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A Sensitive HPLC Method for Determination of Isoniazid in Rat Plasma, Brain, Liver and Kidney

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Abstract

A simple and rapid reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for quantitative determination of Isoniazid (INH) in plasma, brain, liver and kidney samples and in solid lipid nanoparticles (SLNs). Isoniazid was analyzed by using a reverse phase column (Waters, Symmetry shield RP-18, 4.6 mm x 150 cm, 5 microns), with mobile phase consisting of 0.1 M phosphate buffer, pH 5 (pH adjusted with ortho phosphoric acid) and methanol and the detection was made at 254 nm using Photo Diode Array detector at a temperature of 30°C (sample 4°C). The retention time for INH was around 3.5 minutes. The calibration curves were linear (r^2 0.9998) over a concentration range from 250 ng to 25,000 ng/mL. Limit of detection (LOD) was 150 ng/mL and the Limit of quantitation (LOQ) was 200 ng/mL for plasma and tissue homogenates (brain, liver and kidney). Intra and inter-day variability's (RSD) for extraction of INH from plasma and other tissue homogenates were less than 5% and accuracy was ± 5%. The results established selectivity and suitability of the method for pharmacokinetic studies of INH from INH SLNs.

Keywords: RP-HPLC; PDA; Isoniazid; Tissue analysis; Tuberculosis

Introduction

Isoniazid (INH) belongs to the class of nucleoside reverse transcriptase inhibitor and is the one of the first line agents reserved by 'WHO' for the treatment of tuberculosis [1].

Isoniazid is a Biopharmaceutical Classification System (BCS) class III drug (high solubility and low permeability) reported to have aqueous solubility of 140 mg/ml [2] and log P of -0.64 (2008). The low permeability [3] and short t1/2 [2] and fast elimination (50 to 70% [4]) potentiated with a high dose (300 mg/adult) makes it a suitable candidate for delivery by a novel biodegradable drug delivery system, solid lipid nanoparticles (SLNs).

Liposomal systems of isoniazid were reported in late 90's for reducing the dose and dosing frequency [5]. Polymeric nanoparticles and solid lipid nanoparticles of isoniazid are also reported with an aim of reducing the dose and improving the permeability of isoniazid [5-12]. Moreover, ingredients like lipids and fatty acids which mimic the natural cell wall components are reported to help in rapid uptake of the NDDS into the mycobacterium and a consequent release of the drug directly inside the mycobacterium thus achieving both targetability and a facilitative permeation into the mycobacterium [13].

With an aim of improving the bioavailability of isoniazid we prepared solid lipid nanoparticles of isoniazid using a recently patented method by us [14] (results not shown).

Poor retention of polar compounds has been reported earlier on non polar reverse phase HPLC columns earlier [15], however, numerous methods for determination of polar compounds using reverse phase columns specific to isoniazid exists in the literature [16-19]. Establishment of a reproducible analytical method is always prerogative step before the development of a pharmaceutical product. A sensitive HPLC method capable of quantifying INH (upto 10 ng/ with spiking) in bronchoalveolar lavage and plasma in HIV-infected patients using electrochemical detector was reported in late 90's [20]. Gradient methods to detect INH in urine upto 1400 ng/mL was reported in 2002 [18]. In addition, methods to detect INH in the presence of its metabolite (acetyl isoniazid), pyrazinamide, rifampicin and ethambutol are also reported in literature [17]. A gradient method using Methanol–Acetonitrile–Buffer (20 mM of heptanesulfonic acid sodium, pH 2.5) as the mobile phase and C-12/18 column chemistry was able to quantify INH at as low as 100 ng/mL concentration [17]. A USP isocratic method with sodium phosphate buffer (pH 6.8)/acetonitrile as the mobile phase has reportedly been used for determination of INH in presence of pyrazinamide, ethambutol and rifampicin in plasma, brain, liver and kidney with LOQ of 200 ng/mL [21], the method validation was however, not been reported in the literature except the LOD and LOQ. However, majority of these HPLC methods are cumbersome gradient methods and are time consuming (long retention times). No validated isocratic HPLC methods for quantitative determination of INH entrapped in solid lipid nanoparticles or quantification of INH in plasma/tissue samples (brain, liver and kidney) were reported till date with chromatographic conditions described herein. Further, there was a need to develop a HPLC method that could specifically and sensitively detect INH in the presence of plasma/tissue components as well as polysorbate 80 (an essential ingredient for preparing solid lipid nanoparticles).

Experimental

Materials

INH was obtained as a gift sample from Panacea Biotec Ltd. Lalru, Punjab, India. Soya lecithin (Phospholipon 90 H) was received as a gift sample from Lipoid GmBH, Germany. Compritol 888 ATO® was a gift sample from Colorcon Asia Pacific Pvt. Ltd, India. Stearic acid

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and tween 80 were purchased from Central Drug House, Mumbai. Other chemicals and solvents used were of analytical or HPLC grade. Methanol, acetonitrile and orhophosphoric acid were of HPLC grade and purchased from Merck. HPLC grade water was obtained from a Milli-Q analytical deionization system.

Chromatographic conditions

The HPLC system consisted of Waters Alliance (Waters Corporation, MA, USA) equipped with a Waters e-2695 ALLIANCE separation module comprising of a solvent (quaternary gradient mode), auto injector, column oven and a 4 channel in line degasser), a sample management system (sample heater cooler) and a 2998 PDA detector. Chromatographic separation was performed using a Waters, Symmetry shield RP-18, 4.6 mm x 150 cm, 5 microns at 254 nm. Data acquisition was performed by the Empower 2® software. The mobile phase consisted of 0.1 M phosphate buffer, pH 5 (pH adjusted with ortho phosphoric acid) and methanol. The mobile phase ratio was maintained at 50:50 during the first one minute and thereafter at 90:10. The mobile phase was delivered at a flow rate of 0.9 ml/min and the detection of INH was carried out at 254 nm. The injection volume was 20 µL and the analysis was performed at 30°C (sample 4°C).

Preparation of INH SLNs

Solid lipid nanoparticles of INH were prepared using a recently patented method [14,22]. Briefly the lipidic phase (lipid-8%) and the aqueous phase (polysorbate 80, soy lecithin and water) were heated to ~10 degree above the lipid melt temperature. The proportion of surfactant and the volumes of two phases were so adjusted that a microemulsion was formed spontaneously upon mixing the two phases. Hot microemulsion, thus formed was transferred into cold water (~2°C) under constant stirring (WiseTis HG-15 D, 10,000 rpm) to obtain SLNs. The prepared SLNs were used as such for further studies.

Preparation of standard stock solution and working standards

A 100 µg/ml stock solution of INH was prepared in HPLC grade water. The calibration curve was prepared by suitably diluting the stock solution with HPLC grade water to give solutions containing INH in the concentration range of 250 to 25,000 ng/mL. All samples were filtered through 0.20 µm nylon filters before analysis.

Quality-control samples (QCs) were prepared with blank plasma, brain homogenates, liver homogenates and kidney homogenates in low (250, 500, 1000 ng/mL), medium (5,000 ng/mL) and high (25,000 µg/ml) concentrations.

Sample preparation

Solid lipid nanoparticles: Solid lipid nanoparticles were disrupted using chloroform : methanol - 2:1 to quantitate the INH entrapped within them. The clear solution was then filtered through 0.20 µm nylon filters.

Plasma samples: To 150 µL aliquot of plasma, a 300 µL of the deprotinizing agent (methanol) was added and the dispersion was vortexed for 2 minutes. The samples were then centrifuged at 15000 rpm for 10 minutes at 4°C. The supernatant was then collected and an equal volume of water was added to the clear supernatant. The samples were then filtered (0.20 µm nylon filters) and were injected into the HPLC system.

Tissue samples (Brain, liver and kidney): 20% aqueous tissue

Sample type	Concentration added ngml^{-1}	Intraday (n=6)			Interday (n=6)		
		Concentration found (ngml^{-1}) (mean ±sd)	**Precision (% RSD)**	**Accuracy (%RE)**	**Concentration found (ngml^{-1}) (mean ±sd)**	**Precision (% RSD)**	**Accuracy (%RE)**
SLN	**250**	**240 ± 10**	4.2	4.0	**244 ± 10**	4.1	2.4
	500	**490 ± 12**	2.4	2.0	**502 ± 12**	2.4	-0.4
	1000	**998 ± 32**	3.2	0.2	**986 ± 3**	0.3	1.4
	5000	**4980 ± 12**	0.2	0.4	**4985 ± 19**	0.4	0.3
	10000	**9960 ± 23**	0.2	0.4	**9992 ± 12**	0.1	0.1
Plasma	**250**	**239 ± 11**	4.6	4.4	**247 ± 10**	4.0	1.2
	500	**498 ± 23**	4.6	0.4	**503 ± 14**	2.8	-0.6
	1000	**990 ± 42**	4.2	1.0	**999 ± 17**	1.7	0.1
	5000	**4986 ± 12**	0.2	0.3	**4989 ± 13**	0.3	0.2
	10000	**9966 ± 50**	0.5	0.3	**9994 ± 12**	0.1	0.1
Brain	**250**	**247 ± 12**	4.9	1.2	**252 ± 2**	0.8	-0.8
	500	**505 ± 12**	2.4	-1.0	**498 ± 13**	2.6	0.4
	1000	**999 ± 29**	2.9	0.1	**991 ± 21**	2.1	0.9
	5000	**4988 ± 63**	1.3	0.2	**4980 ± 21**	0.4	0.4
	10000	**9999 ± 22**	0.2	0.0	**9978 ± 18**	0.2	0.2
Liver	**250**	**254 ± 7**	2.8	-1.6	**252 ± 11**	4.4	-0.8
	500	**510 ± 13**	2.5	-2.0	**490 ± 10**	2.0	2.0
	1000	**1010 ± 12**	1.2	-1.0	**1002 ± 21**	2.1	-0.2
	5000	**4992 ± 34**	0.7	0.2	**4997 ± 39**	0.8	0.1
	10000	**9993 ± 46**	0.5	0.1	**9990 ± 21**	0.2	0.1
Kidney	**250**	**251 ± 4**	1.6	-0.4	**249 ± 8**	3.2	0.4
	500	**489 ± 8**	1.6	2.2	**510 ± 12**	2.4	-2.0
	1000	**1004 ± 23**	2.3	-0.4	**998 ± 34**	3.4	0.2
	5000	**5012 ± 34**	0.7	-0.2	**4988 ± 23**	0.5	0.2
	10000	**9989 ± 54**	0.5	0.1	**10010 ± 34**	0.3	-0.1

Table 1: Precision and accuracy of the method.

homogenates were prepared in cold 150 mm KCl. The homogenates were then centrifuged at 15000 rpm for 10 minutes at 4°C and the clear supernatant thus obtained was used further. To 150 µL aliquot of the clear tissue homogenates, a 300 µL of the deprotinizing agent (methanol) was added and the dispersion was vortexed for 2 minutes. The samples were centrifuged at 15000 rpm for 10 minutes at 4°C. The supernatant was collected and an equal volume of water was added to the clear supernatant. The samples were filtered (0.20 µm nylon filters) and were injected into the HPLC system.

Method validation

Linearity: Calibration curve was constructed with 5 concentrations ranging from 0.250 to 25.000 µg ml^{-1} in blank SLNs, plasma and tissue homogenates (brain, liver and kidney). The peak area ratio of the drug was considered for plotting the linearity graph. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

Accuracy and precision: Aliquots of INH from the disrupted SLNs/plasma/tissue homogenate were spiked with INH at different concentrations. The concentrations were injected in duplicate and area responses were compared to the reference standard. The value of average deviation and standard deviation were calculated. Accuracy was determined at four different concentrations by comparing the measured value with that of the standard. Percent accuracy of the four drug concentration studied was found to be close to 100.00%. The respective RSD indicating high closeness of the measured value to the true value are shown in Table 1.

Precision, as defined by USFDA, as the "degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of homogeneous samples".

Interday: For the assay of the inter-day precision and the accuracy, five consecutive batches of QC samples were made by the same procedure on five different days. Each day, a freshly prepared calibration curve was constructed when the INH samples were extracted. The precision was reported as the Relative Standard Deviation (RSD, %) and the accuracy as the Relative Error (RE, %).

Intraday: To evaluate the intra-day precision and accuracy, INH samples at low, middle and high concentrations were extracted and replicate (n=6) determinations within the same QC sample was performed.

Limit of detection (LOD) and lower limit of quantification (LOQ): The Limit of Detection (LOD) was defined as the concentration with a signal-to-noise (*s/n*) of at least 2. The Lower Limit of Quantification (LLOQ) was defined as the lowest concentration with a signal-to-noise ratio of 10.

Recovery: The recoveries were assessed by comparing the peak area of the target compounds extracted from QC samples to those of the same concentration of target compounds spiked into blank-plasma post-extracts.

System suitability: The system suitability was assessed by replicate analysis of six injections of the drug at a concentration of 250, 500, 1000, 5000 and 25,000 ng ml^{-1}. The acceptance criterion was ± 2% for the percent coefficient of variation (%CV) for the peak area and retention times for INH. The number of theoretical plates should be >2500 and the tailing factor should be <2.0.

Stability: Stability experiments were carried to assess the stability of the target compounds in stock solutions, post-extracted solutions and plasma samples.

The short-term stability of the target compounds in the plasma was evaluated by leaving QC samples at 4°C temperature for 24 h. The long-term stability was determined by storing QC samples at 4°C for 1 week. The post-extracted stability was measured by testing the extract solution of QC samples kept at 4°C. All QC samples for stability testing included low, middle and high concentrations, and each concentration had five repeats. During each analytical run, a standard curve was constructed to calculate the concentration of the target compounds.

Result and Discussion

The calibration curve was found to be linear in the tested concentration range for INH. The r^2 values indicated that the developed method significantly conformed to the prerequisites of linearity in the studied range (Table 2). The LOD was determined from the software calculated signal to noise ratio (2:1). The value of LOQ represents the concentration which could be quantified reliably with a specified level of accuracy and precision. LOQ was also determined from the software calculated signal to noise ratio (10:1) (Table 3). Recently a validated HPLC tandem mass method to determine isoniazid in plasma and tissue samples with a LOD of 40 ng/mL (tissue) and a LOQ of 110 ng/mL (tissue) has also been reported in the literature [22]. With an aim of improving the LOD and LOQ we have introduced a minor change in the conventional sample preparation steps reported for isoniazid wherein the samples were directly analyzed in the deproteinizing solvents after centrifugation/filtration. A dilution of the clear deproteinizing

SNo.	Sample description	Concentration Range (µg/mL)	Equation	r^2
1	SLN	0.250 to 25.000	Y = 3.50e+001 X - 6.93e+001	0.999
2	Plasma	0.250 to 25.000	Y = 4.49e+001 X + 1.40e+004	0.999
3	Brain	0.250 to 25.000	Y = 5.52e+001 X - 1.95e+004	0.996
4	Liver	0.250 to 25.000	Y = 3.77e+001 X - 5.91e+003	0.990
5	Kidney	0.250 to 25.000	Y = 1.45e+001 X + 2.70e+002	0.997

Table 2: Linearity and range of the developed method.

S. NO.	Sample description	LOD ng ml^{-1}	LOQ ng ml^{-1}
1	SLN	150	150
2	Plasma	150	200
3	Brain	150	200
4	Liver	150	200
5	Kidney	150	200

Table 3: Limit of detection and quantification of isoniazid in SLNs in plasma and in organ homogenates.

Sample type	Concentration added µgml^{-1}	% mean recovery	% RSD
Plasma	0.200	92	4.0
	1.000	96	3.3
	10.000	94	3.2
Brain	0.200	90	1.9
	1.000	92	1.7
	10.000	92	1.1
Liver	0.200	90	2.5
	1.000	92	4.5
	10.000	91	4.9
Kidney	0.200	87	3.4
	1.000	90	2.3
	10.000	88	3.9

Table 4: % mean recovery of INH from plasma and tissue homogenates.

solvent with water resulted in an increase in the polarity of isoniazid resulting in a consequent sharp peak and hence a low LOD and LOQ. Intra and inter-day variability's (RSD) for extraction of INH from plasma and other tissue homogenates were less than 5% and accuracy was within ± 5% (Table 1) and were comparable with other reported methods of isoniazid [15,16,23].The absolute recovery of INH was calculated for replicate spiked QC samples (200, 1000 and10,000 ng/mL) (Table 4). Extraction recovery is calculated by comparing the peak area ratios of INH in spiked samples and INH standard respectively at a concentrations ranging from 200 to 10,000 ng/mL. Results show an overall mean percent recovery of above 90 %. It may be noted that as per the US FDA guidelines of May 2001, recovery pertains to the extraction efficiency of an analytical method within the limits of variability. It is stated that recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Hence the method developed and reported presently though showing a recovery of above 90% may be considered suitable taking into account the consistency and reproducibility of the results obtained upon repetitive evaluation.

System suitability

The % RSD of peak area and retention time for the drug were within 2% for all the samples indicating the suitability of the system. The efficiency of the column was expressed by number of theoretical plates for the 6 replicate injections and was above 4000 and the USP tailing factor was below 1.5 for all the samples.

Stability: The results of all stability tests indicated that the target compounds in plasma and tissue samples were stable during sample storage, extraction and chromatographic analysis. The method can be used for the routine analysis of INH in plasma, brain, liver and kidney (small retention time). No degradation of INH in plasma was observed as reported earlier [19].

The extraction of INH from plasma and tissue homogenates was achieved by simple deproteination with methanol, which resulted in simple, convenient and rapid separation of the analyte. Both INH and its metabolite acetyl isoniazid show very similar spectral characteristic [18]. The chromatograms were evaluated for the peak purity in order to determine interferences from the metabolite of INH. Purity angle to purity threshold ration of 1:50 (and/or above) was observed in all the chromatograms, hence concluding that the peaks were pure and no interference from co eluting peaks.

Conclusion

The developed HPLC method of analysis for INH exhibited well resolved peaks. The short retention times indicated that a large number of samples could be completed in a short duration. Therefore the method can be used for analyzing large number of samples. Significantly low LOD and LOQ values for both the drugs suggest suitability of the method for detecting and quantifying fairly low concentrations of these agents. Low RSD values (i.e., <2.0%) in accuracy, and precision ratify the adequacy of the analytical assay. Thus it may be concluded that the developed method is simple, reproducible, selective and rapid.

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None

Declaration of Interest

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