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Original Article

Development and validation of high performance liquid chromatographic method for the determination of pyrazinamide in human plasma

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ABSTRACT

Background: Pyrazinamide is one of the key APIs used in the combination treatment of tuberculosis recommended by WHO. Since sufficient analytical methods have not been reported officially for the quantitative estimation of pyrazinamide, there is necessity for investigation of new analytical methods for quantitative estimation of pyrazinamide in human plasma.

Objective: A rapid, sensitive, simple and cost-effective high performance liquid chromatographic method for the determination of pyrazinamide by UV detection in human plasma is to be developed and validated.

Materials and methods: The extraction process involved a liquid–liquid extraction using a 70:30% v/v mixture of t-butyl methyl ether and dichloromethane. Both pyrazinamide and the internal standard were eluted under isocratic mode using a 150 x 4.6 mm i.d, 5 μm Phenomenex ODS 2 C18 column. The mobile phase was composed of a mixture of 15:85 % v/v methanol and 0 mM potassium dihydrogen phosphate (pH adjusted to 7.4) at a flow rate of 1.0 ml/min. The wavelength of detection was 268 nm. The injection volume was 20 μl. The runtime of the method was 8 min.

Results: The method showed good linearity in the range of 1.02–50.23 μg/ml. The overall recovery of pyrazinamide was 27.21% with a CV of 2.71% and recovery of internal standard was 83.34% with a CV of 4.38%.

Conclusion: A rapid, sensitive, simple and cost effective method for the estimation of pyrazinamide in human plasma using metronidazole as internal standard was developed and validated according to FDA guidelines.

1. Introduction

Treatment of tuberculosis is now very complex because of the emergence of multi drug resistant bacteria, which are resistant to first-line anti-tuberculosis drugs, pyrazinamide, isoniazid and rifampin.[1] Pyrazinamide (Fig. 1) is used extensively in the treatment of tuberculosis together with rifampicin, isoniazid and ethambutol.[2] The structure of pyrazinamide is given by Fig. 1 and the structure of metronidazole is given by Fig. 2. It has a plasma half-life of 3–4 h, and is quickly absorbed from the

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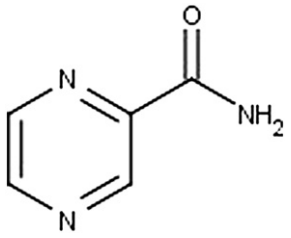

Fig. 1 – Structure of pyrazinamide.

gastrointestinal tract with peak serum concentrations of 6–8 μg/ml occurring 1.5–2.0 h after administration.[3]

The determination of PZA levels in biological fluids was carried out earlier by spectroscopic methods,[4–6] colorimetric methods[7] and gas chromatographic–mass spectrometric technique.[8] A survey of literature revealed that HPLC technique has been used for the determination of pyrazinamide in pharmaceuticals.[9] A HPLC technique reported earlier had a step of very tedious extraction.[10] Another HPLC technique also involved a preliminary extraction of the drug and an internal standard, paracetamol, from acidified rabbit plasma samples (pH 4.2).[11] Since sufficient analytical methods have not been reported for the quantitative estimation of pyrazinamide, there is a necessity for investigation of selective and sensitive new analytical methods for quantitative estimation of pyrazinamide in human plasma. Additionally, pyrazinamide has a strong chromophore showing reddish brown color at wavelength of 268 nm. This chromophore not only allows for successful determination in human plasma by UV detection but also offers acceptable sensitivity as offered by LC-MS/MS detection.

Although LC-MS/MS is a versatile tool, the development of HPLC based separation methods makes it more economical and simpler both in terms of maintenance and data interpretation. The present article describes a simple and sensitive RP-HPLC method with a low LLOQ for UV detection of PZA using metronidazole (Fig. 2) as an internal standard (IS) eluted under isocratic mode which can be directly applied to the successful estimation of rifampicin in a bioequivalence study and to validate the developed method according to FDA guidelines.[12]

2. Materials and methods

2.1. Solvents and chemicals

Pyrazinamide (purity 98.00% w/w) was used as received from Lupin Laboratories Ltd. Metronidazole (MTZ) (used as internal standard, purity 99.0% w/w) is purchased from Sigma Aldrich Inc. HPLC grade methanol and potassium dihydrogen phosphate (purified grade) were purchased from Merck Ltd (Mumbai, India). Deionized water was processed through a Milli-Q water purification system (Millipore, USA). All other chemicals and reagents were of analytical grade.

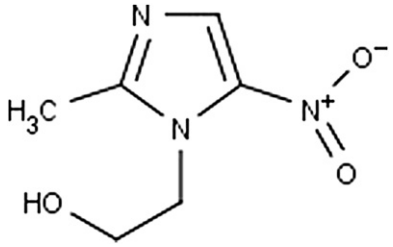

Fig. 2 – Structure of metronidazole.

2.2. Chromatographic system

The chromatographic system consisted of a Shimadzu Class VP Binary pump LC 10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV–Visible Detector. All the components of the system were controlled using SCL-10Avp System Controller. Data acquisition was done using LC Solutions software. The detector is set at a wavelength of 268 nm. Chromatographic separations were accomplished using a Phenomenex C_{18}, 5 μm, 150 mm × 4.6 mm column. The mobile phase was composed of a mixture of 15 parts of methanol and 85 parts of 10 mM potassium dihydrogen phosphate (pH 7.4), adjusted with potassium hydroxide. The mixture was filtered through 0.22 μm membrane (Millipore, Bedford, MA, USA) under vacuum, and then degassed by flushing with nitrogen for 5 min. The mobile phase was pumped isocratically at a flow rate of 1.0 ml/min during analysis, at ambient temperature. The rinsing solution consisted of a mixture of 50: 50% v/v of methanol: HPLC grade water.

2.3. Preparation of standard solutions

A stock solution of pyrazinamide was prepared in diluent solution (mixture of 50:50% v/v of methanol: HPLC grade water) such that the final concentration was approximately 10 mg/ml. Stock solution of metronidazole (approx 5 mg/ml) is prepared in HPLC grade methanol. The solutions were stored at 4 °C and they were stable for two weeks.

2.4. Sample preparation

Aqueous stock dilutions were prepared initially. Aqueous stock dilution, 0.5 ml each, was transferred into a 10 ml volumetric flask. The final volume was made up with screened drug-free K_2EDTA human plasma and mixed thoroughly for 5 min to achieve the desired concentration of calibration curve standards. The final calibration standard concentrations were 0.0 (Blank; no pyrazinamide added), 1.02, 2.04, 4.29, 7.96, 14.09, 28.18, 45.33 and 50.23 μg/ml. Each of these standard solutions was distributed into disposable polypropylene micro centrifuge tubes (2.0 ml, eppendorf) in volume of 0.7 ml and the tubes were stored at −70 °C until analysis. Similarly quality control samples were prepared in plasma such that the final concentrations were 1.03, 2.94, 24.50, 37.36 μg/ml respectively and labeled as lower limit of quantification (LLOQ), low quality control (LQC), median quality control (MQC) and high quality control (HQC) respectively.

The extraction of the plasma samples involved liquid–liquid extraction process. For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 500 μl was then transferred to prelabeled 2.0 ml polypropylene centrifuge tubes. Internal standard dilution, 25 μl (200 μg/ml) was then