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QUALITY CONTROL OF PYRAZINAMIDE IN FORMULATION USING MICELLAR LIQUID CHROMATOGRAPHY

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ABSTRACT: Pyrazinamide is an important anti-tubercular drug which shortens the course of TB chemotherapy. A direct injection liquid chromatographic method is developed for the determination of pyrazinamide in pharmaceutical formulation. The method includes a micellar mobile phase containing 0.15M sodium dodecyl sulphate and 1% butanol (v/v) buffered at pH3, a Princeton SPHER-100 C₁₈ column (250mm × 4.6mm, 5µm particle size) and UV detection was set at 269nm. The Micellar Liquid Chromatography (MLC) method is rapid, precise, sensitive, and robust. In this method pharmaceutical samples were directly injected to the column without pre-treatment step. Under all these conditions, method has very short analysis time of 3.2 min, linearity ($r > 0.998$), limit of detection and limit of quantification is 1.4, 36.5 ng/ml respectively; Intra- and inter-day precision (RSD%) were 1.5, overall recovery in pharmaceutical formulation is 99.4%, 69.5%, 81.25%, 87.9%. The method is suitable for routine quality control analysis. This chromatographic techniques, MLC has the advantage of avoiding sample extraction step from matrices, thus reduces the time of analysis.

INTRODUCTION: Pyrazinamide (pyz) is an anti-tuberculosis drug synthesized in the 50's of last century and formerly used only as salvage therapy. Recent developments have elevated it to a central role in tuberculosis chemotherapy. It is an important sterilizing and front-line drug that shortens tuberculosis (TB) therapy. It also demonstrates a bactericidal property which shows effective preventive activity against *Mycobacterium tuberculosis* *in vitro* at acidic pH¹.

Pyz is a heterocyclic compound (**Table 1**) chemically related to thiosemicarbazones and nicotinamide which is recommended by World Health Organization (WHO) in Directly Observed Treatment Short Course (DOTS) regimen of TB. It is an important component for the current 6 months short course in TB chemotherapy.

Pyz also plays an important role in reducing the therapy period from 9 - 12 months to 6 months because it kills a population of semi dormant tubercle bacilli, residing in an acidic environment (occurring during active inflammation), which are not killed by other anti-tubercular drugs¹. In India pyz is available in 500mg, 750mg and 1000mg dosage in the form of tablets. After oral administration of 500mg pyz, peak plasma

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concentrations attained *i.e.* about 9-12 μ g/ml in first two hours and 7 μ g/ml in 8 hours. The half-life

period of the drug is 9-10 hours²⁻⁴.

TABLE 1: SHOWS THE CHEMICAL PROPERTIES OF THE PYZ

Name	Chem. formula	Structure	Mol. Wt.	Log p	pKa
Pyrazinamide	C ₅ H ₅ N ₃ O		123.11g/mol	-0.60	-0.5

In India pyz is generally given directly to the patient by the government controlled hospitals because the treatment of TB is available only in these hospitals. Sometimes the patients also purchase the same drugs from local pharmacy as prescribed by private medical practitioner. As the number of TB patients in developing countries is very high therefore in order to meet the ever growing demand by hospitals as well as the pharmacy shops, it is produced in bulk and most of the drug are supplied to government controlled hospitals. Hence, it is very important to check and ensure the quality and quantity of the supplied drugs with respect to the shelf life and the stability of the drug. Due to the above fact, there is an urgent need to develop an analytical method which has good quality outcomes, should be inexpensive, simple, rapid, sensitive and selective for the detection and quantification of pyz.

For the determination of pyz in pharmaceutical preparation and biological sample, various analytical methods has been developed and reported using either UV-Vis spectrophotometry^{5, 6}, Colorimetry^{7, 8} or separation technique like chromatographic methods which includes High Performance Thin Layer Chromatography⁹, High-Performance Liquid Chromatography with different detection mode^{10, 11}. A few methods have also been reported for the determination of pyz using chromatography after derivatization to increase its selectivity or sensitivity¹².

Other sophisticated chromatographic technique like UPLC¹³, LC/MS/MS^{14, 15} have also been developed for quantitative and qualitative analysis of pyz in formulations as well as in biological samples. The drawbacks of these techniques are its limited availability, either due to expensive instrumentation or due to high cost of solvents and chemicals used for its operation. Thus, there is an

urgent need for the development of a simple, rapid and cost effective method for the qualitative and quantitative analysis of pyz either in formulation for quality control or in biological fluid for drug monitorization.

In the present work efforts have been made to develop a rapid, selective, and sensitive method for the determination of pyz in pharmaceutical formulations using MLC. The developed method uses surfactant as mobile phase and negligible amount of organic solvent as modifier. As, surfactant has been used in the mobile phase, so direct injection of pharmaceutical formulation as well as biological samples will be explored which will give an edge over existing HPLC methods.

MATERIAL AND METHODS:

Materials: Standard pyrazinamide (purity >99.99%) was provided by Wilcure Remedies Private Limited (Indore, India) a Quality Control Pharmaceutical Company as gift sample, and pyrazine-2- carboxylic acid (a major metabolite of pyz) was purchased from Sigma Aldrich (Banglore, India). Pharmaceuticals formulation like P-Zide 500 from Cadila Pharmaceutical Limited (Gujarat, India), PZA-CIBA Sandoz Private Limited (Novartis, India), Z = pyrazinamide (Lupin Private limited) Pyrazinamide Forecox (Macleods Pharmaceutical Limited). Chemicals, solvents and reagents like sodium dodecyl sulphate (SDS) (99%) were purchased from Merck (Mumbai, India), sodium dihydrogen phosphate, hydrochloric acid, sodium hydroxide were purchased from Himedia laboratories Private Limited (Mumbai, India) for the preparation of mobile phase. HPLC grade pentanol, butanol, methanol used as modifiers were purchased from Ranchem, RFCL Limited (New Delhi, India). Propanol was from Spectrochem Private Limited (Mumbai, India).

The chemicals used in the preparation of mobile phase are of analytical grade and all the solution were filtered through 0.45 μ m nylon membrane from Micron Separation (Westboro, MA, USA) filter paper using different filtration unit prior to chromatographic analysis.

Instrumentation: Shimadzu Prominence HPLC (Shimadzu Corporation, Kyoto, Japan), equipped with an isocratic pump LC-20 AT, an auto-sampler SIL-20AC, and a diode array detector SPD-M20 A (190-800nm) was used. The Column used for the separation SPHER-100 C₁₈ 100A (250mm \times 4.6mm, 5 μ m particle size) was purchased from Princeton Chromatography INC (Cranbury, NJ, USA). Chromatographic signals obtained on the personal computer was evaluated using LC Solution software version 1.22SP1 connected with Shimadzu Prominence HPLC instrument. In order to process data and various statistical calculation Microsoft Office Excel 7 (Microsoft Corporation, Seattle, W.A. USA) and Origin Pro 8 (Northampton, Massachusetts, USA) were used. The standards were weighed using an analytical balance from Mettler Toledo (Mettler Toledo India Private limited). The pH of the prepared solution were measured by using a digital pH meter pH-102/103 (Context, Instruments Private Limited, India). The sample were dissolved using ultrasonic bath (Sonicator) 1.5 Lt (PCI Analytics Private Limited Russia) and for storage of prepared samples a deepfreezer (Remi Electrotechnik Limited, India) was used.

Methods:

Mobile Phase Preparation: Mobile phase was prepared by weighing an appropriate amount of surfactant *i.e.* of SDS and buffer salt which in this case is sodium di hydrogen phosphate (0.01M) directly dissolve in deionized water using magnetic stirrer. The pH of the above prepared solution was adjusted either by using 0.01M NaOH or HCl. In order to improve chromatographic parameters like capacity factor, asymmetry or efficiency a small amount of organic modifier (propanol, butanol or pentanol) was also added in different percentage (v/v).

Preparation of Standard Solution: 10mg of standard pyz was weighed accurately and then dissolved in 100ml of deionized water followed by

sonication using ultrasonic bath and stored in amber colored bottle in a deepfreezer.

Preparation of Marketed Formulation: Different pharmaceutical preparation of pyz tablets were purchased from local pharmacy store. The tablets were weighed accurately and crushed separately into fine powdered using mortar and pestle. As per desired concentration of pyz, the grinded tablets, weighed approximately to 10mg and dissolved in 10ml of deionized water, sonicated for maximum dissolution and stored in amber colored bottle in a deepfreezer.

RESULT AND DISCUSSION:

Optimization of Micellar Chromatographic Condition: In the optimization of micellar chromatographic condition various important factors are to be considered *i.e.* selection of optimum wavelength, selection of pH, concentration of surfactant and percentage of organic modifier. Based on the criteria of minimum analysis time and maximum efficiency, retention time (t_R), efficiency (N) and asymmetric factor (A/B) for the compound was calculated by varying the concentration of surfactant and organic modifier.

Selection of Wavelength: Pyz is a UV active substance due to presence of conjugated saturated and unsaturated bonds as well as heteroatom (three Nitrogen atoms) containing lone pair of electron (**Table 1**). Although the chromatographic detection was carried out using Photo Diode Array (PDA) detector still optimum λ_{max} (wavelength of maximum absorbance) of the compound was recorded using UV-visible spectrophotometer. Appropriate dilution of standard stock solution 100 μ g/ml pyz was prepared in water. Maximum absorbance for pyz was obtained by plotting a scan of absorbance in the wavelength region of 200-400nm. The absorption maximum of pyz was recorded at 269nm. This was also verified from different research article which dealt with UV detection of pyz^{6,7}.

Selection of pH: The pH used during the analysis of an analyte depends upon its pKa. The pH should be chosen in such a way that the drug remains charged. Dissociation constant of pyz is very low **Table 1**.

The pKa value of analyte suggest the suitable pH of mobile phase to be used. As the pka of pyz is - 0.5 any pH in the working range of normal HPLC column (pH 3 - pH 8) will have similar effect on the retention behaviour of the analyte. To study the effect of pH on the capacity factor of the analyte, three mobile phase having 0.15M SDS, 1% butanol of organic modifier were buffered to pH 3, pH 5, pH 7. The retention time obtained for pyz using the above mentioned mobile phase showed negligible change. Thus justifying the fact that the compound generally remains deprotonated throughout the working pH range of the column. Similarly, efficiency and assymetry for pyz obtained were almost similar. Whereas while compairing the three factors *i.e.* capacity factor, efficiency and asymmetric factor for pyz together in the above mentioned mobile phase, pH 3 seems to be better than that of pH 5 or pH 7. Thus, for all future analysis pH 3 was selected as the optimum pH for the chromatographic analysis.

Selection of SDS Concentration and Organic Modifier: Based on the literature and past experience of working on micellar mobile phase, SDS was selected as a surfactant for the chromatographic analysis of pyrazinamide. Three different concentration of SDS was selected in this particular case *i.e.* 0.05M, 0.1M and 0.15M for the preparation of mobile phase. Using the above mention mobile phase chromatographic parameter *i.e.* capacity factor (k), efficiency (N) and asymmetry (B/A) were compared. As it is a well established fact in MLC that by increasing the concentration of SDS the retention time (t_R) decreases like in 0.05 M SDS t_R - 4.5min, 0.1M SDS t_R . 3.91min and in 0.15M SDS t_R . 3.51min and other chromatographic parameters also improves. The same behaviour was obtained for pyz. While using low concentration of SDS the efficiency and assymetry of the analyte were poor,

which shows marked change when the concentration of SDS was increased to 0.15M. It was also evident from literature survey that researcher working on micellar based liquid chromatography generally used 0.15M SDS for obtaining better chromatographic parameters^{16, 17}. It is well known fact about MLC that a small amount of low carbon chain alcohol (propanol, butanol or pentanol) helps to improves the chromatographic peak profile¹⁸.

Based on the above optimization of separation parameters, further chromatographic analysis were performed with the strength of SDS fixed at 0.15 M and a small amount of butanol in the range of 1% (v/v) - 7% (v/v) was added. While using 6% (v/v) butanol the value of k, N, and B/A were 2.9 min, 3511 and 1.62 respectively which changed to value 3.2 min, 3824, 1.13 respectively when the concentration of butanol was changed to 1% (v/v).

It is evident from the above data that when higher amount of butanol (v/v) was used, the efficiency and assymetry of the peak were improved but at the same time the peak of pyz merged with the band eluting at the head of chromatogram (peak of dead volume), thus discarding the use of higher amount of organic modifier. Chromatographic data obtained while using 0.15M SDS and 1% butanol (v/v) as organic modifier were compared with that of 0.15M SDS without any modifier. The data obtained while using 1% butanol (v/v) were slightly better thus promoting the use 0.15 M SDS, 1% butanol (v/v) buffered to pH 3 as mobile phase for further analysis. While using the same mobile phase the major metabolite of pyz that is pyrazine carboxylic acid was also injected and both the compounds resolved in acceptable time (**Fig. 2**). The developed method was also compared with the existstng HPLC method and the present MLC method was found better¹⁹⁻²⁴ (**Table 2**).

TABLE 2: COMPARATIVE CHART OF DEVELOPED HPLC METHOD FOR DETERMINATION OF PYRAZINAMIDE IN A PHARMACEUTICAL FORMULATION

S. No	Mobile Phase	Ratio	LOD, LOQ ($\mu\text{g/ml}$)	t_R (min)
1.	Methanol: Water: Propanol: Acetonitrile: Sodium Acetate ¹⁹	51:42:3:2:2	0.012, -	4
2	Acetonitrile: Phosphate Buffer ²⁰	50:50	2.0,4.0	8.0
3	Acetonitrile: Phosphatebuffer ²¹	75:25	4.92,16.39	24.00
4.	Acetonitrile: Phosphate Buffer ²²	50:50	1.07,-	2.9
5	Phosphate buffer: Methanol ²³	80:20	0.0045,0.013	3.62
6	Phosphate buffer: Methanol ²⁴	50:50	0.036,0.11	5.04

Validation Parameters: The developed method was validated with respect to various parameters such as accuracy, precision, robustness, linearity, limit of detection and limit of quantification as per International Conference on Harmonisation (ICH) guideline²⁵.

Linearity: The linear range of the pyrazinamide was studied by constructing calibration curve using areas obtained from chromatographic peaks. Seven different concentration (0.1, 0.5, 1, 5, 15, 25, 50 µg/ml) of analyte were injected in the optimum mobile phase. Each solution were injected in triplicate (n = 3) and average value of the peak area was plotted against the concentration. The procedure was repeated 5 times on different days (new standard solution were prepared every time before analysis). The slope and intercept calculated was 0.04, 0.39 respectively and regression coefficient was (R^2) near to 1.

Repeatability and Reproducibility: In accordance with the ICH Harmonised Tripartite Guide, accuracy and precision were evaluated. The intra- and inter day precision were performed in three different concentration of standard pyz (5, 1.25, 0.31 µg/ml) dissolved in micellar mobile phase. The intra-day precision was determined by injecting the 3 different concentration of pyz five times in the same day, and inter-day analysis was evaluated by taking the average of five measurement of intra-day values taken on 5 day over a period of two month, performed by different analysts and on different equipment. The (RSD%) intraday precision was 0.52, 0.035, 0.98%, whereas (RSD%) interday precision was 0.38, 1.29, 0.599%. As there was no significant difference between assay result either within days or between days, it implies repeatability and reproducibility of the developed method was good.

Limit of Detection and Quantification: The Limit of detection (LOD) and quantification (LOQ) were determined using 3s criterion and 10s criterion respectively. A series of 10 solutions in the increasing order from the lowest concentration of drug in the calibration range were analyzed. The mean of the standard deviation of the peak area was divided by slope obtained from the calibration plot. Based on above calculation LOD of pyrazinamide was found to be 1.4 ng/ml while LOQ was 36.5 ng/ml. The LOQ obtained could easily be applied

for the routine analysis of pyz in formulation or monetarization in biological samples.

Robustness: Robustness of the developed method was determine by varing some parameter like concentration of SDS, percentage of butanol (v/v), pH and the flow rate of the mobile phase. The study was performed by injecting standard solution using a different concetration in the calibaration range. The RSD (%) of the retention time calculated from the variation was lower than 6.0% **Table 3.** Variation in the flow rate had a stronger influence on the retention time of the pyz. This indicate that the developed method is very much reliable in the identification and quantification of pyz.

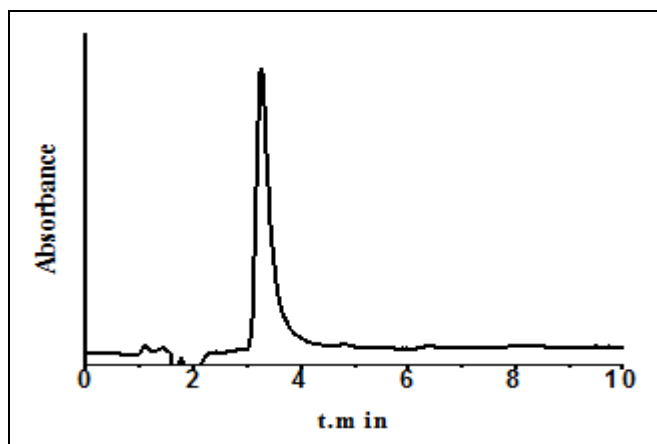


FIG. 1: CHROMATOGRAM OF STANDARD PYRAZINAMIDE (5ppm) IN MOBILE PHASE CONSISTING 0.15M SDS pH 3 1% (v/v) BUTANOL WITH DETECTION AT 269 nm, FLOW RATE 1.0ml/min, INJECTION VOLUME 20µL

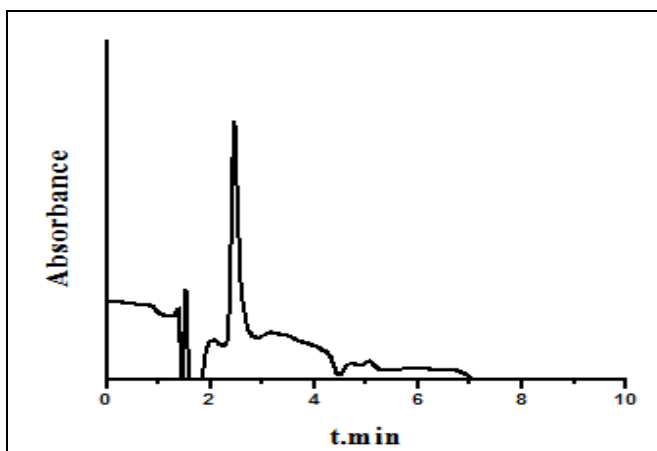


FIG. 2: CHROMATOGRAM OF STANDARD PYRAZINE CARBOXYLIC ACID (5 ppm) IN MOBILE PHASE CONSISTING 0.15M SDS pH 3 1% (v/v) BUTANOL WITH DETECTION AT 269nm FLOW RATE 1.0ml/min, INJECTION VOLUME 20µL

TABLE 3: ROBUSTNESS EVALUATION OF THE DEVELOPED MLC METHOD

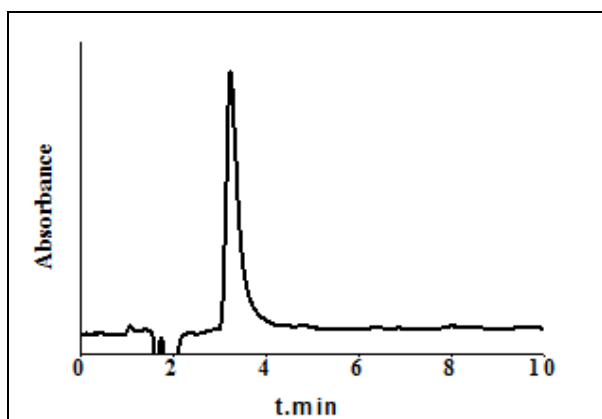
Chromatographic Changes variation	Pyrazinamide t_R (min)	N	B/A
Flowrate (ml/min)			
0.9	3.591	3785	1.08
1.0	3.214	3824	1.13
1.1	2.831	3819	1.10
Mean \pm SD	3.212 \pm 0.3	3809 \pm 21	1.10 \pm 0.25
RSD (%)	11.8	0.55	2.28
pH			
2.8	3.211	3612	1.11
3	3.214	3824	1.13
3.2	3.216	3511	1.11
Mean \pm SD	3.213 \pm 0.0025	3649 \pm 159.74	1.11 \pm 0.011
RSD (%)	0.07831	4.377	1.034
Butanol (% v/v)			
0.5	3.213	3745	1.09
1	3.214	3824	1.13
1.5	3.21	3498	1.12
Mean \pm SD	3.212 \pm 0.0020	3689 \pm 170	1.11 \pm 0.020
RSD (%)	0.064842	0.0461	1.86
SDS (M)			
0.14	3.219	3721	1.07
0.15	3.214	3824	1.13
0.16	2.945	3645	1.11
Mean \pm SD	3.126 \pm 0.156	3730 \pm 89.83	1.31 \pm 0.33
RSD (%)	5.015	2.4085	25.50

Pharmaceutical Formulation Analysis: The developed method was applied to four different pharmaceutical products available in the market coded as a, b, c, and d. The entire process follows the same protocol of analysis which was adopted for standard. Except for filtration no other pre-treatment step was performed and the

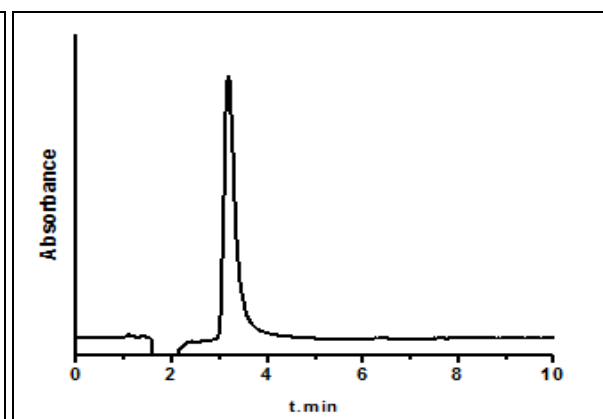
pharmaceutical products were injected directly into the chromatographic system **Fig. 3a-d**. Recoveries obtained by injecting different pharmaceutical compound were found to be in accordance with the amount mentioned on the pharmaceutical products **Table 4**.

TABLE 4: ABSOLUTE RECOVERIES OF PYRAZINAMIDE IN VARIOUS PHARMACEUTICAL FORMULATIONS

S. no	Pharmaceutical	Found ($\mu\text{g/ml}$) Mean \pm S.D	Absolute Recoveries (% Mean)
1.	P-Zide 500	49.42 \pm 1.4	99.4%
2.	Z=Pyrazinamide	347.5 \pm 5.1	69.5%
3.	PYZ-CIBA 750	40.62 \pm 0.6	81.25%
4.	FORECOX Pyz 750	43.95 \pm 0.4	87.9%



(A)



(B)

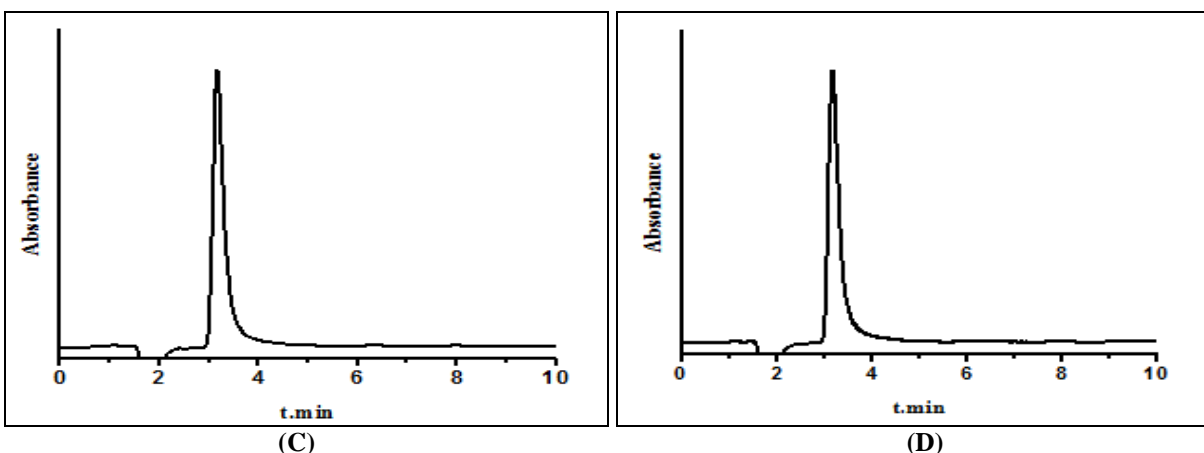


FIG. 3: CHROMATOGRAMS CORRESPONDING TO PYRAZINAMIDE FROM VARIOUS COMPANIES: (A) Z = PYRAZINAMIDE (B) P-ZIDE 500 (C) PYZ-CIBA 750 (D) FORECOX PYRAZINAMIDE 750. CHROMATOGRAPHIC CONDITION 0.15M SDS-1% (v/v) BUTANOL 0.01M PHOSPHATE BUFFERED AT pH 3 AND DETECTION AT 269nm

CONCLUSION: The present method developed for the determination of pyz in pharmaceutical formulations is rapid, selective, reliable and robust which can be applied for routine analysis by any pharmaceutical industry. As well as the method can be applied by any drug quality control laboratory working under law governing agency. Since the parent drug (pyz) is well separated from metabolite (pyrazine carboxylic acid) and as both do not elute out in the dead time therefore the developed method can be applied by hospital's clinical drug monitorizing unit. Apart from the benefit of direct injection, due to which there is minimum loss of analyte in real sample, the developed method is very fast (within five minute) reducing the overall cost of analysis.

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