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Determination of pyrazinamide and its main metabolites in rat urine by high-performance liquid chromatography

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Abstract

A new high-performance liquid chromatograhic procedure for simultaneous determination of pyrazinamide (PZA) and its three metabolites 5-hydroxypyrazinamide (5-OH-PZA), pyrazinoic acid (PA), and 5-hydroxypyrazinoic acid (5-OH-PA), in rat urine was developed. 5-OH-PZA and 5-OH-PA standards were obtained by enzymatic synthesis (xanthine oxidase) and checked by HPLC and GC–MS. Chromatographic separation was achieved in 0.01 *M* KH_2PO_4 (pH 5.2), circulating at 0.9 ml/min, on a C_{18} silica column, at 22°C. The limits of detection were 300 μg/l for PZA, 125 μg/l for PA, 90 μg/l for 5-OH-PZA and 70 μg/l for 5-OH-PA. Good linearity (r^2>0.99) was observed within the calibration ranges studied: 0.375–7.50 mg/l for PZA, 0.416–3.33 mg/l for PA, 0.830–6.64 mg/l for 5-OH-PZA and 2.83–22.6 mg/l for 5-OHPA. Accuracy was always lower than ±10.8%. Precision was in the range 0.33–5.7%. The method will constitute a useful tool for studies on the influence of drug interactions in tuberculosis treatment. © 1997 Elsevier Science B.V.

Keywords: Pyrazinamide

1. Introduction

Quite frequent use of pyrazinamide (PZA) in the chemotherapy of tuberculosis [1,2], warrants either pharmacokinetic or metabolic disposition studies of PZA. The fact that the drug is harmful often restricts the scope of research to studies on patients and/or laboratory animals.

In order to study pyrazinamide metabolism in rats, we developed a simple HPLC method for determination of PZA and its principal metabolites in rat urine.

In vivo, PZA is extensively metabolized (Fig. 1) [3–5]. The metabolites are pyrazinoic acid (PA), produced by the action of the liver microsomal deamidase, PA is further hydroxylated to 5-hydroxypyrazinoic acid (5-OH-PA) by xanthine oxidase (XO). PZA hydroxylation to 5-hydroxypyrazinamide (5-OH-PZA) is also mediated by XO, but it has never been demonstrated that this metabolite, 5-OH-PZA, is produced exclusively by this enzyme [6,4,7]. One minor part of the PZA metabolic pathway consists of conjugation of PA with glycine to give pyrazinuric acid (PU).

As two of the three PZA main metabolites, 5-OH-PA and 5-OH PZA, are not commercially available,

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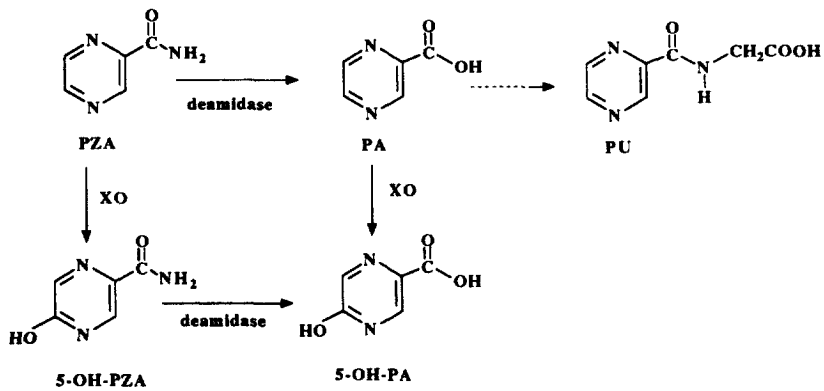

Fig. 1. Metabolic pathways of PZA.

they must be synthesized. For both, the method which is widely utilized, consists of in vitro synthesis by XO action [6,8], and for 5-OH-PA, organic synthesis is another possibility [9]. After relatively simple in vitro biosynthesis, metabolites must be isolated and purified. Despite the structural diversity of PZA and its metabolites, their polarity and lipophilicity characteristics are not sufficiently different to enable their separation by simple organic extraction, without significant loss of material. Most frequently, column chromatography and pH gradient elution were used for this purpose [4,8,10], which requires quite large amounts of the compounds, freeze drying apparatus and of course, utilization of equivalent amounts of expensive enzyme. To rationalize this step we made it by preparative thin layer chromatography (TLC).

The characteristics of PZA and of its metabolites already mentioned impose a simultaneous determination by direct chromatography of diluted urine samples, and utilization of a buffered, fully aqueous mobile phase for their separation. Till now, only two HPLC methods [8,10] concerning simultaneous determination of PZA and its metabolites (in human urines) were published. The disadvantage of the first HPLC method [10] includes a questionable separation and the use of an acidic mobile phase, whose pH is near the lower limit (i.e. 2.45) of the recommended range for many C_{18} columns. Due to the requirement of a special equipment, the utility of the second HPLC method [8] is severely restricted.

2. Experimental

2.1. Chemicals

Pyrazinamide (purity:99%) and pyrazinoic acid (purity:97%) were purchased from Aldrich (Milwaukee, WI, USA). Other chemicals were purchased from Merck (Darmstadt, Germany), organic solvents HPLC purity grade were from Prolabo (Paris, France), and water for injections, purity grade, were from Fresenius (Louviers, France).

5-OH-PZA and 5-OH-PA were prepared by reacting (36 h at 37°C) PZA (0.5 mmol) or PA (0.35 mmol) dissolved in 9 ml or 7 ml phosphate buffer pH 7.0, respectively, with xanthine oxidase (26.07 U/3 ml), Sigma (St.Louis, MO, USA). The reaction was monitored by TLC (on 5 cm×7.5 cm aluminium sheets, Silicagel 60 F_{254}; Merck, in a solvent system I: chloroform–methanol (70:30), and II: chloroform–methanol–ammonia (20:20:1), respectively. The new compounds (detected on UV, 254 nm), presented a R_f of 0.5 (5-hydroxypyrazinamide) and 0.055 (5-hydroxypyrazinoic acid), compared to R_f=0.64 and 0.55 of pyrazinamide and pyrazinoic acid. Their purity was checked by HPLC and GC. After incubation, reaction mixtures were deproteinized by continuous addition of methanol. Clear supernatants (after centrifugation at 1350 *g*, 15 min) were concentrated with a stream of nitrogen to the volumes appropriate for application to several TLC plates (Silicagel 60 F_{254}, Merck), as 15×0.25 cm bands.

Table 1
Study of the repeatability, reproducibility, accuracy and limit of detection of the rat urine with added amounts of PA, 5-OH-PA, 5-OH-PZA

Compound	Concentration (mg/l)	Repeatability R.S.D.(%)	Reproductibility R.S.D.(%)	Accuracy (%)	Quantification limit (μg/l)	Detection limit (μg/l)
PA	0.416	1.08	6.75	110.80		
	0.832	0.76	2.98	94.70		
	1.665	1.31	2.29	95.73	416	125
	2.21	0.79	2.28	99.39		
	3.33	5.7	6.58	102.15		
5-OH-PA	2.825	1.57	4.35	97.91		
	5.65	0.33	1.81	99.93		
	11.3	1.00	2.37	101.32	233	70
	15.05	0.5	2.38	100.60		
	22.6	1.25	1.50	102.92		
5-OH-PZA	0.83	0.75	3.10	103.61		
	1.66	0.59	4.21	101.20		
	3.32	0.34	1.16	100.00	306	92
	4.42	0.64	1.03	100.00		
	6.64	0.99	1.54	100.16		

The plates were chromatographed (two times) in solvent systems I and II, respectively, and the bands corresponding to 5-hydroxypyrazinamide and 5-hydroxypyrazinoic acid were scraped, and the compounds eluted from silicagel with methanol by repeated sonication. After centrifugation (2400 *g*, 20 min), the UV spectra of the clear methanol eluates, as well as identically treated scraped silicagel blanks, were registered. Mass spectra of methanolic eluates obtained by gas chromatography–mass spectrometry (GC–MS) confirmed the structure of the compounds. Eluates were filtered, after reduction of the volumes, in a stream of nitrogen, ultrafiltered (pore size, 0.45 μm), evaporated and dessicated to give several milligrams which were used as chromatographic standards.

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of an isocratic pump Beckman Model 110A (Beckman, San Ramon, CA, USA), a sample injector with 20-μl loop (Rheodyne, Cotati, CA, USA), and variable wavelength UV detector Spectra 100 (Spectra-Physics), connected to the Beckman analog interface module 406. The chromatography column Nucleosil 100–5 μm, C_{18}, 250×4 mm (Macherey-Nagel, Düren, Germany) was protected by a guard cartridge (11×4 mm) packed with the same material. The mobile phase was 0.01 *M* KH_2PO_4, ajusted at pH 5.2 by K_2HPO_4, circulating at a flow-rate of 0.9 ml/min at room temperature (22±2°C). The data recording system consisted of an IBM personal computer PS/2 Model 8550 Z with System Gold software (version 5.1 Beckman).

The GC–MS system consisted of a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA, USA) equipped with a split/splitless inlet system. The split ratio was 1:3. The fused-silica capillary column (25 m×0.25 mm I.D., 0.23 μm film thickness) (Macherey Nagel) was coated with a crosslinked 5% phenyl–95% methyl silicone gum. The carrier gas was helium C at an inlet pressure of 62 kPa. A Hewlett-Packard 5970 MSD mass spectrometer, operated in electron impact (70 eV) was directly interfaced with the gas chromatograph by the capillary column and was used in full scan mode between 50 and 550. The chromatographic conditions were identical for each metabolite. The oven temperature was maintained at 110°C during 2 min after injection and then increased at a rate of 10°C/min to 210°C. The final temperature was held for 11 min. Injector and transfer line (interface between gas chromatograph and mass spectrometer) were maintained at 240°C. The data recording system consisted of a HP

9000 300 computer equipped with a 599 70C Workstation system version 3.1.

2.3. Standard solutions and calibration

Aqueous PZA and PA stock solutions were prepared at concentrations of 300 mg/l and 319 mg/l. After dilution they gave a calibration concentration range of 0.375–7.5 mg/l or 0.416–3.33 mg/l, respectively.

Aqueous 5-OH-PZA and 5-OH-PA stock solutions at concentrations of 3110 mg/l or 2716 mg/l respectively, gave the calibration concentration ranges of 0.83–6.64 mg/l for 5-OH-PZA or 2.83–22.6 mg/l for 5-OH-PA.

Known quantities of PZA, PA, 5-OH-PZA and 5-OH-PA, reflecting the compound ratio found in treated rat urine samples, were added in a blank urine sample and those solutions were subjected to the validation procedure. The quantification of the metabolites is presented in Table 1.

The stability of the standard solutions and of the spiked diluted urine samples, at room temperature, was tested by repeated analyses, separated by 8-h periods. The results differed by less than 1%. Refrigerated stock solutions, and frozen urine samples were stable up to two months. The results of repeated analyses differed less than 5%.

2.4. Sample preparation

2.4.1. HPLC sample preparation

A 40-mg (325.2 μmol) PZA aqueous solution (48.57±3.81 mg/kg) was administered orally to eight female Sprague–Dawley rats (body mass 414.0±32.6 g), using curved cannula 60/10 (Carrieri). The rats were placed in metabolic cages (Pajon) with access to food and water ad libitum. Urine (24 h) was collected and measured (range 7–51 ml) and 4-ml portions were centrifuged and stored at −20°C until analyzed.

Before the determination, urine samples were thawed, appropriately diluted with water (50–350 times), ultrafiltered, and 20-μl aliquots injected into the HPLC column. Blank urine samples, as well as urines with added known quantities of compounds, were treated identically.

2.4.2. GC–MS reference sample preparation

With the gas capillary column used, pyrazinamide and 5-OH-PZA could both be analysed in solution in methanol without any derivatization, but in order to analyse rat urine, and to facilitate signal detection in urine, it was preferable to practice derivatization of each compound. A 100-μl aliquot of each compound solution in methanol was evaporated to dryness, at room temperature, under a stream of nitrogen. The residue, reconstituted with 50 μl of pyridine and 100 μl of a silylating mixture consisting of 99% BSTFA (*N,O-bis*(trimethylsilyl)trifluoroacetamide) and 1% TMCS (trimethylchlorosilane) was added. The solution was heated at 80°C for 30 min, and then chromatographed.

2.4.3. GC–MS rat urine samples preparation

In order to avoid significant loss of product caused by the classical simultaneous extraction of each compound, 20 μl of rat urine were evaporated in a vial, at room temperature, under a stream of nitrogen. In order to simultaneously silylate all urinary compounds, especially PZA and its metabolites, residue was reconstituted exclusively with 200 μl of the silylating reagent previously described. After vigorous agitation, the solution was submitted to identical heating, as was described in Section 2.4.2 for standard solutions, and then analysed.

2.5. Quantitation of HPLC data

Quantitation was based on the comparison of peak-area ratios for each compound, the sample analysis and in standard curves, taking into account the dilution factor.

2.6. HPLC method validation

HPLC method validation was performed using quality control urine samples.

Validation was performed according to the procedure described by Caporal-Gautier et al [11]. Six criteria were studied: repeatability, reproducibility, accuracy, linearity limit of detection and limit of quantitation.

For PA, 5-OH-PA, 5-OH-PZA, repeatability was

studied for five different concentrations of aqueous solutions mixture. Each solution was injected five times. Reproducibility was studied for the same concentrations as those studied for repeatability at three differents days, and so was evaluated for fifteen injections. Results corresponding to these two criteria were expressed by the relative standard deviation:

$$\text{R.S.D.}\% = \frac{S}{X} \times 100$$

where S is the standard deviation and X is the mean.The accuracy of the assay was studied for PA, 5-OH-PA, and 5-OH-PZA by evaluating the recovery defined by the ratio (found/added) expressed in percentage.

Detection limit and quantitation limit correspond to concentrations presenting a signal-to-noise ratio of 3 and 10, respectively.

3. Results and discussion

3.1. GC–MS characterisation of 5-OH-PZA and 5-OH-PA

Fig. 2 presents mass spectra of PZA and its metabolites after derivatization treatment. Although electron impact was used as ionisation mode, each mass spectrum presents mass peak: PA: $[M^{+\cdot}]$ 195; 5-OH-PZA: $[M^{+\cdot}]$ 283; PA: $[M^{+\cdot}]$ 196; 5-OH-PA: $[M^{+\cdot}]$ 284.

It is well known that mass spectral data of silylated compounds present a very abundant fragment corresponding to the loss of a methyl group. This was observed especially for the PA spectrum for which the abundance of the mass peak was very weak compared to that for $[M-CH_3]^{+\cdot}$, but for all the other compounds, $[M-CH_3]^{+\cdot}$ was more abundant than $[M]^{+\cdot}$.

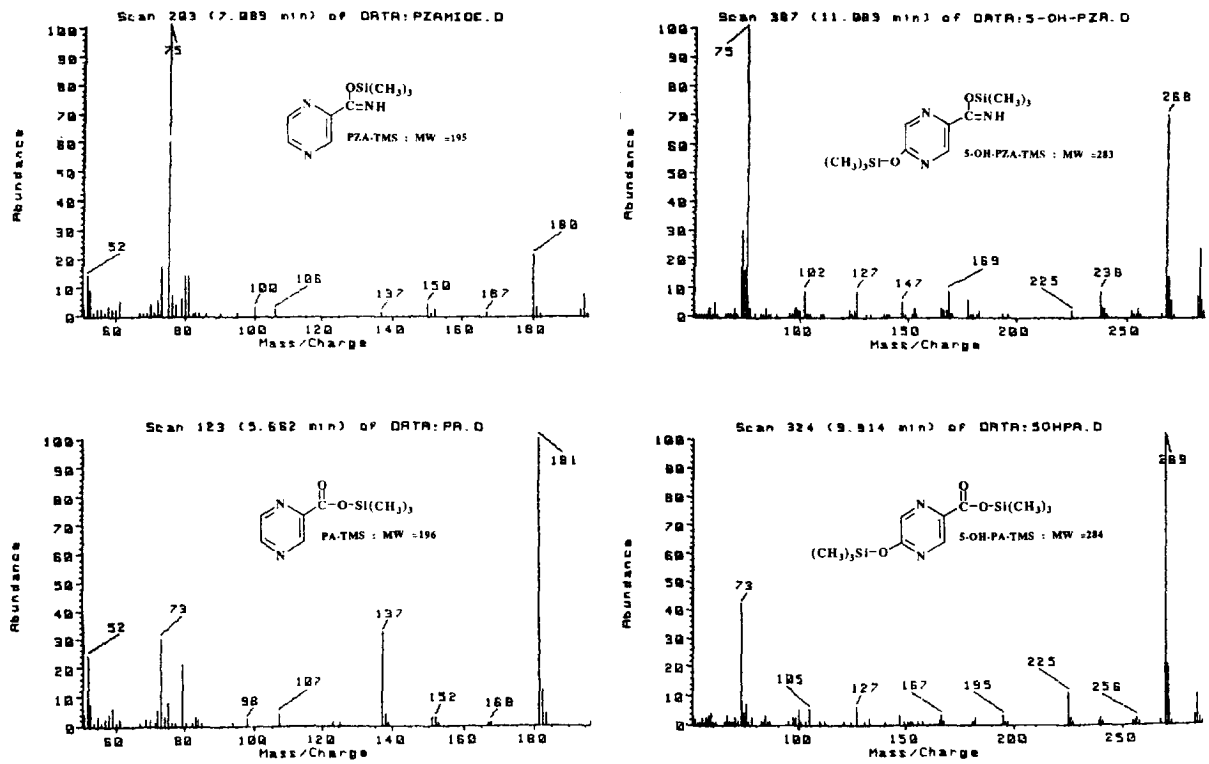

Fig. 2. Mass spectra of PZA, 5-OH-PZA, PA and 5-OH-PA after silylation.

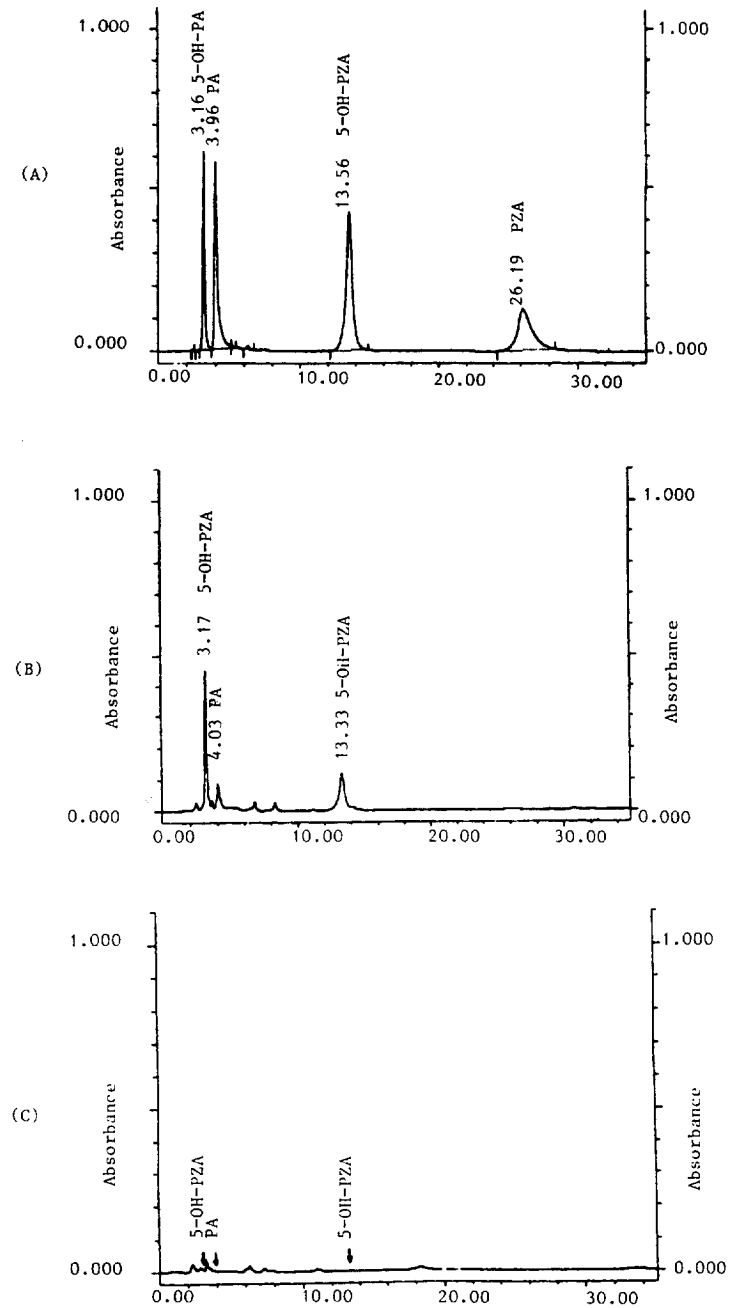

Fig. 3. Chromatograms of (A) a water solution mixture containing 5-OH-PA, PA, 5-OH-PZA and PZA, (B) diluted rat urine treated with pyrazinamide, (C) blank rat urine sample after dilution.

Retention times of derivatized compounds can also contribute to their identification by comparison of the values obtained in pure solutions with those observed for urine of PZA-treated rats.

Retention times of PZA, 5-OH-PZA, PA, 5-OH-PA derivatives are respectively, 7.089 min, 11.083 min, 5.662 min, 9.914 min.

3.2. HPLC data

The chromatograms are presented in Fig. 3: (A) of the water solution mixture containing 5-OH-PA (10.90 mg/l), PA (8.64 mg/ml), 5-OH-PZA (15.84 mg/l), and PZA (9.01 mg/l); (B) of rat urine sample containing 5-OH-PA (7.55 mg/l), PA (1.5 mg/l), 5-OH-PZA (4.2 mg/l); and (C) of identically treated blank rat urine sample.

3.3. Results of validation

The results of the validation for repeatability, reproducibility, accuracy, limit of detection and limit of quantitation are summarized in Table 1.

Linearity was studied for PA, 5-OH-PZA and 5-OH-PA and gave the regression line slope equations: $y=17.9549x-2.2948$, $r^2=0.995$ for PA, $y=9.1158x-1.729$, $r^2=0.999$ for 5-OH-PA; $y=13.9740x-1.13$, $r^2=0.999$ for 5-OH-PZA (y and x represent area and concentration respectively. For each compound, the five concentrations referred to in Table 1, were studied. Each solution was injected five times.

For PZA, linearity was tested and the regression equation was $y=17.4443x-6.6293$, $r^2=0.999$ and limit of detection was 300 mg/l. Concentrations in rat urine were below the detection limit.

3.4. Data concerning rat urine samples

The amounts of pyrazinoic acid, 5-hydroxypyrazinoic acid, and 5-hydroxypyrazinamide recovered in 24-h rat urine collections are presented in Table 2. The standard deviation allows the relative homogeneity of the results for 8 different animals to be noted.

Table 2
Amounts of 5-OH-PA, PA and 5-OH-PZA recovered in 24-h rat urines after administration of 40 mg (325.2 μmole) PZA

Rat	5-OH-PA (μmol)	PA (μmol)	5-OH-PZA (μmol)	Total (μmol)
1	111.42	50.16	57.12	218.70
2	111.30	50.46	57.41	219.17
3	102.85	40.06	77.69	220.61
4	122.03	47.50	97.59	267.12
5	100.13	37.92	76.27	214.32
6	123.56	46.39	70.00	239.95
7	114.26	46.70	100.52	261.48
8	120.85	47.40	70.79	239.04
Mean	113.30	45.82	75.92	235.05
S.D.	8.70	4.51	16.19	20.44

3.5. Discussion

Previous quantification was carried out by Whitehouse et al. in a male Wistar rat urine [12], using TLC for separation and liquid scintillation counting for quantification of compounds in urine after administration of radiolabelled PZA. 74.3% of a dose was recovered in 12-h urines, in the form of three main metabolites, of which 5-OH-PA, PA and 5-OH-PZA accounted for 33.51, 47.24 and 19.24% respectively. In 24-h urine collection from dogs [3], 70% of a PZA dose was excreted in the form of metabolites, of which PA accounted for 27.14% and other metabolites for 72.86%. By application of the method presented here, in 24-h urine collection from Sprague–Dawley female rats, 72.28±6.28% was recovered in the form of three main metabolites, of which 5-OH-PA, PA and 5-OH-PZA accounted for 48.31±2.97, 19.58±2.24 and 32.31±4.89% respectively. Small amounts of PZA were detected but could not be quantified.

4. Conclusion

This simple and efficient HPLC method allowed simultaneous separation and quantification of PZA and its metabolites in rat urine. The analytical assay method was successfully validated.

It will constitute a useful tool for studies on the influence of drug interactions in tuberculosis treatment.

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