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# Standardization of the method for estimation of ethambutol in pharmaceutical preparations and biological fluid

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A simple column chromatographic method for determination of ethambutol (EMB) in pharmaceutical preparations containing EMB in combination with other anti-TB drugs is presented. The method involved extraction of EMB into an organic solvent. followed by basification and column chromatographic separation on Amberlite CG 50 (100-200 mesh) and elution with suitable eluants and estimation at a wavelength of 270 nm. The assay was linear from 25 to 400 µg/ml. The relative standard deviations of intra and inter day assays were lower than 5%. Ethambutol was recovered from human urine quantitatively and stable for a period of atleast one week in urine stored at-20°C.

**Keywords**: Ethambutol, Amberlite CG-50, Rifampicin, Isoniazid. Pyrazinamide

Ethambutol (EMB), dextro-rotatory isomer of 2,2'–(ethylene diimino)-di-1-butanol dihydrochloride is the most frequently used for the short course treatment of tuberculosis along with rifampicin (RMP), isoniazid (INH) and pyrazinamide (PZA)[1] and it is bacteriostatic to both intracellular and extracellular organisms[2-8]. Many reports have documented the efficacy of ethambutol for the treatment of bronchopulmonary, urogenital and osteoarticular tuberculosis and *Mycobacterium avium* complex[9].

Successful treatment of tuberculosis requires adequate concentration of the chosen drug in biological fluids namely blood, cerebrospinal fluid (CSF), saliva or urine and in this respect varying results have been published for ethambuto[10-12]. Microbiological and gas liquid chromatographic (GLC) methods are available for assaying ethambutol concentrations in serum and CSF. In microbiological assay, when EMB is present along with other drugs in plasma, it cannot be assayed without the interference of other drugs[13]. Gas liquid chromatography methods using capillary columns are available but they involve a series of steps for preparation of the sample before analysis and needs internal standard[14]. Moreover, all laboratories cannot afford to have such a sophisticated equipment. Hence, pharmacological studies of ethambutol require specific and simple method for estimation of the drug in biological fluids containing other anti-TB drugs. One such method has been standardized and presented in this paper. In addition, checking for the stated content of a drug in pharmaceutical preparations prior to administering to patients; cannot be over-emphasized. Previous experience with cycloserine[15] and with a syrup formulation of isoniazid[16] has shown that drug preparations could lose their potency when stored. For this reason, it is now customary to routinely assay the content of anti-tuberculosis drugs in various pharmaceutical preparations.

Ethambutol has been routinely assayed using a non-aqueous titration method[17,18]. It was observed that with certain pharmaceutical preparations, the values were higher than the stated contents. Another method for estimation of ethambutol content in pharmaceutical preparations involves reaction with cupric chloride and measurement of the absorption in the visible range, which is again a time consuming procedure[19,20]. In drug formulations containing EMB in addition to other anti-TB drugs, EMB has to be exclusively estimated without the interference of other drugs.

A method that estimates EMB alone by HPLC has been reported by Lacroix *et al.*,[21]. This was modified and this procedure involves ion exchange chromatography that enables specific estimation of EMB in the presence of other anti-TB drugs.

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Monitoring of EMB concentrations in body fluids may be valuable to study drug-drug interactions, if any, when co-administered with other antituberculosis drugs. It is also possible to specifically estimate EMB in urine without the interference of other anti-TB drugs.

The aim of the study was to develop and validate a simple method for the estimation of EMB in fixed dose combinations (FDCs) containing EMB along with other anti-TB drugs and also in biological fluid viz., urine, collected from human volunteers after the administration of RNTCP regimens which includes ethambutol (Ethics committee clearance was obtained, informed written consent from each subject was obtained).

## Materials and Methods

*Chemicals and reagents*—Pure rifampicin, ethambutol, pyrazinamide and Amberlite CG-50 (100-200 mesh) from Sigma Chemical Company, St. Louis Missouri, USA, pure isoniazid from May & Baker Ltd, Detroit, USA, were used. Ammonia (specific gravity 0.91%). absolute ethanol, copper sulphate, hydrochloric acid, sodium hydroxide and sodium tetraborate were of analytical grade.

Alkaline ethanol (9 ml of absolute ethanol + 1 ml of 10 *N* NaOH), 0.05 *M* of borate buffer (pH 8). 10% aqueous ammonia, ammonical copper sulphate reagent (50 mg $CuSO_4$ and 6.33 ml of absolute ammonia made up to 100 ml with water) were prepared freshly on the day of assay.

*Chromatographic system*-Amberlite CG-50 (10 g)-weakly acidic cation exchanger (mesh size 100-200) was washed 5 times with double distilled water and allowed the beads to swell in 50 ml of 1 *N.* NaOH over night for basification. The soaking of the resin in NaOH should not exceed 24 hr. The *p* H of the resin was broughtdown to 7.5-9.5 by washing with water. Then, the resin was packed in the column of 1 cm diam. to a height of 7.5 cm from the tip plugged with cotton. The resin was prevented to dry by keeping water level above the resin bed. Plasticine was used to control the flow rate.

*Preparation of standard solution* — A stock solution (1mg/ml) was prepared by dissolving EMB in 0.1 *N* HCl. Ethambutol standards were prepared in serial concentrations ranging from 25 to 400µg/ml in 0.1N HCl as well as normal pooled urine, which contained RMP (250µ/ml), INH (l00µg/ml) and PZA (600µg/ml). From the standard graph, the quantification of EMB in FDCs as well as in urine collected from the patients after the drug administration was carried out.

*Formulation containing EMB along with other anti-TB drugs*—A total of 15 tablets with EMB alone (ethambutol hydrochloride-single drug-200, 400 and 800 mg of each), 4 tablets with RMP and INH (Macox plus 150-double drug formulation without EMB), 6 tablets containing EMB and INH (Combunex 800-double drug formulation without RMP), 11 tablets containing RMP, EMB and INH (AKuriT 3-triple drug formulation-without PZA) and 26 tablets containing RMP, EMB, INH and PZA [AKuriT 4, and AKuriT FD-Quadruple drug formulations containing all the 4 drugs of 2 different brands; (17 and 9 of each)] were processed by the method standardised currently. In brief, the weight of each tablet was noted, crushed and made up to a known volume with 0.1 *N* HCl and filtered. Suitable dilutions were made so that the concentrations of EMB from each tablet was around 400 µg/ml. To 5 ml of sample, 15 ml of chloroform was added and kept on gyrotary shaker for 30 min. Four ml of chloroform free phase was basified with 0.4 ml of alkaline ethanol. Loaded 2 ml of the mixture onto the packed column. The loaded columns were washed sequentially with 3-5 ml of water, 5.0 ml of ethanol, 15 ml of borate buffer (0.05 M; *p* H 8.0) and 15 ml of 10% aqueous ammonia solution. Two aliquots of ammonia eluates (5 ml) were collected, centrifuged for 5 min and to 2.2 ml of the eluate, 0.8 ml of ammonical copper sulphate solution was added and the optical densities were recorded at 270 nm using lcm cells, in a unicam spectrophotometer.

*EMB in urine*—Ethambutol was estimated by this procedure in urine collected over a period of 0-8 hr from 12,13,12 patients suffering from HIV, TB, HIV/TB respectively. The patients investigated were receiving RNTCP regimen containing RMP (450 mg), EMB(1200 mg), INH (600 mg) and PZA(1500 mg). The study was presented before the ethics committee and clearance was obtained. In addition written consent was obtained from study subjects. This is just to check for the application of this methodology in the estimation of EMB in urine in the presence of other drugs.

*Sensitivity*—The sensitivity of the method was estimated after setting up serial concentrations of EMB (50–400µg/ml).

*Precision*—The precision of the method was evaluated by analyzing pooled human urine samples (These are normal urine samples collected and pooled

from volunteers (staff members) from TRC. Since the volunteers were willing to give the samples, ethics committee clearance was felt unncessary.) containing four different concentrations of EMB. The inter and intra-day variations were determined by assaying each sample in triplicate for 3 days.

*Recovery*—For the recovery experiment, the pooled human urine samples containing previously determined concentrations of EMB, were spiked with 50, 100 and 200 µg/ml of EMB and assayed. The percentage of recovery was calculated by dividing sample differences with the added concentrations.

*Stability*—Stability of EMB in urine was evaluated by analyzing pooled human urine samples containing 50, 100,200 and 400 µg/ml of EMB along with other anti-TB drugs in the proportions mentioned above. The samples were prepared and stored for 10 days at –20°C. The stability was checked on 0, 1,4,7 and 10 days after storage.

**Results and Discussion**

Ethambutol is widely used for the treatment of mycobacterial diseases. It was developed in response to increasing bacterial resistance to the first line agents[22]. Ethambutol, being bacteriostatic, is rapidly taken up by the organism and inhibits mycobacterial cell wall synthesis [23]. Also, tuberculosis requires effective chemotherapy, which can be achieved with regimens consisting of a number of drugs given either individually or in FDC(s) containing RMP, INH and PZA, in addition to EMB. In the routine management of patients with this drug as in the case with other drugs, it is necessary to have information on the levels attained in biological fluids. Several HPLC and GLC-mass spectrometry methods have been reported to measure and to quantify EMB levels in plasma and urine[14]. The methodologies described in these reports are complex and lengthy involving pre-preparation of the samples and the usage of expensive columns for GLC. Chemical methods are tedious, time consuming and are not efficiently sensitive. A microbiological assay [13] has been reported that it is time consuming as results are available only after 48 hr. Taking the leads from the earlier workers, a procedure for EMB in urine has been standardised. The method described in this report presents the results on the standardisation of ethambutol in formulations and biological fluid, defining the optimal conditions. It has been found to be very simple, satisfactory and reproducible. The methodology also defined several essential criteria to be fulfilled for the satisfactory results. For example, use of Amberlite CG-50 with 100-200 mesh size seem to be highly essential. The present method has the advantages of being rapid, involving simple steps like dilution and extraction into chloroform, adsorption of EMB onto the basified column leaving behind other drugs, and further, elution with suitable solvent system without any loss of analyte at any step. The *p* H has been found highly critical at which the elution was maximal and other anti-TB drugs did not interfere. The time taken for chromatography was also shorter.

Figure 1 gives mean values for EMB in 0.1 *N* HCI and pooled normal urine (collected from different volunteers). The concentration standards ranged from 50 to 400 µg/ml in 0.1 *N* HCI (7 occasions) or pooled normal (drug free) urine (10 occasions). Under these operating conditions, the lowest detection limit was 25 µg/ml. Table 1 gives the per cent recovery of EMB from pooled normal human urine and was found to be 90-100% in the presence of other anti-TB drugs. The sensitivity of the method. indicated that the lowest concentration (25 µg/ml) of EMB could be estimated by this procedure.

Ethambutol in HCI or urine was estimated in the presence of other anti-TB drugs (RMP, INH and PZA) and these drugs did not interfere and was specific for EMB.

The method described was applied for determination of EMB excreted in urine upto a period of 8 hr collected from the patients HIV (12), TB (13) and HIV/TB (12). who were given RNTCP regimen containing RMP, EMB, INH and PZA. Based on the excretion of the drug in urine (0-8 hr), % dose excreted was determined for EMB. The specificity in relation to other anti-TB drugs was a matter of concern, since the assay was based on the adsorption of EMB onto Amberlite CG 50 (100-200 mesh size) under basic conditions where other anti-tuberculosis

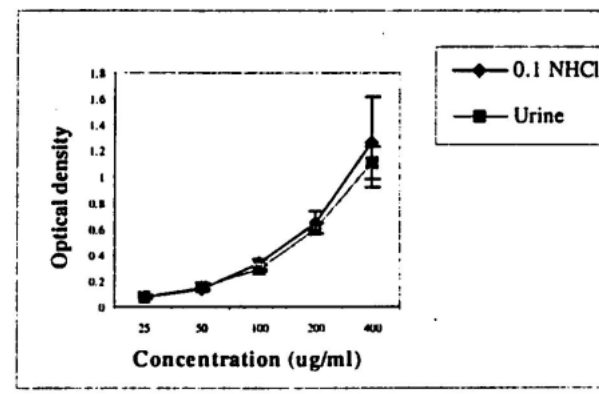


Fig. 1 - Ethambutol in 0.1 *N* HCI and urine

Table 1 — Recovery of EMB from Urine

| Concentration estimated already (µg/ml) | Added concentration (µg/ml) | Total concentration (µg/ml) | EH* | | EHR* | | EHRZ* | |
|---|---|---|---|---|---|---|---|---|
| | | | Concentration (µg/ml) | % | Concentration (µg/ml) | % | Concentration (µg/ml) | % |
| 50 | 50 | 100 | 104.3 | 104 | 98.1 | 98 | 94.0 | 94 |
| 100 | 50 | 150 | 135.2 | 90 | 146.2 | 97 | 147.2 | 98 |
| 200 | 50 | 250 | 255.8 | 103 | 257.1 | 103 | 260.1 | 104 |
| 50 | 100 | 150 | 157.3 | 105 | 160.6 | 105 | 153.8 | 102 |
| 100 | 100 | 200 | 182.5 | 91 | 196.8 | 98 | 204.0 | 102 |
| 200 | 100 | 300 | 296.0 | 99 | 315.5 | 105 | 311.3 | 103 |
| 50 | 200 | 250 | 256.8 | 103 | 260.1 | 104 | 247.3 | 99 |
| 100 | 200 | 300 | 296.7 | 99 | 292.1 | 97 | 305.6 | 102 |
| 200 | 200 | 400 | 396.7 | 99 | 395.8 | 99 | 411.8 | 103 |

*H-100 µg/ml, R-250 µg/ml. Z-600 µg/ml

Table 2 — Stability of EMB in urine

| Concentration (µg/ml) | Optical Density | | | | |
|---|---|---|---|---|---|
| | 0 day | 1st day | 4th day | 7th day | 10th day |
| 50 | 0.154 | 0.110 | 0.148 | 0.142 | 0.152 |
| 100 | 0.314 | 0.302 | 0.259 | 0.300 | 0.276 |
| 200 | .0.650 | 0.669 | 0.609 | 0.629 | 0.663 |
| 400 | 1.332 | 1.218 | 1.218 | 1.242 | 1.346 |

drugs namely RMP, INH and PZA did not interfere. The % dose of EMB excreted in urine was almost similar in patients under investigation. However, this needs to be confirmed on a larger sample size.

Differential concentrations of EMB were assayed in the presence of other anti-TB drugs on 0, 1, 4, 7 and 10 days after storage. The results showed that there was no deterioration of EMB even after storage upto 10 days (Table 2) and was 80-100% stable.

Since for TB, monotherapy is not advocated, and the patients are always given combination regimens, checking for both the quality of the drug as well as for the presence of exact quantity of the stated content in pharmaceutical preparations, are highly essential, in turn, to assess the bioavailability of the drug in human subjects. This could be achieved only after applying reliable, accurate and specific method to quantitate the drug in the formulation without the interference of other drugs. It is now possible to estimate in urine containing EMB in addition to other drugs and their metabolites. Therefore, fixed dose formulations containing EMB alone and in combination with other anti-TB drugs need to be examined by this method to validate the specificity. A total of 15 tablets of ethambutol hydrochloride containing EMB alone, 6 tablets of combunex 800 containing INH in addition to EMB, 11 tablets of AKuri T3 containing RMP, INH in addition to EMB, 17 tablets of AKuri T4 containing RMP, INH, PZA and EMB and 9 tablets of AKuriT FD containing RMP, INH, PZA along with EMB, were processed by this method to quantify EMB (Table 3).

The results obtained in urine excreted during 0-8 hr in patients administered RNTCP regimen showed that there was a significant difference in the % dose of EMB excreted between TB (control) and HIV group. However, TB vs HIV/TB and HIV vs HIV/TB were not significant (Table 4).

Based on the findings obtained with different formulations (FDCs) as well as with urine, it was concluded that EMB could be quantitatively assayed without the interference of other anti-TB drugs. Similarly, EMB could be determined in urine samples excreted over 0-8 hr collected from human subjects who were administered EMB along with other drugs. Dissolution of the drugs in pharmaceutical preparations, which is an indirect measure of the drug release in stomach, was carried out using the standard equipment. Tablets containing EMB alone were run through the dissolution apparatus and the release of EMB was estimated by the present procedure to check for completeness of release. Similarly, FDC containing 2, 3 or 4 drugs (containing EMB in addition to other drugs), were also processed by the dissolution apparatus and assayed for EMB.

Thus, this methodology is highly useful to assess the bioavailability of EMB either in pharmaceutical preparations or in biological fluid in the presence of other anti-TB drugs. The present method has the advantages of being simple and rapid. The technique involved steps like dilution and extraction into

Table 3 — Estimation of EMB in tablets

[Values are mean ± SD]

| EMB alone (mg) | | | EMB in FDCs (mg) | | | | |
|---|---|---|---|---|---|---|---|
| Ethambutol hydrochloride (E) | | | Macoxplus 150 (RH) | Comunex 800[e] (EH) | AkuriT3[f] (ERH) | AkuriT4[f] (ERHZ) | AkuriT FD[g] (ERHZ) |
| 200[a] | 400[b] | 800[c] | | | | | |
| 201.42 ± 6.50 | 392.66 ± 14.69 | 806.57 ± 34.69 | — | 801.67 ± 29.25 | 273.75 ± 8.02 | 275.42 ± 7.76 | 264.85 ± 13.04 |

Acceptable range —

[a]190-210 mg: [e]760.0-840.0 mg;
[b]380-420 mg: [f]261.2-288.7 mg;
[c]760-840 mg; [g]253.6-280.3 mg

Table 4 — % dose of EMB excreted in urine of patients with TB. HIV and HIV/TB

| Group | Mean ± S.D. |
|---|---|
| TB (n=13) | 41.5 ± 13.4 |
| HIV (n=12) | 22.4 ± 10.8 |
| HIV/TB (n=12) | 30.9 ± 13.5 |

TB Vs HIV ($p < 0.05$)-S; TB Vs HIV/TB - NS; HIV Vs HIV/TB - NS

chloroform, adsorption onto the column and elution with suitable solvent system and during these steps there was no loss of the analyte. The *p*H is highly critical at which the elution is maximal and other anti-TB drugs do not interfere and the results are available with shorter chromatographic adsorption and clution time.

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