Original Article

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Extraction/Isolation and Detection of Ethambutol in Blood Using High Performance Thin Layer Chromatography Plate

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Abstract

Ethambutol is 1,2-diamino alcohol, being a medication it is primarily used to treat tuberculosis. It plays an important role in antitubercular agent inhibiting the synthesis of spermidine in myobacteria and also inhibit transfer of mycolic acids into cell wall of tubercle bacillus. The route of administration is oral and is easily available in market. This is readily absorbed by gastrointestinal tract and 50% excreted unchanged by urine. Being a powerful chemotherapeutic agent it is specifically effective against actively growing microorganism of myobacterium and tuberculosis. Its analysis is usually done from biological samples such as blood and urine which are the main sample of choice in management and medicolegal cases. There are several techniques such as Gas chromatograph (GC), High Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass spectrometry (GC-MS), Liquid Chromatography-Mass Spectrometry (LC-MS) for its analysis. These techniques are not only costly but also time consuming and require more sophisticated instruments handling. An attempt has been made to develop a new method for analysis of ethambutol in blood using High Performance Thin Layer Chromatography plate. Ethambutol was extracted from blood using liquid-liquid extraction method and analyzed by using High Performance Thin Layer Chromatographic plate. For chromatographic separation various solvent systems were used as mobile phase. After the development of plates they were examined under UV light followed by spray of chromogenic reagents such as ninhydrin and iodine fumes which successfully increase the sensitivity without dispensing with the simplicity of the method. Present method is simple, non destructive, reproducible and repeatable which can easily be performed in any laboratory.

Keywords: Ethambutol; Ninhydrin; Iodine Vapours; HPTLC Plate; Liquid-Liquid Extraction etc.

Introduction

Ethambutol (EMB) is a medication frequently used for the treatment of Mycobacterium tuberculosis as well as used drug for infections caused by Mycobacterium avium complex. Being synthetic, bacteriostatic, antitubercular agent it inhibit the synthesis of spermidine in myobacteria and also inhibit transfer of mycolic acid into cell wall of bacteria. Ethambutol is given in combination with other tuberculosis drugs, such as isoniazid, rifampicin and pyrazinamide. It is on the World health oragnisation's list of essential medicines. It is sold under the trade names 'myambutol'and 'servambutol'.Its mechanism of action is not completely known. Ethambutol exert its bacteriostatic activity by inhibiting arabinosyl transferase, an enzyme that polymerizes arabinose into arabinan and finally to arabinogalactoid a constituent of myobacterial cell wall [1-3] .

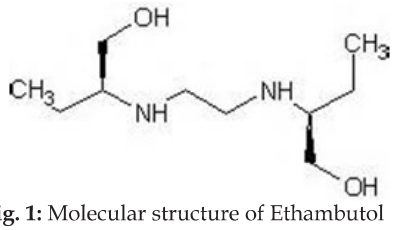

Fig. 1: Molecular structure of Ethambutol

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Molecular formula of ethambutol is $C_{10}H_{24}N_2O_2$ with molecular weight of 204.31g/mol. Both ethambutol and isoniazid are rapidly absorbed from the gastrointestinal tract following, oral adminisration.absoption rate get reduced when ethambutol and isonizid are administered with food. Its biological half life in normal renal function is 3 to 4 hours while in case of impaired renal finction it is upto 8 hours [4-5].

Fig. 2: Ethambutaol hydrochloride tablets on the trade name Myabutol

Five (5) mcg/ml are generally considered resistant to ethambutol. Ethambutol is widely distributed to most tissues and body fluids except cerebrospinal fluid (CSF). CSF Concentration are 10 to 50% of the corresponding serum concentration. Ethambutol also get distributed into breast milk. In normal person it cannot penetrate meniniges, but patients with tuberculous meningitis, it can penetrate 10 to 15% into meniniges .Its analysis can be done with various analytical sophisticated techniques such as LC-MS, GC, GC-MS, IR etc. An attempt has been made to develop an alternative sensitive method for detection of ethambutol on HPTLC plate using ninhydrin and iodine fumes as spraying reagent [6-8].

Experimental

Reagents/material: Ethanol, ammonia, methanol, chloroform from Merk India and ultra pure water from Rion India were used.

Glasswares: Beaker, HPTLC plate, glass chromatographic chamber, separating funnel, volumetric flask, fine capillaries form Bolrosil India were used.

Preparation of standard solution: The 1000 ppm solution of ethambutol was prepared by dissolving 10mg of ethambutol in 10 ml of ethanol.

Preparation of Spray Reagent

1. Ninhydrin reagent: 0.1g of crystals of ninhydrin was dissolved in 25 ml of acetone.
2. Iodine fume: 10g of iodine sublimate is kept in beaker and properly covered with parafilm.

Extraction from Blood

A 10 ml of blood was taken in the conical flask with 1 g of sodium tungstate and 1 ml of conc. Sulfuric acid was added and mixed. This mixture was then heated for 2-3 minute at 60°C. Contents were then cooled and filtered using filter paper. The filtrate is transferred to separating funnel and 20 ml diethyl ether was added to it and shaken for few minutes. The ether layer (organic layer) was separated .

The aqueous acidic layer was made alkaline by adding ammonium hydroxide (pH should be approximately 9-10) in a separating funnel and extracted with 20 ml of ether: chloroform (3:1) mixture and shaken for few minutes. The ether layer was separated (BE1). The aqueous layer was again extracted with 15 ml and 10 ml ether: chloroform (3:1) mixture repectively. The ether layers were separated (BE2 & BE3). All three organic extract BE1, BE2 and BE3 were combined and passed through a pad of anhydrous sodium sulfate over a funnel and then evaporated to dryness.

Spotting of samples and standard on HPTLC plates: Ethambutol extracted from blood was loaded on TLC plates along with the standard.

Development of HPTLC plates: The plates were kept in different solvent system (Table 1) which was taken in different ratios. After developing of TLC plates, the plates were taken out of the solvent chamber and dried in air then the plates were subjected to UV light at 254nm, followed by spraying reagent.

Visualization of HPTLC plates: Dried HPTLC plates were sprayed with ninhydrin reagent and than kept in oven at 110C for 10 minutes. After 10 minutes HPTLC plates were taken out and pink colour spots were observed. Another dried HPTLC

plates were a subjected to iodine fumes, yellow colour spots were observed.

Result and Discussion

Dried TLC plates were sprayed with ninhydrin reagent and then kept in oven at 110°C for 10 minutes. After 10 minutes HPTLC plates were taken out and pink colour spots were observed in both sample and standard. Another dried HPTLC plates was also subjected to iodine fumes, yellow colour spots were observed. The mobile phase used gives a compact spot. The limit of detection was 5µg with ninhydrin reagent and 2µg with iodine fumes. Rf of separation of ethambutol in all solvent system was analyzed and presented in table 1.

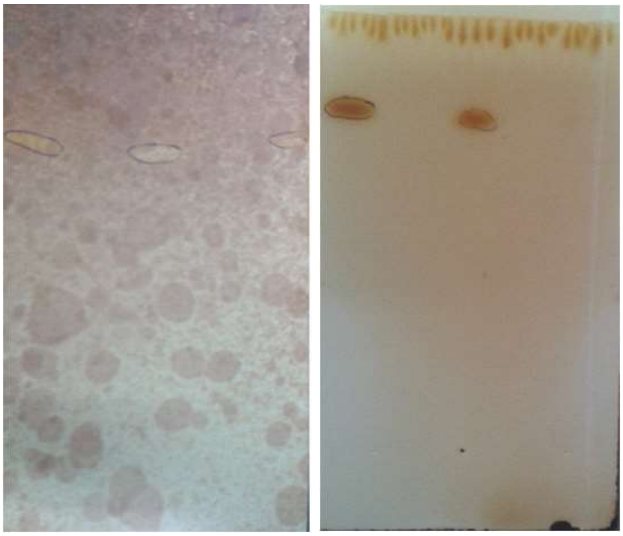

Fig. 3: Developed and dried plate is exposed with (a) Ninhydrin spray (b) Iodine fumes

Conclusion

The method developed for analysis of ethambutol in blood using liquid-liquid extraction followed by thin layer chromatography (TLC) is a rapid, simple and economical technique and can be performed in very less time in comparison to other sophisticated instruments.

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Table 1: Different solvent systems with ratio, colour, Rf. etc.

Solvent system	Ratio	(Run time (Min	Spraying reagents	Colour of spots	Rf
Chloroform: ethanol: ammonia	8:8:1	35	Ninhydrin Iodin Fume	Pink Yellow	0.65 0.64
Chloroform: ethanol: ammonia	4:4:2	30	Ninhydrin Iodin Fume	Pink Yellow	0.71 0.69
Ethanol: acetic acid: water: ammonia	4:1:5:0.5	60	Ninhydrin Iodin Fume	Pink Yellow	0.59 0.57
Ethanol: water: ammonia	12:2:1	30	Ninhydrin Iodin Fume	Pink Yellow	0.67 0.68
Ethanol: acetic acid: water: ammonia: n-hexane	4:1:5:0.5:4	55	Ninhydrin Iodin Fume	Pink Yellow	0.56 0.55
Methanol: chloroform	5:5	30	Ninhydrin Iodin Fume	Nil Nil	Nil Nil
Chloroform: methanol	8:2	30	Ninhydrin Iodin Fume	Nil Nil	Nil Nil
Ethanol	1:0	35	Ninhydrin Iodin Fume	Pink Yellow	0.17 0.15

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