



Evaluation of ^{99m}Tc -Ethambutol Radiopharmaceutical Using High Performance Liquid Chromatography with Radioactive Detector (Radio-HPLC)

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Abstract

^{99m}Tc -ethambutol is a radiopharmaceuticals used to determine the location of *Mycobacterium tuberculosis* that causes TB disease. To prepare this radiopharmaceuticals, supporting compounds such as mannitol as filler, SnCl_2 as a reducing agent and sodium pyrophosphate as co-ligand are required in addition to ethambutol and radionuclide compounds. The radiopharmaceuticals is made in the form of a lyophilized kit without ^{99m}Tc radionuclides. The aim of this study was to determine the effect of reducing agents on ^{99m}Tc -ethambutol preparation using the High Performance liquid chromatography (HPLC) equipped with radiodetector (radio-HPLC) to the quality of radiopharmaceuticals. In the previous study, the determination of radiochemical purity only used TLC by determining the amount of impurities TcO_2 and TcO_4^- . This study was carried out more profoundly by using radio-HPLC to ensure the quality of radiopharmaceutical products. The test were performed using paper chromatography, electrophoresis, radio-HPLC, and stability test of ^{99m}Tc -ethambutol radiopharmaceuticals. The results showed that the radiopharmaceutical kit of ethambutol is performing good physical characteristics and high radiochemical purity of ^{99m}Tc -ethambutol in analysis using radio-HPLC as well as paper chromatography and electrophoresis. The retention time (t_r) of ^{99m}Tc -ethambutol were found at 7.97 minutes using UV and radioactive detector, C18 column, isocratic system using phosphate buffer 0.2 M pH 7.4 and Acetonitrile (90 : 10), flow rate 0.8 mL/min. The radiochemical purity of ^{99m}Tc -ethambutol is still remain high ($91.44 \pm 0.92\%$) after 27 days of kit were made. From the results, it was concluded that radio-HPLC analysis provides better information on radiochemical purity as well as chemical purity of the labeling of ^{99m}Tc -ethambutol.

Keywords: ^{99m}Tc -ethambutol, Radio-HPLC, TB disease, *Mycobacterium tuberculosis*, Radiopharmaceuticals.

1. Introduction

The performance of a radiopharmaceutical kit is determined not only by radiochemical/chemical purity but also by physical appearance. Stability testing is termed as a complex process because several factors that influencing the stability of a pharmaceutical product ¹. A pharmaceutical product may undergo change in appearance, consistency, content uniformity, clarity (solution), moisture contents, particle size

and shape, pH, package integrity thereby affecting its stability. Such physical changes may be caused by impact, vibration, abrasion, and temperature fluctuations such as freezing, thawing or shearing. The chemical reactions like solvolysis, oxidation, reduction, racemization that occur in the pharmaceutical products may lead to the formation of degradation product, loss of potency of active pharmaceutical ingredient (API), loss of excipient activity like antimicrobial

preservative action and antioxidants ². The radiopharmaceutical stability is defined as the time during which the radioisotope can be safely used for the intended purpose. The radiochemical stability studies must be conducted to represent the effective use of the product ³. The physical-chemical characteristics of a radiopharmaceutical determine its pharmacokinetics, i.e., its fixation on the target organ, metabolism and excretion of the organism, whereas the physical characteristics of the radionuclide determine the application of the compound in diagnosis or therapy ³.

High Performance Liquid Chromatography (HPLC) is a popular analytical technique used for the separation, identification and quantification of each constituent of mixture ⁴. In this research, the HPLC uses two different detectors, the UV-Vis detector and radioactivity detector, hence it called radio-HPLC. The UV-visible absorbance detector is the most common HPLC detector in use today since many compounds of interest absorb in the UV (or visible) region (from 190–600 nm). Radioactivity detectors (sometimes referred to as radiometric or radio-flow detectors) are used to measure radioactive analytes as they elute from the HPLC column ⁴. Most of the HPLC systems currently available are controlled via a suitable software, which is also used to acquire and process signals coming from detectors ⁵. The radioactivity detector can be very sensitive and is extremely useful for the detection of radiolabeled compounds in toxicological, metabolism, or degradation studies ⁶. Analysis of radiochemical purity using HPLC is recommended in order to quantify the radiochemical purity of the preparation before its administration to patients ⁷.

Previous research that using HPLC as a modality for determination of labeled compound was conducted by Mihon, et al. for detection of Na¹⁸F ⁸, ⁶⁸Ga-labeled protein by Irina Velikyan ⁹, Tian-Ze Zhou conducted determination of ^{99m}Tc-pertechnetate using HPLC ¹⁰ and also Van Hemert et.al using HPLC to quantify the quality of ^{99m}Tc-

mertiotide (Mag-3) ⁷.

This study was conducted to evaluate the quality of the radiopharmaceutical kit of ethambutol made in packs of two vials (reducing agents and ligands) using paper chromatography/TLC, electrophoresis and radio-HPLC to determine radiochemical purity as well as chemical purity and also determine the stability of the radiopharmaceutical kit.

2. Materials and methods

2.1. Materials

Ethambutol-HCl, Na-pyrophosphate (Sigma-Aldrich), SnCl₂·2H₂O (Wako), Na^{99m}TcO₄ (Polatom), Acetone (Merck), Acetonitrile (Merck), phosphate buffer pH 7.4 0.2M, Aquabidest (IPHA), ITLC-SG (Agilent), Whatman 31ET, Whatman 1, cellulose acetate paper, pH indicator universal (Merck), metal free vials, syringe (Terumo), analytical balance (Mettler Toledo), vortex stirrer (lab companion), micropipette (Eppendorf), tip, oven (Heraeus), Chromatography chamber, paper electrophoresis set (Mojo science), microtube, lead container, HPLC equipped with GABY-star detector (Agilent), Single Channel Analyzer (SCA) (Ortec), Dose Calibrator (Victoreen) and TLC scanner (Bioscan AR-2000).

2.2. Methods

2.2.1. Labeling of ^{99m}Tc-ethambutol

A total of 3.5 mg of Na-pyrophosphate was dissolved in 1 mL of aquabides, then 1.4 mg of SnCl₂·2H₂O was added (Vial A). In another vial (Vial B), 3.5 mg ethambutol and 5 mg Manitol were dissolved in 0.5 mL aquabidest. In to vial B was added 0.5 mL of solution in vial A and 1 mL of Na^{99m}TcO₄ (2-5 mCi). The radiochemical purity testing was carried out by using two chromatographic systems, first eluent is acetonitrile 50% and whatman 31 ET as a stationary phase and the second is dry acetone as an eluent and ITLC-SG as stationery phase.

2.2.2. Electrical charge determination of ^{99m}Tc-ethambutol

Using paper electrophoresis
Determination of electrical charge was done through cellulose acetate paper

electrophoresis method. Cellulose acetate paper was immersed in a phosphate buffer pH 7.4 0.2 M for 30 minutes and dried by tapping it with tissue paper. Then cellulose acetate paper is placed in an electrophoresis device. The cellulose acetate paper was added by 2 μ L ^{99m}Tc -ethambutol at zero point, then electrophoresed for 1 hour with a 5A current/paper and a potential of 200 V.

2.2.3. Analysis of impurity using radio-HPLC

The purity of ^{99m}Tc -ethambutol were done by the reverse phase method using C18 column. The elution system is isocratic and the mobile phase is mixture of phosphate buffer 0.2 M pH 7.4 and Acetonitrile (90:10). The flow rate 0.8 mL/minute for retention time of 30 minutes. The detectors used were GABI star for radioactive and UV with wavelength of 202 nm

2.2.4. Stability determination of ^{99m}Tc -Ethambutol

Chemical stability of a drug is the length of time for a drug to maintain the chemical integrity and its potential as stated in the label within the prescribed time limit. Physical stability is evaluating changes in the physical properties of a product depending on the time (storage period). The examples of physical changes include the change in color, taste, smell, texture or appearance. Evaluations of physical stability tests include organoleptic examination, homogeneity, pH and specific gravity. Stability testing is

carried out by determining the radiochemical purity of ^{99m}Tc -ethambutol over a certain time period. In addition to radiochemical purity, stability testing is also carried out on the physical conditions of the preparation and the kits obtained. The test was carried out at 0-6 hours after labeled with technetium-99m and for 30 days using paper chromatography.


3. Results

The result in table 1. described the physical and chemical performance of radiopharmaceutical kit of ethambutol. From the result, it is shown that both the radiochemical purity of ^{99m}Tc -ethambutol and its impurities also the pH were met the requirements as a good radiopharmaceutical.

^{99m}Tc -ethambutol radiopharmaceuticals consisted of several ingredients which are ethambutol, pyrophosphate, and also mannitol. The conventional tests to determine the radiochemical purity of ^{99m}Tc -ethambutol is using paper chromatography. The paper/TLC chromatography method cannot separate the ^{99m}Tc -pyrophosphate impurities which may be formed during the labeling process. Therefore the radio-HPLC were used to determine radiochemical purity as well as chemical impurities.

The electrophoresis method were done to assign the electrical charge of the ^{99m}Tc -ethambutol, from the result from figure 1., it has shown that ^{99m}Tc -ethambutol has a negative charge and it was correspond to previous result ¹¹.

Table 1. Performance of radiopharmaceutical kit of ethambutol

Parameter	Result
Radiochemical purity	92.61% \pm 4.59%
TcO ₂	1.55% \pm 0.97%
TcO ₄ ⁻	5.83% \pm 4.47%
pH	8,5-9
Physical appearance	 Clear

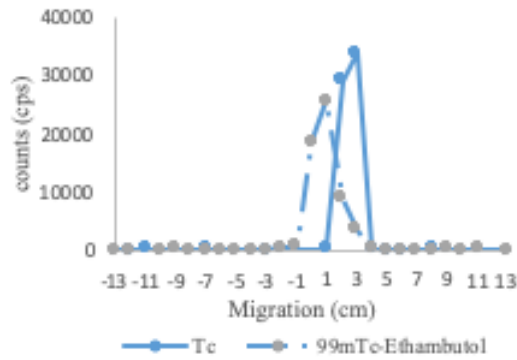


Figure 1. Cellulose acetate paper electrophoresis chromatogram of ^{99m}Tc-ethambutol

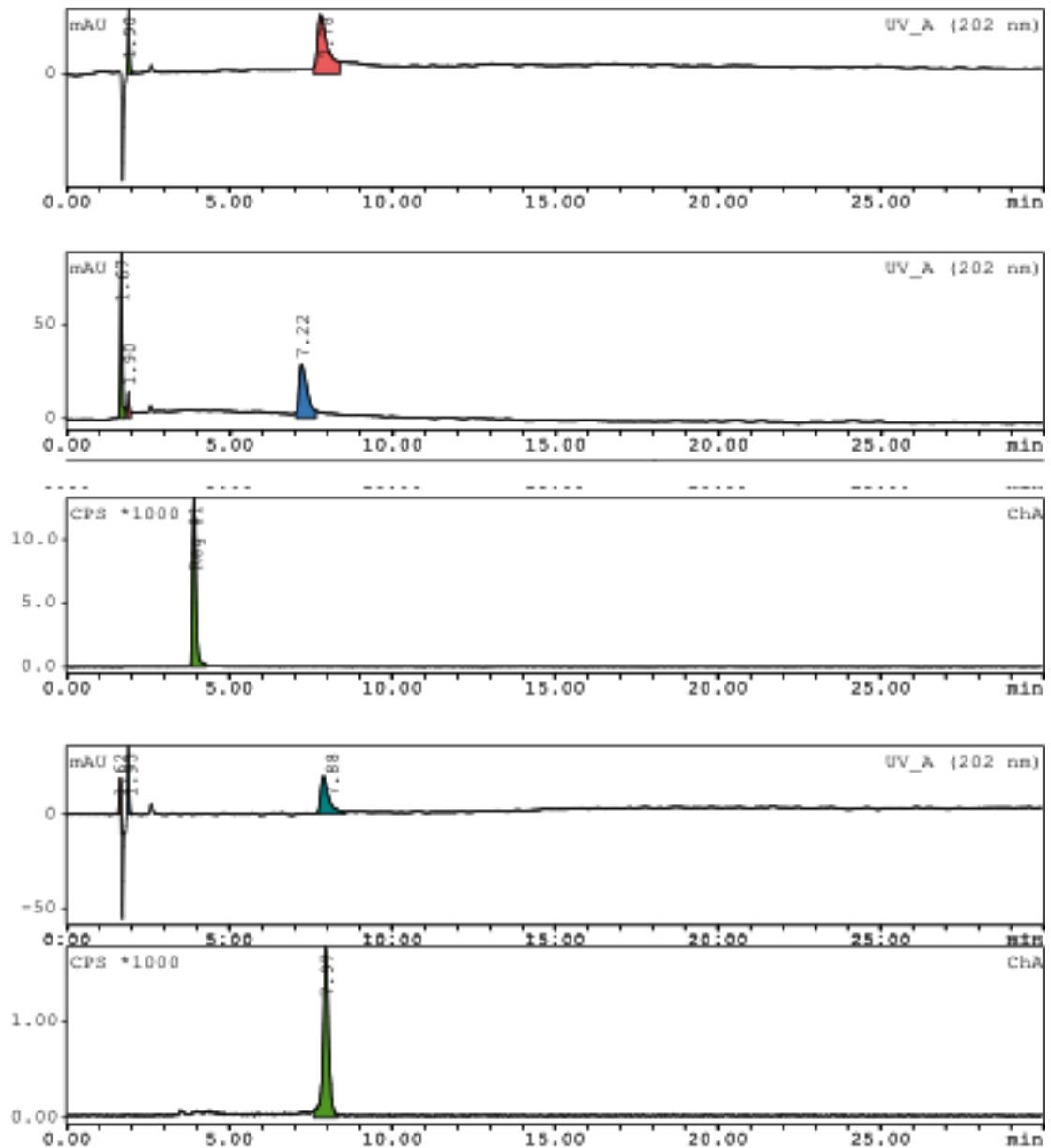


Figure 2. High Performance Liquid Chromatography (HPLC) pattern of (a) ethambutol, (b) mixture of Sn, pyrophosphate and ethambutol (c) TcO_4^- and (d) ^{99m}Tc-ethambutol

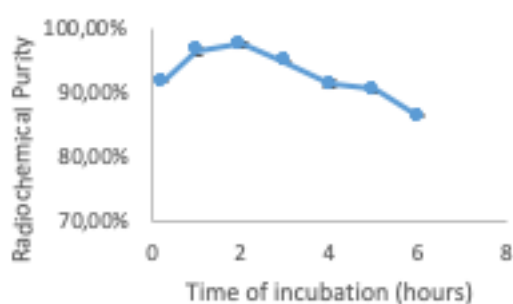
The result from HPLC detection of each compounds can be seen in Figure 2. The retention time (t_r) of ethambutol is at peak at t_r 1.6 and 1.9 min representing the

Sn-Pyrophosphate, while t_r 7.22 represent ethambutol. The radiopharmaceutical kit of ethambutol appears in two different detector, UV and GABI-star. The UV detector shown three peaks with the same t_r as the cold kit, whereas in the GABI detector there is only one peak appears at t_r 7.5 represent the ^{99m}Tc -ethambutol. t_r value can be seen in the Table 2.

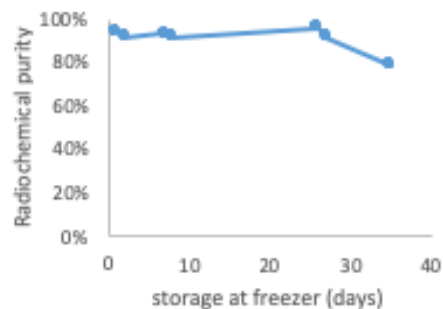
Since the radiopharmaceutical kits were made in the form of the frozen kit, the stability testing is a parameter that is needed to ensure the kit is in a good quality or not. It was conducted by determining the radiochemical purity and arranged during the labeling process as well as after storage within a certain time period. The test results can be seen in Figure 3.

Table 2. t_r Value of kit in the HPLC

Compounds	Tr UV detector (min)	Tr GABI detector (min)
Ethambutol	7.78	-
Sn Pyrophosphate ethambutol	1.69 1.90 7.22	-
$\text{Na}^{99m}\text{TcO}_4$		3.90
^{99m}Tc -ethambutol	1.62 1.93 7.97	7.88



(a)



(b)

Figure 3. Percentage of ^{99m}Tc -ethambutol radiochemical purity in the time of incubation (a) and in the time of storage (b)

4. Discussion

The physical and chemical performance of radiopharmaceutical kit of ethambutol was described at table 1. The radiochemical purity of ^{99m}Tc -ethambutol was $92.61 \pm 4.59\%$. This result is still met the requirement of a good radiopharmaceuticals. These values also correspond to the results obtained by Nanny K (2007).

The obtained pH result was different with the study obtained by Nanny et al (2007) due to the different amount of reducing agent. Since the amount of Sn-pyrophosphate was higher, the pH of the solution increased

because the pH of Sn-pyrophosphate solution was alkaline (9-10). Thus, it is still met the requirement for injected solution of pharmaceutical, which have the values 3-10.5.

Figure 2 shows that all ethambutol have been labeled by ^{99m}Tc . It was assigned by there was only one peak that appear in the chromatogram of GABI star detector. The chromatogram also indicates that the labeling process is effective and does not produce any other impurities. A small shift in t_r value indicates caused by different pH¹². The structure is not significantly changed and proves that the reaction takes place through

the mechanism of exchange labeling where molecules of pyrophosphate replaced by 2 molecules of ethambutol formed the ^{99m}Tc -ethambutol chelate complex. The structure is shown in Figure 4.

Based on the results obtained in Figure 3 (a), it can be seen that 5 hours after the incubation time, the radiochemical purity of ^{99m}Tc -ethambutol becomes less than 90%. It

means that the preparation cannot be used after 5 hours of labeling because the quality is not met the requirement and caused imaging results to be false. The testing also conducted to the radiopharmaceutical kit of ethambutol that stored in the frozen form. The result in figure 3 (b) shown that the radiochemical purity of ^{99m}Tc -ethambutol decreased after 27 days of storage.

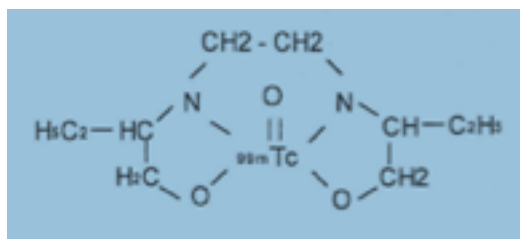


Figure 4. Structure of ^{99m}Tc -ethambutol ¹¹.

5. Conclusion

The radiopharmaceutical kit of ethambutol is performing good physical characteristics and high radiochemical purity of ^{99m}Tc -ethambutol both in analysis using paper chromatography, electrophoresis and radio-HPLC. The ^{99m}Tc -ethambutol shown the peak at 7.97 minutes using UV and radioactive detector, C18 column, isocratic system using phosphate buffer 0.2 M pH 7.4 and Acetonitrile (90:10), flow rate 0.8 mL/min. The radiochemical purity of ^{99m}Tc -ethambutol is $91.44 \pm 0.92\%$ after 27 days of kit were made. From the results, it was concluded that radio-HPLC analysis provides better information on radiochemical purity as well as chemical purity of the labeling of ^{99m}Tc -ethambutol.

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