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ORIGINAL ARTICLE

Quantification of Pyrazinamide in Human Plasma by Validated High Performance Liquid Chromatography Method

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ABSTRACT

Objective: To be able to accurately determine the quantity of Pyrazinamide (PZA) in different tablet preparations and human plasma using an Ultra violet detector equipped high performance liquid chromatography (HPLC).

Study Design: Experimental study

Place and Duration of Study: Department of Bioequivalence Studies, University of Veterinary and Animal Sciences Lahore and the Department of Pharmacology, University of Health Sciences, Lahore the from 1st April 2017 to 31st March 2018.

Methodology: Two mobile phases were used, the first compromised of disodium hydrogen phosphate buffer having a pH of 6.8 and acetonitrile in the proportion of (95:5) and the second was a combination of aforesaid substances in equivalent proportion (50:50 v/v). The gradient for the first 5 min was exclusively Mobile phase "a" after which 5-6 min Mobile phase "b" was raised from 0 to 100% and was kept at 100% till the completion of the cycle**.** The flow of mobile phase was kept at 1000 µl/min. Determination of PZA was done using a ultraviolet detector at a wavelength of 238 nm. Amount of sample injected was 40 µl. Procedure was done by using Shizmadu Chromatographic System, Japan equipped with a SIL-20AC HT auto-sampler, SPD-M20A, CTO 20 AC, a LC-20AT VP pump, and CBM 20A controller unit**.** A C_{18} column was used as well.

Results: Retention time of PZA was 6.1±2%. Precision was 0.46 to 2.20% relative standard deviation for intra assay and for inter assay we obtained 0.29 to 34.45% RSD for all quality control levels. The overall recovery of PZA was 96.75%.

Conclusion: High selectivity for PZA was seen and no other spikes from drugs present in FDC regimen were observed at the time when PZA is detected in blank plasma samples

Key words: Chromatography, High pressure liquid. Pyrazinamide. Tuberculosis

INTRODUCTION

Pyrazinamide is one of the four first line drugs used to treat tuberculosis (TB). Alongside rifampicin, isoniazid, and ethambutol, it is given for the first 8 weeks of treatment.[1] While rifampicin and isoniazid are considered the most effective drugs in the treatment of tuberculosis pyrazinamide also has a very important role. It is used to prevent emergence of resistance along with cover in case of primary resistance to rifampicin and isoniazid. But it also has one more important role that is it kills latent bacteria and is therefore reduces the overall treatment duration from 9-12 months to only 6 months.[2]

Pyrazinamide is related to nicotinamide structurally, it is primarily used for the treatment of TB. It is slightly soluble but stable in water. At a pH of 7 it is inactive, but active at a pH of 5.5. Inhibition of tubercle bacilli starts with a blood concentration of 20 µg/ml.[3] Macrophages take up the drug where it exerts it effect on mycobacteria present inside the acidic lysosomes.

Pyrazinamide is the inactive form of the drug. It is converted by mycobacterial pyrazinamide to its active form pyrazinoic acid (POA). Pyrazinamidase is encoded by pncA.[4] Cell membranes of mycobacterium are disrupted along with transport functions as well by POA. Dosing at 25mg/kg/day orally achieves a peak serum concentration within 1-2 hours of about 30-50 µg/ml.[5]

From the gastrointestinal tract PZA is well absorbed following which it is distributed widely in the body including in meningitis. The half-life is 8–11 hours. Pyrazinamide is hepatically broken down, following which it is eliminated through the kidneys. Patients taking twice or thrice weekly treatment regimens, 40-50 mg/kg dose is used.[6]

Susceptibility of isolates of *Mycobacterium tuberculosis* (MTB) to PZA is dependent on pyrazinamidase activity, and loss of pyrazinamidase activity has been associated with PZA resistance. Pyrazinamidase and nicotamidase have been found to be lacking in PZA-resistant strains of MTB and therefore are cross resistant with nicotinamide is seen. Prevention of conversion of PZA to its active form due to pncA mutations or failure to uptake PZA has been attributed to the cause of resistance.[7]

Hepatotoxicity (1-5%) is one of the major adverse effects alongside hyperuricemia, drug fever, nausea and vomiting. Even though hyperuricemia is seen consistently with the use of PZA this alone does not warrant to stop its use. An acute attack of gouty arthritis may result due to the elevated uric acid levels. Intermittent dosing (two to three times a week) lets uric acid to be removed in the meanwhile preventing accumulation and hence adverse effects due to hyperuricemia.[8]

MATERIALS AND METHODS

This research was performed at department Bioequivalence Studies, University of Veterinary and

Animal Sciences, Lahore and the Department of Pharmacology, University of Health Sciences, Lahore from 1st April 2017 to 31st March 2018. Shazo Zaka pharmaceuticals provided working standard of PZA with a purity of 98%. Acetonitrile, disodium hydrogen phosphate, water and methanol were of HPLC grade. All chemicals were procured from Merck Ltd.

A C_{18} column (250 mm x 4.6 mm, particle size 5000 nm) from Merck limited, Germany was employed. The HPLC was carried out at ambient temperature using Shizmadu Chromatographic System, Japan equipped with a SIL-20AC HT auto-sampler, SPD-M20A, CTO 20 AC, a LC-20AT VP pump, and CBM 20A controller unit. All preparations were mixed using a Vortex mixer, model no. M37610-33, Barstead international, USA. Separation was carried out using a centrifugation machine, EBA20, Germany

Stock solution was prepared by taking 50mg of PZA, accurately weighed, in a 50ml volumetric flask then added 5 ml methanol which was sonicated for 5 minutes. Then, using mobile phase "a", the volume was made up to the mark. This made a standard solution containing of 1000 µg of PZA per ml. By diluting with blank plasma, strengths of 1000,2000,4000,6000,8000 and 10000 ng/ml were made. Similarly, quality control standard was made of 3000, 5000 and 9000 ng/mL.[9,10]

Disodium hydrogen phosphate solution of 0.01M was made by weighing 1.4 g of Disodium hydrogen phosphate and dissolving it in 0.20 L of HPLC grade distilled water which was then made up to 1.0 L. Orthophosphoric acid was used to adjust the pH to 7.0. Filtration of all chemicals was done via filtration device with filter paper having a pore size of 450 nm.

A pair of mobile phases were utilized. The first, Mobile phase "a", combined disodium hydrogen phosphate buffer and acetonitrile (95:5 v/v) and the second, mobile phase "b", combined the aforesaid substances in equal proportion (50:50 v/v). The gradient for the first 5 min was exclusively Mobile phase "a" only after which 5-6 min Mobile phase "b" was raised from 0 to 100% and was kept at 100% till the completion of the cycle. During the run the flow rate was maintained at 1000 µl/min. Determination was done using a UV detector at a wavelength of 238 nm. The amount of sample injected was 40 µl.

For preparation of samples, frozen plasma was thawed ambiently. After reaching ambient temperature the plasma was put through a vortex mixer for a minute. In an Eppendorf tube we took 1ml of the thawed plasma along with 700 micro liters of HPLC grade methanol. Using a vortex mixed we mixed the mixture for 5 minutes following which centrifugation of the mixture was done for 10 min at 5000 rpm. The clear liquid above the precipitate was then removed and filtered using 0.2 µm syringe filter and shifted into HPLC vials. The HPLC vial were placed into the autosampler to be injected into HPLC system. Validation was performed following the guidelines laid down under the International Council for Harmonization Of Technical Requirements for Pharmaceuticals for Human Use guidelines and Food and Drug Administration guidance for industry.[9,10]

Pyrazinamide exhibited reasonable retention as well as reasonable peak shape. Typical PZA chromatogram is given in Fig. 1.

To assess the anticipated range of the drug linearity was used. Pyrazinamide standard solutions ranging from 1000-10,000 ng/ml were utilized to evaluate linearity. Calibration curve was constructed of concentration versus area ratio. Least squared regression analysis was employed to estimate linearity and concentrations

To estimate system correctness six replicate injection results were used. Precision (intraday) and Precision between days (inter-day) was also done to asses nearness of replicate in the determination of PZA in the drug assay. Accuracy was calculated by estimating percentage of drug recovered.

Selectivity was confirmed by finding no interfering peaks in the retention time of PZA after inserting samples. By relating the new samples results with light explored, freeze and thaw at ambient, 24 hours and 48 hours ambient temperature sample results stability was judged.

To examine the recovery Low, medium and high-Quality control checks of plasma concentration were used. Percentage mean recoveries were determined by analyzing the responses between solvent and extracted quality control samples.

RESULTS

Pyrazinamide exhibited reasonable retention as well as reasonable peak shape. Characteristic chromatogram of PZA in plasma and PZA in solvent are shown in figures 1 and 3. Pyrazinamide was separated under optimized HPLC conditions and a retention time 6.1-6.3 was observed. Accuracy of 68.94%-116.02% was seen overall with relative standard deviation (RSD) under 5% for three consecutive batches of calibration curves. A range of 1000-10,000 was applied for PZA. ng/ml (Table 1).

The procedure was found to be highly precise and accurate. Intraday samples obtained an accuracy of 96.3 to 101.88% and 76.33 to 98% for inter assay accuracy. Precision for all quality control levels was 0.46 to 2.20% RSD and 0.29 to 34.45% for intra and inter assay respectively (Table 2)

Table 1: Precision and accuracy from three validation batches of calibration standards of pyrazinamide in human plasma

Analysis batch	Theoretical concentration (ng/ml)					
	1000	2000	4000	6000	8000	10,000
	Concentrations measured (ng/ml)					
A	724	2345	3960	6156	8183	10099
B	662	2311	3978	6159	8019	9868
C	681	2304	3819	6091	8032	9889
N	3	3	3	3	3	3
Mean	689	2320	3919	6135	8078	9952
RSD (%)	3.797241	0.775987	1.81223	0.511291	0.920041	1.04507
Accuracy	68.94	116.02	97.99	102.26	100.97	99.52

No significant interference peaks were seen in PZAs retention region in all six replicates of blank matrix samples tested as shown in chromatogram of blank plasma (figure 2). This shows our method is selective for PZA. Recovery results were consistent for all QC levels. Overall the recovery of PZA was found to be 96.75% (Table 3). Table 4 shows the stability results. Samples were reinjected after storage at room temperature for 24 and 48 hours and two freeze/thaw cycles. The results show the ruggedness and robustness of the method.

Table 2: Results of Precision and accuracy for high-pressure liquid chromatography procedure

Analysis batch	Theoretical concentration (ng/ml)		
	3000	5000	9000
Precision day 1			
N	6	6	6
Intra assay mean	2890	5090	9150
RSD	2.20	0.67	0.46
Accuracy	96.33	101.88	101.67
Precision day 2			
N	6	6	6
Intra assay mean	2290	5900	8800
RSD	34.45	0.29	1.25
Accuracy	76.33	98.00	97.78

Table 3: Recovery for pyrazinamide at low, medium and high-quality control levels

Qc levels	Statistics	Pyrazinamide
Low (3 µg/ml)	RSD	0.94/0.97
	recovery	90.4%
Mid (5 µg/ml)	RSD	1.17/1.30
	Recovery	99.2%
High (9 µg/ml)	RSD	0.38/0.94
	Recovery	100.65%
Overall	Recovery	96.75%

Table 4: Stability of high pressure liquid chromatography method under varying conditions

Statistics	Pyrazinamide	
	Low QC (3000ng/ml)	High QC (9000ng/ml)
Room temp 24 h		
N	3	3
RSD	1.52	0.52
Accuracy (%)	98.44	101.37
1st freeze thaw cycle		
N	3	3
RSD	0.28	0.41
Accuracy (%)	95.67	100.67
2nd freeze thaw cycle		
N	3	3
RSD	0.94	0.05
Accuracy (%)	92.55	100.18

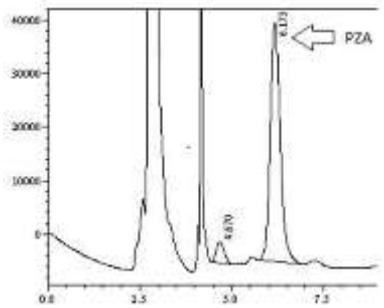

Fig. 1: Typical chromatogram of pyrazinamide

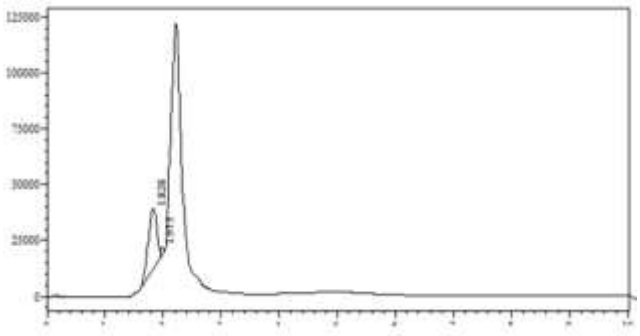
Fig. 2: Blank plasma

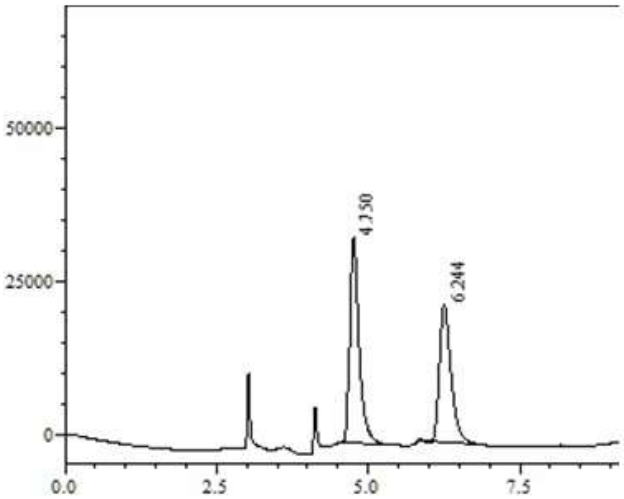

Fig. 3: Pyrazinamide in solvent

DISCUSSION

In this research a HPLC method was developed and validated for plasma containing PZA. A single step LLE extraction process involving methanol as the extracting solvent was used. We employed LLE as these render the sample clean of any polar interferences.

Noise is usually minimum or absent as compared to solid phase extraction or precipitation. Volume of plasma needed is very small for this assay and requires easily available and simple to use reagents and laboratory equipment. Compared to other methods where multiple steps requiring precipitation centrifugation drying under nitrogen stream and then reconstitution ours is simple and easy to adopt.[11]

A pair of mobile phases were utilized. The first, Mobile phase “a”, was disodium hydrogen phosphate buffer and acetonitrile (95:5 v/v) and mobile phase “b” was a combination of aforesaid substances in even proportion (50:50 v/v). Other methods using 2 mobile phases involved mobile phase “a” having 0.1% trifluoracetic acid in water and mobile phase “b” was 0.1% trifluoracetic acid in methanol with a gradient of 2% of 2 till 0.5min increased to 15% at 1.7 min brought up to 85% and kept till 2.9 min after which only 2% of mobile phase “b” is used.[11] Our flow rate throughout was kept at 1000 µl/min which is in keeping with other studies. Overall accuracy for the calibration curves

was 68.94%-116.02% with relative standard deviation below 5%. The results are similar to other studies.[11]

Precision and accuracy were 96 to 101% with an RSD of 0.4 to 2.2%. Other studies showed accuracy of 96 to 114 with an RSD of 1.4 to 10.2%. Recovery of PZA was 96.75% which is comparable to other studies which show PZA having a recovery of 101%.[11,12] Stability was seen to be 92 to 98% at low QC and 100 to 106% at high QC. This is comparable to other studies that show 95% at low QC and 87% at high QC levels. [11] This HPLC method is simple, highly accurate, precise, and sensitive. A retention time of 6.1 min ± 2% was seen for PZA which is similar to other retention times.[12]

Tuberculosis is endemic in Pakistan its effective treatment is very crucial. Pyrazinamide is one of the major drugs that is used in the treatment of TB.[13] By adding PZA to the regimen it reduces the treatment duration form 9-12 months to 6 months. After knowing therapeutic levels of PZA are achieved in the blood or not can have a major impact on duration of therapy. Therefore, we set out to develop and validate a cheap and relatively simple method for PZA estimation in plasma. The method was found to be fully validated according to the SOPs laid down under ICH guidelines. Only PZA was evaluated in present study due to financial constraints

CONCLUSION

A rapid and robust method was developed for the anti-tubercular drug pyrazinamide. The method is fully validated and can be used directly to analyze patient's samples.

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