered and Group 2, to whom DPI of INH loaded LPN and CIP HCl loaded LPN (in combination)were administered by pulmonary route. The animals were bled at several time points. The plasma was obtained from each mice and deproteinized with 100 ml of acetonitrile (ACN), vortexed for 5 min and centrifuged at 5000 × g for 20 min at 4-8^{o}C. The supernatant was used for the analysis of INH and CIP HCl. The drugs were analyzed by HPLC and compared with calibration graphs (obtained by analyzing pooled blank mice plasma spiked with known drug amounts) to obtain the plasma drug concentration versus time profile. Peak plasma concentration (C_{max}) and time taken to reach C_{max} (T_{max}) were determined. Elimination rate constant (K_{el}), elimination half life ($t_{½}$), Mean Residence Time (MRT), Systemic Clearance (CL), Volume of distribution at steady state (V_{ss}) and area under plasma drug concentration over time curve ($AUC_{0-\infty}$) were calculated using a Thermo Kinetica Version 5.0. The ratio area under moment curve (AUMC)/area under curve (AUC) (AUMC/AUC), yielded the mean residence time (MRT)[19]. For the organ distribution study, the visceral organs (lung, liver, kidney and spleen) of the dissected mice were excised at predetermined time intervals (2, 8, 12& 24 hrs) after administration of the formulations. After isolation of organs from the mice, they were washed with distilled water and blot dried using tissue paper and kept in isotonic saline solution and homogenized with PBS pH 7.4 by using tissue homogenizer. This homogenized tissue matrix was then centrifuged at temperature 24^{o}C for 10000 RPM in refrigerated centrifuge. Supernatants of different tissue matrixes was separated in other freshly chemically cleaned centrifuged tube and stored in deep freezer for further study. After 30 min of freeze thaw at room temperature, an aliquot of 100µL of tissue matrix was taken to a chemically clean eppendorf tube, and vortex-mixed for 5 min. After mixing 1.6 mL of precipitating agent i.e. acetonitrile (ACN) was added and vortex-mixed for 5 min and centrifuged for 10 min at 10,000 RPM at 24^{o}C in refrigerated centrifuge and filtered using Rocker 410 vacuum filter assembly with 0.2 micron membrane filters. Then 0.2mL of supernatant was taken and poured in a fresh chemically clean eppendorf tube and reconstituted it by adding 1.8 mL of mobile phase. The supernatants from successive extracts of an organ from each mice were pooled and the sample solution was injected into the rheodyne injector of an HPLC system for analysis. The amount of drug in each organ was calculated as percent drug recovered from the respective organ at different time intervals.

RESULTS AND DISCUSSION

In the present study, a RP-HPLC method for the simultaneous estimation of INH and CIP HCl in mice plasma and in organ homogenates was investigated and validated, according to the principles of current good laboratory practices (CGLP). Chromatographic conditions were optimized for the simultaneous estimation of INH and CIP HCl within a short analysis time (<5 min) and an acceptable peak resolution (R_s > 2) as shown in figure 2. With the advent of the expected usefulness of INH and CIP HCl in the therapeutic regimen of tuberculosis, their quantification in the biological matrix in conjunction with ascertaining the stability of samples during extraction and analysis is an important assignment.

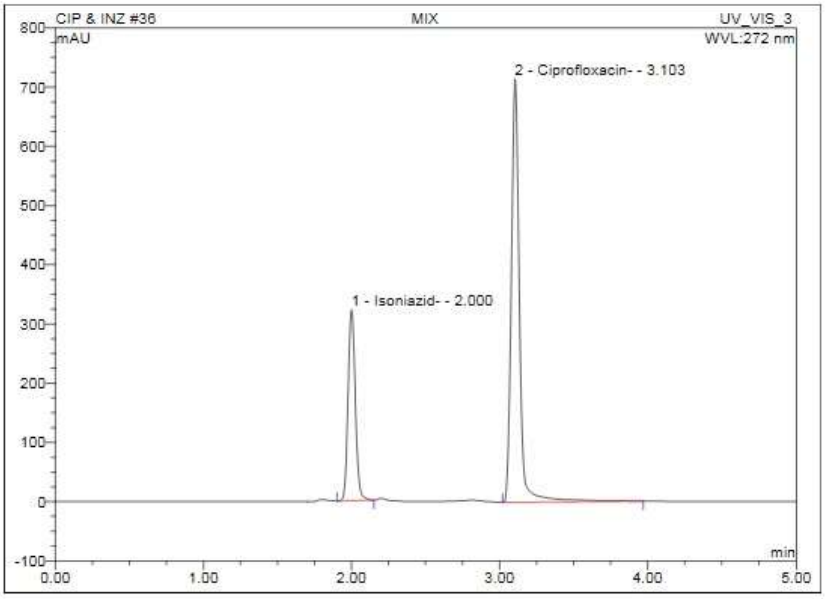

Figure 2: Chromatogram of INH and CIP HCl

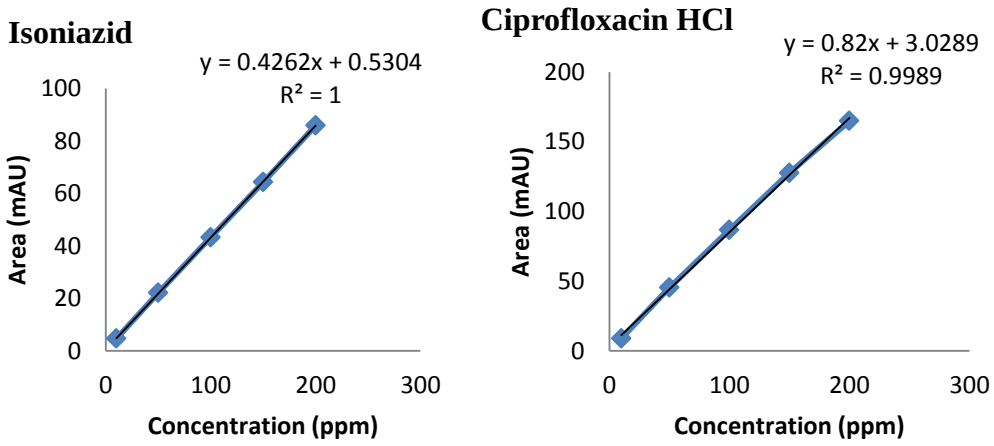

Figure 3: Linearity plot for Isoniazid and Ciprofloxacin HCl (in mobile phase)

Preparation of calibration curve in mobile phase

The linearity of the calibration curves for INH and CIP HCl was calculated and constructed by least square regression method. The correlation coefficient (r^2) for the standard calibration curves for INH and CIP HCl were 1 and 0.998, respectively. This indicates linearity of peak area ratio of INH and CIP HCl in the range of 10-200 µg/mL for both INH and CIP HCl respectively (Figure 3).

Preparation of calibration curve in plasma

The linearity of the calibration curves for INH and CIP HCl was calculated and constructed by least square regression method. The correlation coefficient (r^2) for the standard calibration curves for INH and CIP HCl were 0.9905 and 0.991, respectively. This indicates linearity of peak area ratio of INH in the range of 100-350 µg/mL and for CIP HCl linearity in the range of 50-300 µg/mL respectively (Figure 4). This RP-HPLC bio-analytical method was used for estimation of Isoniazid and Ciprofloxacin HCl in the LPN. Figure5 represents the overlay spectra of Isoniazid andCiprofloxacin HCl. The mobile phase used was a mixture of 70 volumes of 0.1% v/v of trifluoroacetic acid and 30 volume of acetonitrile, flow rate of 1.0 ml/min with the retention time of 2.584 min for Isoniazid and 3.781 min for Ciprofloxacin HCl respectively. The diluent used was mobile phase.

Isoniazid **Ciprofloxacin HCl**

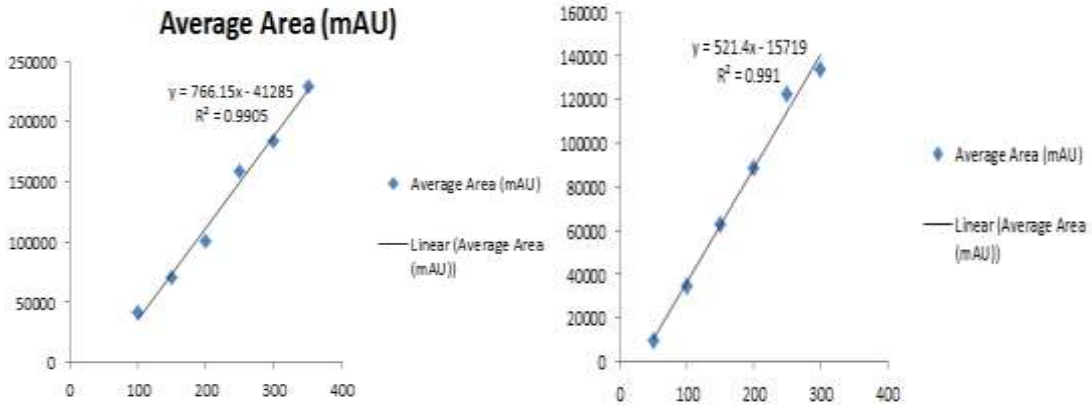

Figure 4: Linearity curve of Isoniazid and Ciprofloxacin HCl (in plasma)

METHOD VALIDATION

System Suitability

The system was found to be suitable for the determination of drugs, INH and CIP HCl under optimized chromatographic conditions. Average peak area per injection was determined at each time point and relative standard deviation (RSD) which was0.374 % for INH and 0.270 % for CIP

HCl (Table 1). As per USFDA bioanalytical method validation guidelines, 2001, accuracy of the developed analytical procedure should be high and its RSD should be less than 5 %. Column efficiency was calculated from the number of theoretical plates, which were found to be 7025.4 for INH and 10722.1 for CIP HCl.

Table 1: System Suitability Study

Parameter	R.T.	P.A.	Asymmetry	T.P.	R.T.	P.A.	Asymmetry	T.P.	Resolution
	Isoniazid				Ciprofloxacin HCl				
Average	2.008± 0.004	39.347 ±0.147	1.044± 0.018	7025.4± 109.363	3.139± 0.029	85.206 ±0.230	1.02±0 .02	10722.2± 624.068	10.392 ±0.061
% RSD	0.228	0.374	1.740	1.557	0.953	0.270	1.960	5.820	0.590

R.T. = Retention Time,P.A. = Peak Area,T.P. = Theoretical Plates, RSD = Relative Standard Deviation

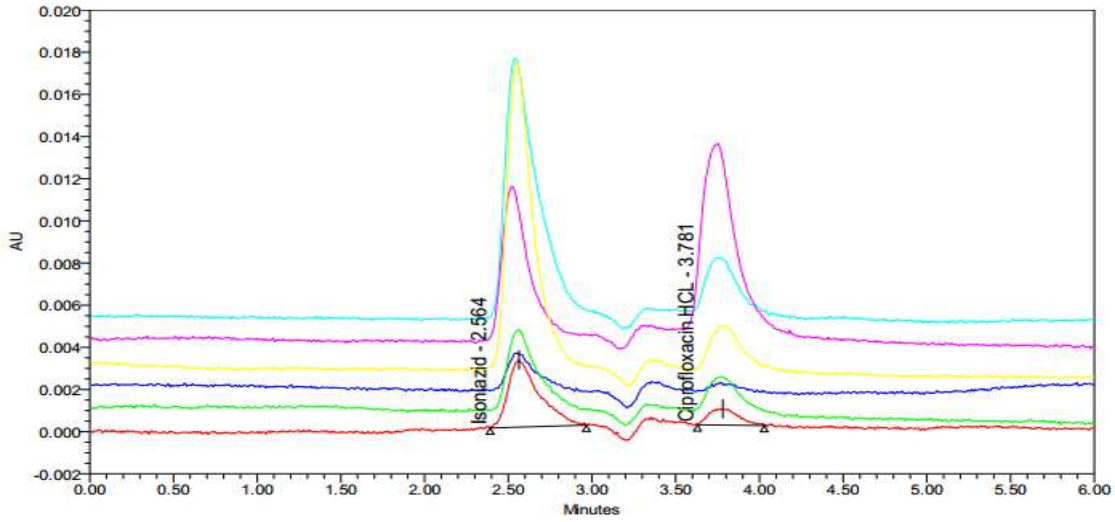

Figure 5: Chromatogram of Isoniazid and Ciprofloxacin HCl in plasma

Specificity

Any potential interference (overlapping peaks) components were within 2-3 min only, later on there was no significant interference that affected the response of Isoniazid & Ciprofloxacin HCl indicated high specificity of the developed method. Further, chromatogram obtained from the spiked plasma samples were found to be specific for INH and CIP HCl as shown in figure 1.

Sensitivity

LOD and LOQ values for INH were found to be 1.584 µg/ml and 4.802 µg/ml respectively. Similarly, LOD and LOQ values for CIP HCl were found to be 9.514 µg/ml and 28.831 µg/ml respectively.

Recovery or Accuracy

Accuracy or percentage recovery data of the analyzed samples in the present study were in the range from 96.12 to 102% shown in Table 2.The absolute recovery was computed from the peak area of INH/CIP HCl standard solutions to those containing INH/CIP HCl LPNs at different concentrations. It was determined by comparing the peaks areas obtained after extracting plasma standards with areas obtained for corresponding stock standards. The study was accomplished at three concentration levels (50, 100, and 150 ug/ml) for both the drugs (n = 3).

Table 2: Accuracy or percentage recovery data

Sample	Amount added (µg/ml)			Amount Recovered (µg/ml)			% Recovery		
	80 %	100%	120%	80%	100%	120%			
INH	0.64	0.8	0.96	1.382	1.618	1.721	96±1.2	98.9±1.13	97.8±1.76
CIP	2.56	3.2	3.84	5.696	6.19	7.18	98.9±1.42	96.8±0.78	102±0.92

Entrapment efficiency and % recovery were measured in order to demonstrate the applicability of HPLC method for the simultaneous estimation of INH and CIP HCl in LPNs. To calculate the entrapment efficiency of INH and CIP HCl in LPNs, free unloaded compounds were separated from the INH and CIP HCl loaded LPNs using Centrisart filter assemblies. The concentration of the free unloaded INH and CIP HCl in the filtrate, as measured by HPLC method was 0.011 and 0.025 mmol%, respectively, which corresponds to an entrapment efficiency of 98.95 % (±0.37) for INH and 97.52 % (±0.67) for CIP HCl. The absolute recovery was computed by comparing the peak area of the INH/CIP HCl standard solutions to those containing INH-CIP HCl LPNs at different concentrations. The results of this experiment are given in table 2. The % recovery of INH and CIP HCl from LPNs was in the range from 96 to 97.8 % and 98.9 to 102 %, respectively. This indicates the suitability of the developed method in simultaneously quantifying the concentration of both the drugs in LPNs.

Inter-day and Intra-day precision

Precision for the QC samples during the intra- and inter-day run are shown in Table 3 and Table 4 respectively. As observed from the table, data obtained was within the acceptable criteria (i.e. 5%). Inter-day precision was assessed over a period of 3 days. Inter-day as well intra-day replicates of Isoniazid & Ciprofloxacin HCl resulted % R.S.D. below 1.7 and 1.6 respectively as show in Table 3 and Table 4(should be less than 15 % according to USFDA guidelines for bio-analytical method validation), revealed that the proposed method is highly precise. Intra-day precision and inter-day variation of method was determined using three replicate injections of three concentration levels and analyzed on same day for three times and three different days. The inter-day and Intra-day results of the INH and CIP HCl are shown in figure 6.

Table 3: Intra-day precision of INH and CIP HCl

Nominal concentration (µg/mL)	Average Area	% RSD
INH (50)	22.005±0.963	1.375
INH (100)	43.203±1.161	1.688
INH (150)	65.992±1.798	1.724
CIP HCl (50)	45.206±0.637	1.409
CIP HCl (100)	86.561±0.535	0.618
CIP HCl (150)	130.788±1.638	1.234

Table 4: Inter day precision from INH and CIP HCl

Nominal Concentration(µg/mL)	Day 1		Day 2		Day 3	
	Average	% RSD	Average	% RSD	Average	% RSD
INH (50)	22.038±0.431	1.143	22.038±0.789	0.913	21.939±0.592	0.928
INH (100)	43.270±0.199	0.461	43.243±0.923	1.125	43.094±0.628	1.045
INH (150)	66.021±0.354	0.536	66.574±0.786	0.947	65.401±0.821	0.826
CIP HCl (50)	45.224±0.373	0.826	45.301±0.689	1.271	45.092±0.736	1.192
CIP HCl (100)	86.628±0.214	1.247	86.641±0.816	0.895	86.416±0.911	0.849
CIP HCl (150)	130.950±0.684	0.522	131.350±0.781	1.218	130.064±0.592	0.597

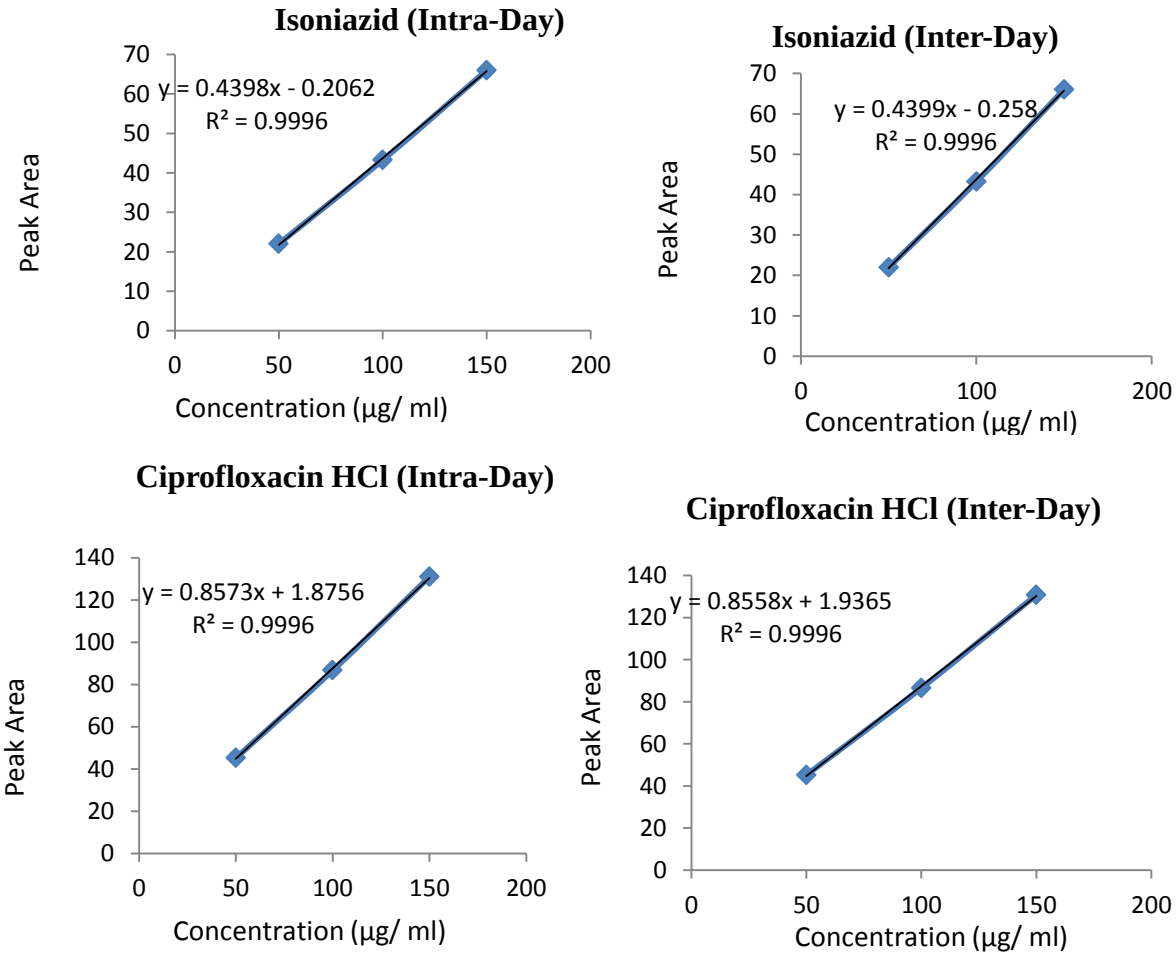

Figure 6: Diagrammatic Representation of Inter-day and Intra-day graph of INH and CIP HCl

Stability Study

Three sets of plasma samples at low, medium and high concentrations of QC were analyzed in all stability tests and the results indicated that the drugs were stable. Processed plasma and organ samples (i.e. lung, liver, spleen and kidney samples) were stable for at least 24 hr at room temperature in the autosampler. These samples were also stable after three freeze-thaw cycles. These results showed that plasma and organ samples could be thawed and refrozen without compromising the integrity and accuracy of the samples (Table 5). The bench top stability was investigated to ensure that drugs remained stable in plasma and organsof interest (i.e. lung, liver and kidney) at room temperature for 4 hr.

Table 5: Stability data for INH and CIP HCl in plasma and lung samples

Stability	Nominal concentration(ng/mL)	Measured concentration(ng/mL)	Precision (% RSD)	Accuracy (%)
Plasma				
Bench top 4 hr	50	50.21	2.14	100.42
	100	97.94	3.31	97.94
	150	152.28	2.56	101.52
Autosampler 24 hr	50	49.85	3.62	99.7
	100	100.72	2.57	100.72
	150	149.37	3.25	99.58
Freeze-thaw three cycles	50	48.40	2.46	96.8
	100	98.56	3.24	98.56
	150	151.64	3.17	101.09
Liver				
Bench top 4 hr	50	51.15	2.46	102.3
	100	98.67	2.67	98.67
	150	152.64	2.36	101.76
Autoosampler 24 hr	50	50.75	3.24	101.5
	100	104.05	2.84	104.05
	150	156.8	3.19	104.53
Freeze-thaw three cycles	50	52.59	2.35	105.18
	100	105.15	2.58	105.15
	150	154.24	3.02	102.83
Lungs				
Bench top 4 hr	50	50.45	2.46	100.9
	100	99.69	3.58	99.69
	150	150.47	3.91	100.31
Autoosampler 24 hr	50	49.87	3.06	99.74
	100	100.46	2.37	100.46
	150	149.79	2.09	99.86
Freeze-thaw three cycles	50	50.24	2.56	100.48
	100	99.87	2.37	99.87
	150	149.35	2.58	99.57

Kidney				
Bench top 4 hr	50	51.2	2.47	102.4
	100	103.94	2.36	103.94
	150	145.69	2.73	97.13
Autoosampler 24 hr	50	48.32	2.57	96.64
	100	104.47	2.59	104.47
	150	147.31	3.28	98.21
Freeze-thaw three cycles	50	48.19	2.41	96.38
	100	97.64	3.57	97.64
	150	154.96	3.51	103.31
Spleen				
Bench top 4 hr	50	51.1	2.17	102.2
	100	102.34	1.96	102.34
	150	146.79	2.63	97.86
Autoosampler 24 hr	50	48.19	2.93	96.38
	100	105.37	3.50	105.37
	150	147.37	2.41	98.24
Freeze-thaw three cycles	50	47.38	2.36	94.76
	100	98.21	2.77	98.21
	150	153.46	2.57	102.3

Pharmacokinetic studies

Various pharmacokinetic parameters were evaluated to determine any differences between free versus (vs) nano encapsulated anti-tuberculosis drugs. Pharmacokinetic (PK) parameters obtained after non-compartmental analysis of the plasma concentration vs time data are summarized in table 6. Free INH and free CIP HCl reached C_{max} at approximately 5 hours and 6hour respectively, INH and CIP HCl loaded LPN reached C_{max}at approximately 3 hours and 4 hour respectively.$AUC_{0-\infty}$ for free INH and free CIP HCl was 1399891 ± 0.34 µg/ml*min and 1123272 ± 0.23 µg/ml*min respectively. Similarly, AUC for INH and CIP HCl loaded LPN was 523187 ± 0.14 µg/ml*min and 458483 ± 0.72 µg/ml*min respectively. Thus, it was observed that the area under the curve (AUC) estimated after pulmonary administration of the developed formulation (LPN) was significantly lower than from the free drug administration, suggesting sustained release of drug. However, half-life ($t_{1/2}$) of these drug loaded formulations (LPN) were significantly longer ($p \leq 0.0001$) as compared to free drug at 1.324± 0.23 hrs (free INH) and 0.181 ± 0.32 hrs (free CIP HCl), respectively. INH and CIP HCl loaded LPN maintained controlled and sustained drug release over 24 hrs and free drugs only for 12hrs, which was also observed from tissue distribution study. Thus, it provided a clear distinction between LPN encapsulated drug releases versus free drug release.

Table 6: Plasma drug analysis for freeand DPI of INH and CIP HCl loaded LPNs in mice (n=3)

Parameters	Ciprofloxacin Hydrochloride		Isoniazid	
	CIP-HCl Free	CIP-HCl loaded LPN	INH Free	INH loaded LPN
AUC_{0-t} (ug/ml*min)	1156 ± 0.12	436 ± 0.27	151 ± 0.12	451 ± 0.54
K_{el} (λ) (min^{-1})	0.0612 ± 0.32	0.0000	0.0408 ±0.93	0.0000
$AUC_{0-\infty}$ (ng/ml*min)	1123272 ± 0.23	458483 ± 0.72	1399891 ± 0.34	523187 ± 0.14
$t_{1/2}$ (hr)	0.181 ± 0.32	3.54 ± 0.27	1.324 ± 0.23	6.013 ± 0.21
C_{max} (ng/ml)	56342 ± 0.82	51200 ± 0.18	38713 ± 1.21	30741 ± 0.13
T_{max} (min)	340 ± 0.29	240 ± 0.26	280 ± 1.04	180 ± 0.24
CL (ml/min)	11.15 ± 0.46	5.71 ± 0.61	18.2 ± 0.94	9.1 ± 0.31
$AUMC_{0-t}$ (ng/ml*min^2)	16422123 ± 0.82	5368267 ± 0.25	738220 ± 0.64	6552213 ± 0.82
MRT (min)	14.2 ± 0.24	12.3 ± 0.34	4.8 ± 0.14	14.5 ± 1.01
V_{ss} (L)	0.014 ± 0.82	0.018 ± 0.32	0.1 ± 0.04	0.1 ± 0.01

Values are mean ± SD, n=3. $p < 0.005$ with respect to free drugs, according to Two-way ANOVA.

LPN: Lipid Polymer Hybrid nanoparticles; AUC_{0-t}: area under the concentration-time curve for time 0 to time t; k_{el} (λ): terminal elimination rate constant; $AUC_{0-\infty}$: area under the concentration-time curve for time 0 to infinity (∞); $t_{1/2}$=elimination half life; C_{max}= peak plasma concentration; T_{max}= time taken to reach C_{max}; $AUMC_{0-t}$: area under the first moment curve; MRT=mean residence time; V_{ss}: volume of distribution at steady state.

Pharmacodynamic study

Further, DPI of the drug loaded spray dried LPNs (SD LPN) were observed to study the distribution of the drug localized in different organs, namely lungs, liver, kidney and spleen against the free drug inhalation. The free drugs and LPN encapsulated drugs were administered into the lung in powdered form through pulmonary route using dry powder insufflators (DPI). The developed DPI formulations of INH and CIP HCL loaded LPN (in combination) showed greater accumulation in the lungs as compared to the free drug inhalation (INH and CIP HCl combination). In the case of free drug inhalation, only 24.341 ± 1.210 % of Isoniazid and 22.371 ± 1.020 % of Ciprofloxacin HCl were found in the lungs at 2 hr post-administration and were not detectable in the lungs after 24 hr. They readily distributed to systemic circulation from where they were rapidly taken up by liver and cleared (digested) by the tissue macrophages there in. However, in the case of DPI formulation of LPN, 54.121 ± 2.010 % of the administered dose was found in the lungs at 2hr post-administration for INH loaded LPN and 52.231 ± 1.213 % of the administered dose was found in the lungs at 2 hr for DPI of CIP HCl loaded LPN. INH and CIP HCl loaded LPNs were observed over 24 hrs in the lungs which suggests good drug accumulation in the lungs using drugs encapsulated LPN. Different graphs areplottedbetween % drug content in

different organs vs. different time intervals. The percent drug content in lungs, liver, spleenand kidney of plain drug suspension and their DPI form are shown in figure 7,

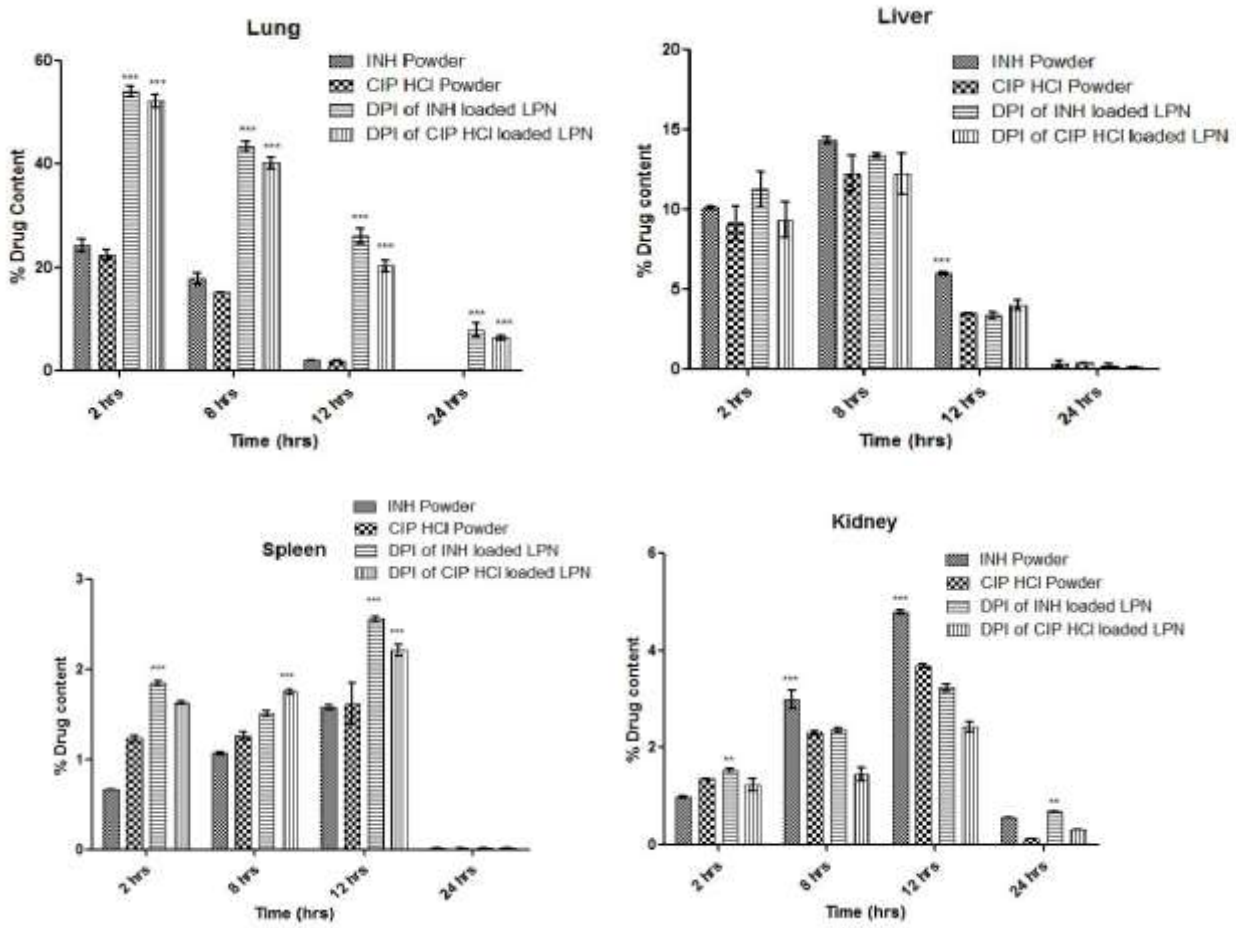

Figure 7: Comparative percent drug recovered from different organ after administration of various formulations. Values are mean ± SD, n=3. p < 0.001 with respect to free drugs, according to Two-way ANOVA.

the graphs plotted by bonferroni post-test showed significant p values (p <0.0001) for lung. These significant increase in "p values" in case of lungs showed that the drugs were reached in high concentration in the lung and remain in the lung for longer duration when given in the form of DPI, thus accomplishing our aim to achieve maximum internalization of the vesicles in the target site. The improved pharmacokinetic as well as tissue distribution profile were achieved through the use lipid polymer hybrid nanoparticles. This system leads to reduction in dose as well as dosing frequency, thus reduced toxicity could be achieved. INH and CIP HCl loaded LPN maintained controlled drug release over several hours as compared to free drugs. All these factors lead to cumulative increase in patient compliance.These observations suggest that the DPI of INH and CIP HCl loaded LPN are not only effective in rapid attainment of high-drug concentrations in alveolar

macrophages (lungs) but could also maintain the drug concentration over a prolonged period of time when compared against the free drug. These results showed that maximum amount of drug reached in lungs when given in the form of DPI form of LPN by pulmonary route.

CONCLUSION

In the light of results obtained, we can say that the developed HPLC method was rapid, sensitive specific and accurate in simultaneous estimation of INH and CIP HCl in free form and in encapsulated form into the lipid nanoparticles. This method produced better results in term of sensitivity and resolution. This method is advantageous of being specific for both the drugs without the need for additional sample preparation and the proposed method can be adopted in LCMS as the mobile phase selected is volatile in nature.

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