Determination of Ethambutol Hydrochloride in the Combination Tablets by Precolumn Derivatization

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Abstract

A new high-performance liquid chromatography method for the quantitative determination of ethambutol hydrochloride in combination tablets is presented. Ethambutol is derivatized with phenylethylisocyanatate at room temperature (22 ± 2°C) for 5 min. Separation is performed by a C_{18} column using methanol–water–glacial acetic acid (70:30:0.2, v/v/v) as the mobile phase. The method is linear for drug concentrations in the range of 20–120 µg/mL (r = 0.9995). The intra- and inter-day precisions are lower than 1.46% and 2.22%, respectively. The average recovery of the samples at three levels is 99.8%. The results show that derivatization of ethambutol is stable at 30°C for 24 h. This method is simple, rapid, and stable in the presence of common excipients and antituberculosis drugs in the tablets.

Introduction

Tuberculosis has been a scourge of mankind for thousands of years and remains one of the largest health problems in the world today (1). The fixed-dose combinations (FDCs) containing ethambutol (EMB) hydrochloride can simplify treatment and virtually eliminate the risk associated with monotherapy (i.e., the development of drug-resistant strains of Mycobacterium tuberculosis). To improve tuberculosis treatment, 2- and 4-drug FDCs were recommended by the World Health Organization (2).

The assay method of EMB hydrochloride in the tablets given in the Chinese Pharmacopoeia (CHP) involves repeated chloroform extraction from a basic aqueous solution and subsequent non-aqueous titration (3). The CHP method was found not applicable to the quantitative analysis of EMB in the combination tablets because of the interference of other compositions. A gas chromatography (GC)–mass spectrometric (MS) method has been used for the determination of EMB in tablets (4). The EMB in the human plasma by liquid chromatography (LC)–MS has been reported (5). The determination of EMB by reverse-phase (RP) high-performance liquid chromatography (HPLC) is difficult because of its high pose, small molecule, and its lack of strong absorption by UV. EMB derivatives have a strong UV absorbance and can be measured by RP-HPLC. A number of methods for the derivatization of EMB have been reported. M. Breda et al. (6) described HPLC using 4-fluoro-7-nitrobenjo-2-oxa-1,3-diagole as a derivatization agent at 80°C for 30 min. Philippe Chenevier et al. (7) developed the method of the pre-column derivatization with phenylethylisocyanatate (PEIC) after chloroform extraction. However, the derivatives have not been studied in detail and there is no specific method for analysis of the tablets. In this paper, a new simple method based on pre-column derivatization is presented for the determination of EMB in the combination tablets without organic extraction and heating. The main derivatives were detected by LC–MS, which was composed of 1 EMB molecule and 2 PEIC molecules.

Experimental

Materials and reagents

Standards of EMB hydrochloride and rifampicin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). PEIC, isoniazid, and pyrazinamide were purchased from Sigma Chemical (Dikma Ltd, Beijing, China). The combination tablets were obtained from the Shenyang Hongqi pharmaceutical factory. Methanol and acetonitrile were of HPLC grade, and all other reagents were of analytical grade. Distilled, deionized water prepared in this laboratory was used to prepare the mobile phase.

Apparatus and chromatographic conditions

The HPLC system used was HP series, which included a pump (510), UV detector (486), chromatogram station (N2000), and i.d. hypersil C_{18} column (250 * 4.6-mm, particulate size 5 µm). The mobile phase was methanol–water–glacial acetic acid (70:30:0.2, v/v/v). The flow rate was 1.0 mL/min with UV 210 nm at room temperature. An LCQ data processing system was used. The LCQ LC–MS (Finnigan, San Jose, CA) was equipped with an electrospray ionization (ESI) source.

Solutions

Stock of PEIC (2 mg/mL) was prepared in acetonitrile. The standard stock solution of EMB hydrochloride (1 mg/mL) was prepared in acetonitrile containing 3% triethylamine. The test

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solution (100 µg/mL) and calibration curve solutions were obtained by diluting EMB hydrochloride standard stock solution in acetonitrile.

Preparation of sample solution

Twenty tablets were weighed and finely powered. A portion of the powder, equivalent to approximately 100 mg of EMB hydrochloride, accurately was transferred to a 100-mL volumetric flask adding approximately 90 mL of acetonitrile containing 3% triethylamine. The solution was sonicated for 5 min, then diluted with acetonitrile to volume and mixed. This solution was filtered though a dry filter into a flask, discarding the first portion of the filtrate. One milliliter of the subsequent filtrate was piped into a 10-mL volumetric flask, diluted with acetonitrile to volume, and mixed.

Derivative conditions

Derivative procedure

A 0.4-mL volume of EMB hydrochloride test solution was added into a tube containing 0.1 mL of PEIC (2 mg/mL) and mixed. It was shaken at room temperature for 5 min and evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in 100 µL of mobile phase, and 20 µL was injected into the HPLC system.

Selected medium

EMB hydrochloride solutions (100 µg/mL) were obtained by diluting EMB hydrochloride standard stock solution in acetonitrile, methanol, and water, respectively. Proceeding derivations were in the same way as described previously.

Derivative reaction time

A 0.4-mL volume of EMB hydrochloride standard test solutions were added into the tubes (containing 0.1 mL of PEIC stock solution) and shaken for different time (1, 5, 10, and 30 min) following the previously described procedures.

Molar ratio

The EMB hydrochloride standard test solution (0.4 mL) were added into the tubes containing the different molar ratios of PEIC to EMB, respectively. The derivative procedures were the same as described.

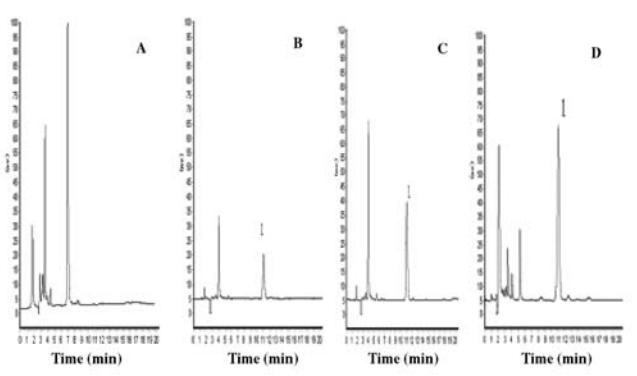

Figure 1. Typical chromatograms of the derivatives in four kinds of medium: blank PEIC (A), aqueous solution (B), methanol (C), and acetonitrile (D). The peak of each derivative is labeled with a 1.

Results and Discussion

Optimization of derivatization condition

Derivative medium

The chromatographic spectra of the PEIC–EMB derivatives from three kinds of medium are presented in Figure 1. The reaction mediums were found to have a strong influence on the derivative yield. The optimal medium for the reaction was found in acetonitrile. The PEIC–EMB derivatives were poor in an aqueous solution because of the degradation of PEIC. Thus, an aqueous solution must not be used.

Derivative reaction time

The derivatives could be reacted rapidly and consistently on different runs at room temperature, which was not significant at different shaking time. Therefore, the shaking reaction time was chosen at room temperature for 5 min.

Molar ratio

The molar ratios of PEIC–EMB from 1:1 to 9:1 were observed. The yield of derivatives was directly related with the molar ratio. When increasing the ratio of PEIC to EMB, the yield of the derivatives rose. The yield was maximum and constant when the ratios were more than 5:1. The PEIC–EMB derivatives were injected into the LC–MS system. The ESI mass spectrum of the derivatives was obtained and is shown in Figure 2. The fake molecule ion [M+Na$^+$] peak at *m/z* 521 shown in Figure 2 indicated the main derivatives was composed of 1 EMB molecule and

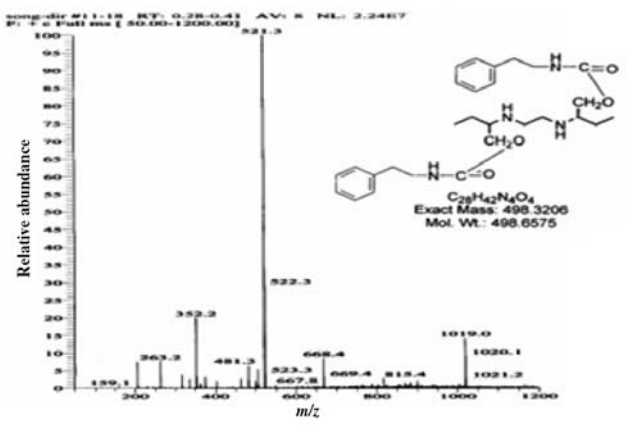

Figure 2. LC–MS of derivatives show the fake molecular ion [M+Na$^+$] peak at *m/z* 521 in positive ionization mode with the voltage set at 4.5 kV.

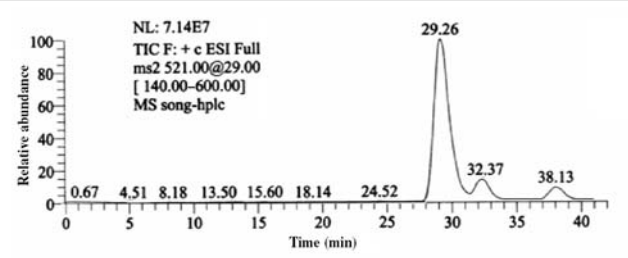

Figure 3. LC–MS–MS chromatograms of *m/z* 521 of derivatives. There were three peaks at 29.26, 32.37, and 38.13 min, respectively.

2 PEIC molecules.

Three peaks appearing in the chromatograms at *m/z* 521 of the derivatives were at 29.26, 32.37, and 38.13 min, respectively, and are shown in Figure 3. The ion was scanned at different times by MS^2 . The results showed that the main fragment ion was always at *m/z* 374 (Figure 4).

It suggested that there should be three isomeric compounds of 1 EMB molecule with 2 PEIC molecules. Among three isomeric compounds, the isomeric compound at 29.26 min had the strongest abundance and the biggest chromatographic peak, and it separated well with the other two isomeric compounds; thus, this isomeric compound was chosen for the quality standard of EMB hydrochloride. There were other weak molecular ion peaks at *m/z* 688 and *m/z* 815 in the mass spectrum of the MS^1 scan of derivatives (Figure 2). It could be concluded that the derivatives may consist of 1 EMB molecule and 3 or 4 PEIC molecules. For assuring the plenitude and completeness of the derivatization reaction, the molar ratio should be more than 6:1.

Validation of the method

Specificity

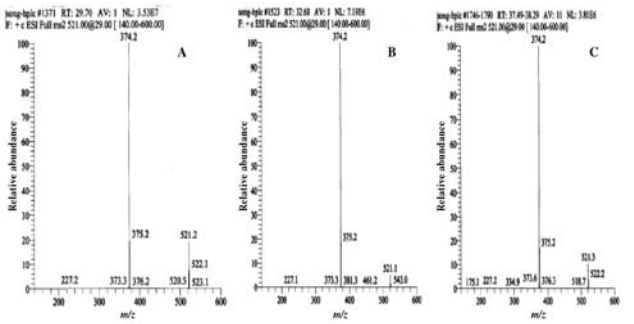

Figure 4. LC–MS–MS of *m/z* 521 of the derivatives with the same fragment ions at *m/z* 374, but different retention times: 29.70 (A), 32.68 (B), and 38.29 min (C).

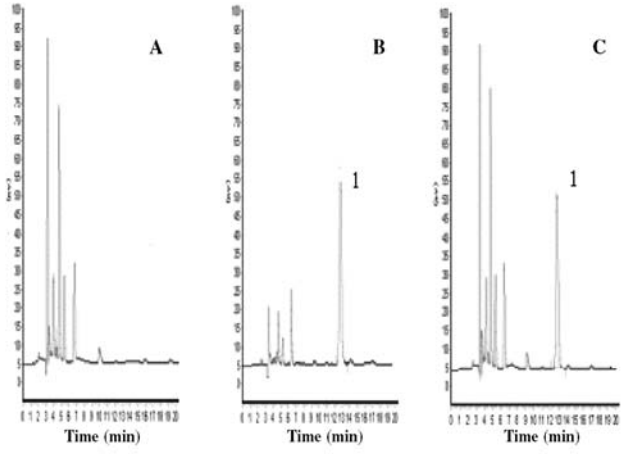

Figure 5. Typical chromatograms of the derivatives of the interferences with common excipients and three kinds of antituberculosis: common excipients, three kinds of antitubeculosis drugs, and PEIC (A); the derivatives (B); and the derivatives, common excipients, and three kinds of antitubeculosis drugs (C). The peak of each derivative is labeled with a 1.

Putative interferences with polyvinylpyrrolidone, stearic acid, lactose, and starch and with three antituberculosis drugs (isoniazid, pyrazinamide, and rifampicin) were in-vestigated. EMB hydrochloride added to the substances described was derivatized according to the previous method. Interference tests are presented in Figure 5. There was no interfering peak at the retention time for the PEIC–EMB derivative. Those excipients and three kinds of antituberculosis drugs did not affect the derivatization and the determination of EMB hydrochloride.

Linearity

Standard curves were constructed by adding appropriate volumes of EMB hydrochloride standard stock to yield the concentrations ranging from 20–120 µg/mL. Linearity solutions were injected in triplicate. The calibration graphs were obtained by regression analysis of the peak area compared with the concentration of EMB hydrochloride. The equations of calibration curves were found to be linear in the range of 20–120 µg/mL. Regression parameters for the calibration curves of EMB hydrochloride are shown in Table I.

Table I. Results of the Linearity Study

Linearity (µg/mL)	Slopes* (n = 3)	Intercepts* (n = 3)	Coefficients of correlations
20–120	20752 (224.0)	88995 (195.1)	0.9995
1–6	3287.9 (5.41)	326.0 (8.26)	0.9997

* Standard deviation shown in parentheses.

Table II. Precision and Accuracy of the HPLC Method

Theoretical concentration (µg/mL)	Intra-day measured concentration (mean ± SD)	CV (%)	Inter-day measured concentration (mean ± SD)	CV (%)	% Accuracy
40	40.0 ± 0.59	1.46	40.2 ± 0.89	2.22	100.2
80	80.2 ± 0.86	1.08	79.4 ± 1.12	1.41	99.9
120	118.6 ± 1.13	0.95	118.5 ± 1.13	0.96	98.9

Table III. Recovery of EMB Hydrochloride in the Combination Tablets

Added (n = 3) (mg)	Found (n = 3) (mg)	% Recovery*	(%) Average recovery†
80.1	79.6	99.4 (0.95)	99.8
100.2	100.4	100.2 (0.70)	
119.6	119.2	99.7 (0.40)	

* Relative standard deviation shown in parentheses.
† Average recovery = the average of three levels and nine determinations.

Precision and accuracy

The precision was determined by three EMB hydrochloride concentrations of 40, 80, and 120 µg/mL. Intra-day precision was assessed by analyzing the three concentrations on the same day, and each concentration had six replicates. Inter-day was assessed at the three concentrations on six different days. Accuracy was assessed at the same concentration and expressed as the percent deviation from theoretical value. The precision and accuracy of the method are presented in Table II. Coefficients of variation (CVs) were lower than 2.22% at the studied concentration range.

Recovery test

Recovery of the samples was studied by recovery experiments. The recoveries were performed at three level samples (80%, 100%, and 120%), which is equivalent to 100 mg of EMB hydrochloride, simultaneously added excipients, and three kinds of antituberculosis drug according to prescription proportion. The samples were prepared in the same way as the derivatization procedure previously described. The percentage recoveries were calculated from the calibration curves in the range of 99.4% to 100.2%. The average recovery of three levels was 99.8% (Table III)

Stability

The stability of PEIC–EMB derivative was observed at 15°C, 20°C, 25°C, and 30°C for 24 h. The results of stability were compared with the peak response area of a freshly prepared PEIC–EMB derivative at 20°C as 100%. Under the condition, no degradation could be evidenced. The quantity of the derivatives was constant when the derivatization reaction was for 5 min at 15–30°C and was stable for at least 24 h.

Determination of the limits of detection and quantitation

To determine the limit of detection (LOD) and the limit of quantitation (LOQ), a specific calibration curve was constructed by diluting the EMB hydrochloride standard stock solution in the range of 1–6 µg/mL. Regression parameters for the calibration curves of EMB hydrochloride are shown in Table I. The LOD, defined as the concentration of analyte on-column able to produce a signal-to-noise ratio (S/N) of 3, was 0.2 µg/mL. The LOQ was 1 µg/mL.

Determination of EMB hydrochloride in the tablets

The contents of EMB hydrochloride in the combination tablets were determined by the proposed method using the calibration curve. The determinations were done in two sets, and six samples were prepared for each set. The label amounts of two sets were 99.8% and 100.6%, respectively.

Conclusion

A specific, rapid, and reliable determination method for EMB hydrochloride in the combination tablets has been developed. The quantity of derivatives was constant and stable. The method was simple, easily operable, and consistent with the routine quality control analysis.

Acknowledgments

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