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An improved LC–MS/MS method for the simultaneous determination of pyrazinamide, pyrazinoic acid and 5-hydroxy pyrazinoic acid in human plasma for a pharmacokinetic study

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ABSTRACT

In the present work the plasma levels of PZA and its two active metabolites, pyrazinoic acid (PA) and 5-hydroxy pyrazinoic acid (5-OH PA) were determined by a sensitive and rapid LC–MS/MS method. The analytes and their labeled internal standards were extracted from 200 μL plasma samples by liquid-liquid extraction with methyl *tert*-butyl ether: diethyl ether (90:10, *v/v*) under acidic conditions. Their separation was achieved on a Zorbax Eclipse XDB C18 (100 × 4.6 mm, 3.5 μm) column using methanol and 0.1% acetic acid (65:35, *v/v*) as the mobile phase within 4.0 min. Detection and quantitation were done by multiple reaction monitoring on a triple quadrupole mass spectrometer following the transitions, *m/z* 124.1 → 81.1, *m/z* 125.0 → 80.9 and *m/z* 141.0 → 81.0 for PZA, PA and 5-OH PA respectively in the positive ionization mode. All the analytes were baseline resolved with a resolution factor of 3.3 and 6.4 between PZA and its metabolites, PA and 5-OH PA respectively. The calibration curves were linear from 0.100–30.0 μg/mL, 0.03–9.00 μg/mL and 0.002–0.600 μg/mL for PZA, PA and 5-OH PA respectively with $r^2 \geq 0.9980$ for all the analytes. The intra-batch and inter-batch accuracy and precision (% CV) across quality controls varied from 93.5–106.7% and 1.10–4.57 respectively for all the analytes. The mean extraction recovery of PZA, PA and 5-OH PA was 83.7%, 89.2% and 80.8% respectively, which was consistent at higher as well as lower concentration levels. The% change in the stability of analytes under different storage conditions ranged −6.7 to 7.1 for all the analytes. The method was applied to assess the comparative bioavailability of a 500 mg PZA test and reference formulation in healthy subjects. The assay reproducibility was also tested by reanalysis of 22 incurred subject samples.

1. Introduction

PZA, an amide derivative of pyrazine-2-carboxylic acid is considered by World health Organization (WHO) to be a pivotal sterilizing drug for the treatment of TB in combination with other drugs for short-course chemotherapy [1]. The combination of PZA with isoniazid and rifampicin offers strong synergistic and accelerating effect for the treatment and therefore it is highly recommended in every combination therapy [2]. Due to the presence of PZA in the combination therapy there is significant reduction in the duration of current chemotherapy regimens [3]. Although the mode of action of PZA is not completely known, it is likely that during the early phase of inflammation PZA targets bacilli residing in acidified compartments of the lung [2]. PZA enters *Mycobacterium tuberculosis* by passive diffusion and through an ATP-dependent transport system [4]. This intracellular accumulation takes place because of an inefficient efflux system unique to *M. tuberculosis* [5].

PZA is a pro-drug and gets converted to its active form pyrazinoic acid (PA) by the enzyme pyrazinamidase. PZA is well absorbed orally and mainly metabolized by the liver. The plasma half-life of PZA is about 3–4 h. It is rapidly absorbed from the gastrointestinal tract to achieve peak serum concentration of 6–8 μg/mL at 1.5–2.0 h after oral administration [6]. Another important metabolite of PZA is 5-hydroxy pyrazinoic acid (5-OH PA) which is formed by the action of xanthine oxidase on PA via oxidation process. The same enzyme is also responsible for the conversion of PZA to 5-hydroxy pyrazinamide (5-OH PZA). One more but less significant metabolite pyrazinuric acid (PZU) is produced when PA combines with glycine. In the treatment involving PZA, measurement of PZA and PA in human plasma is essential to prevent or minimize the risk of

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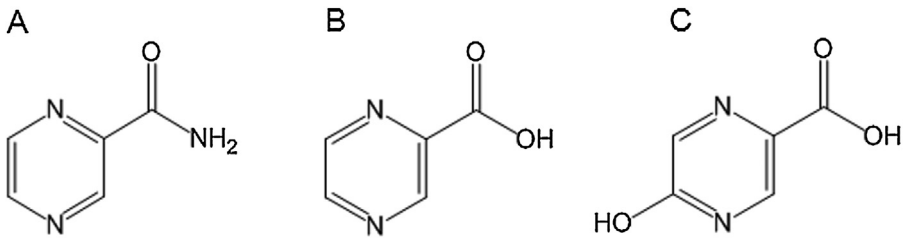

Fig. 1. Chemical structures of the analytes (A) pyrazinamide, (B) pyrazinoic acid and (C) 5-hydroxy pyrazinoic acid.

side-effects, hepatic toxicity and hyperuricaemia which are associated with higher dosages. At the same time estimation of 5-OH PA is also considered to be an important part of drug monitoring studies especially in the patients with xanthinuria [6].

Several chromatographic methods are reported for the quantification of PZA as a single analyte in a variety of matrices such as human plasma [7–9], broncho alveolar lavage and alveolar cells [7], bone tissue [10], rat blood, brain and bile [11]. Similarly, methods pertaining to simultaneous determination of PZA with other antiTB drugs in binary [12–14], ternary [14–17] and in multiple drug combinations [18–25] are also available in literature. However, there are few reports for the analysis of PZA and its metabolites in biological fluids [26,27]. Only one report discusses the separation mechanism of PZA and its metabolites using ion-pair HPLC [27].

Thus, in the present work an improved, fast, sensitive and selective LC–MS/MS method has been developed and validated as per the USFDA guidelines for simultaneous measurement of PZA, PA and 5-OH PA (Fig. 1) in human plasma using labeled internal standards. The developed method was successfully applied to support a pharmacokinetic study with 500 mg PZA tablet formulation in 6 healthy subjects. The reproducibility in the measurement of study data has been established by reanalysis of incurred samples.

2. Experimental

2.1. Chemicals and materials

References standards of drugs and their labeled ISs namely pyrazinamide (PZA, 99.5%), pyrazinoic acid (PA, 97.8%), 5-hydroxy pyrazinoic acid (5-OH PA, 98.0%), pyrazinamide-d3 (PZA-d3, 98.5%), pyrazinoic acid-d3 (PA-d3, 96.0%), 5-hydroxy pyrazinoic acid-13C3 (5-OH PA-13C3, 98.4%) were purchased from Toronto Research Chemicals Inc. (Ontario, Canada). HPLC grade methanol was obtained from Mallinckrodt Baker S.A. de C.V. (Ecatepec, Mexico). Analytical reagent grade methyl *tert*-butyl ether (MTBE), diethyl ether (DEE) and acetic acid (AA) glacial (100%) were procured from Merck Specialties Pvt. Ltd. (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at −70 °C until use.

2.2. Liquid chromatography and mass spectrometry conditions

A Shimadzu Nexera X2 LC-30 AD UHPLC system (Kyoto, Japan) was used for setting reversed-phase liquid chromatographic conditions. The separation of PZA, PA and 5-OH PA was carried out on an Agilent, Zorbax Eclipse XDB-C18 (100 × 4.6 mm, 3.5 μm) column maintained at 40 °C in a column oven. The mobile phase consisted of methanol and 0.1% acetic acid in water (65:35, *v*/*v*). For isocratic elution, the flow rate of the mobile phase was kept at 0.8 mL/min with 50% flow splitting; flow directed to the ion spray interface was equivalent to 400 μL/min. The autosampler temperature was maintained at 5 °C and the pressure of the system was 1450 psi.

The UHPLC system was connected to a triple quadrupole mass spectrometer MDS SCIEX API-4000 (Toronto, Canada). The MS/MS system was equipped with a turbo ionspray ion source and operated in positive ESI mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transitions of drug and their metabolites. The optimized source parameters and compound dependent mass parameters for analytes and their labeled internal standards (ISs) are summarized in Table 1. Quadrupole 1 and 3 were maintained at unit mass resolution. In order to control all parameters of UHPLC and MS, an Analyst software version 1.5.2 was used.

Table 1
Optimized mass spectrometer parameters, MRM transitions and chromatographic performance.

Parameters	PZA	PZA-d3	PA	PA-d3	5-OH PA	5-OH PA-13C3
Mass spectrometry parameters						
Source dependent						
Ion spray voltage (kV)				5500		
Turbo heater temperature (°C)				300		
Nebulizer gas (Gas 1; psig)				60		
Heating gas (Gas 2; psig)				55		
Collisional activation dissociation (psig)				8		
Curtain gas nitrogen (psig)				35		
Compound dependent						
Entrance potential (V)	10	10	10	10	10	10
Collision energy (eV)	22	22	15	15	31	31
Dwell time (ms)	100	100	100	100	100	100
MRM transition (*m*/*z*)	124.1/81.1	127.1/84.1	125.0/80.9	128.0/83.9	141.0/81.0	144.0/84.0
Chromatography characteristics						
Retention time (min)	3.21	3.22	2.40	2.41	1.61	1.60
Capacity factors (*k*)	3.94	3.95	2.69	2.71	1.48	1.47
Theoretical plates	5088	5120	2845	2868	1280	1264

PZA: Pyrazinamide; PZA-d3: Pyrazinamide-d3; PA: Pyrazinoic acid; PA-d3: Pyrazinoic acid-d3; 5-OH PA: 5-hydroxy pyrazinoic acid.
5-OH PA-13C3: 5-hydroxy pyrazinoic acid-13C3.