Flow injection analysis of ethambutol in synthetic urine using a graphite-polyurethane composite electrode as an amperometric detector

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Received 20 February 2013; Accepted 23 May 2013

Abstract: Ethambutol (ETB) is a first-line antitubercular drug effective against actively growing Mycobacterium tuberculosis. Resistance of the mycobacterium to ethambutol among tuberculosis (TB) patients results from inadequate or inappropriate dosing of treatment or using low quality medication. It is therefore necessary to develop reliable methods for determining ethambutol metabolic profiles of patients at point of care for proper dosing. Herein an efficient ETB sensor device is illustrated. It consists of a graphite-polyurethane composite electrode.

In order to characterise the electrochemical behaviour of ethambutol at pH = 8.0 voltammetric studies were performed. The detector was assembled in a flow injection apparatus and operated at +1.2 V (vs. Ag/AgCl(NaCl sat.)). The influence of sample volume and flow rate was studied. The linear response for the method was extended up to a 1.1 mmol L⁻¹ ethambutol solution with a detection limit of 0.0634 mmol L⁻¹. The reproducibility of current responses for injections of 0.7 mmol L⁻¹ ethambutol solution was evaluated to be 5.1% (n = 30) and the analytical frequency was 161 determinations h⁻¹. Two different samples were successfully analysed and the results were in good agreement with those obtained using capillary zone electrophoresis (CZE).

Keywords: Graphite-polyurethane electrode • Ethambutol • Synthetic urine • Flow injection analysis

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1. Introduction

Tuberculosis, or TB, is an infectious bacterial disease caused by Mycobacterium tuberculosis, which most commonly affects the lungs. It is transmitted from person to person via droplets from the throat and lungs of people with the active respiratory disease [1].

In healthy people, infection with Mycobacterium tuberculosis often causes no symptoms, since the person’s immune system acts to “wall off” the bacteria. The symptoms of active TB in the lung are coughing, sometimes with sputum or blood, chest pains, weakness, weight loss, fever and night sweats. Tuberculosis is treatable with a six-month course of antibiotics [1].

Despite the availability of highly efficacious treatment for decades, TB remains a major global health problem. In 1993, the World Health Organization (WHO) declared TB a global public health emergency, at a time when an estimated 7–8 million cases and 1.3–1.6 million deaths occurred each year. In 2010, there were an estimated 8.5–9.2 million cases and 1.2–1.5 million deaths (including deaths from TB among HIV-positive people) [1].

Ethambutol (ETB) is a first-line antitubercular drug effective against actively growing Mycobacterium tuberculosis. While the precise mechanism of action of ETB is unknown, the drug has been shown to inhibit the incorporation of mycolic acid into the mycobacterial cell
A number of methods have been described for determination of ETB in various matrices (such as human plasma, urine and pharmaceutical formulations), from simple analytical techniques such as titrimetry [2] to more modern and refined procedures such as mass-spectrometry [3,4]. Most of these procedures focus on analytical separations under chromatographic [3–7] or electrophoretic [8–11] conditions.

Furthermore, many other ways to quantify ethambutol have been described. Spectrophotometric procedures using different reagents such as copper [12,13] and 2,4-dinitro-1-fluorobenzene [14] have been reported. Electroanalytical methods have also been cited using potentiometric [12,15] and voltammetric sensors [16,17].

Regardless of the advantages of electroanalysis, few articles have studied such applications for the pharmaceutical analysis of ethambutol compared with more expensive techniques. Concerning the use of carbon-based composites it is possible to conclude that, although composite sensors offer many advantages such as low costs, simple preparation, stability and the potential for use with many different media and supporting electrodes, only one record of such an application has been reported, by our group [18].

In the present paper the electrochemical behaviour of ethambutol on a graphite-polyurethane composite surface is introduced and an analytical device for quantification of ETB in synthetic urine is described.

2. Experimental procedure

2.1. Reagents and chemicals

All chemicals were analytical grade and were used as received. Solutions were prepared by dissolving solids or concentrated solutions in deionised water processed through a water purification system (Milli-Q, Millipore, USA). Ethambutol dihydrochloride was obtained from Sigma-Aldrich (St. Louis, MO, USA). Aqueous phosphate buffer (pH 8.0) used in all experiments was prepared by dissolving Na2HPO4 and NaH2PO4 in suitable proportions. Acetic acid and copper(II) sulphate pentahydrate were purchased from Impex (Diadema, SP, Brazil) and Reagen (Rio de Janeiro, RJ, Brazil), respectively. Salts of urea, glucose, uric acid and sodium hydroxide were obtained from Vetec (Rio de Janeiro, RJ, Brazil) and Sigma-Aldrich (St. Louis, MO, USA). Ethanol, lactate and all stock solutions were prepared in phosphate buffer to obtain appropriate concentrations. Aqueous acetate/sodium acetate buffer (HAc/NaAc) (pH 4.6) (buffer solution-BS) stock solution at a concentration of 600 mmol L−1 and a 50 mmol L−1 aqueous copper(II) sulphate pentahydrate (CuSO4·5H2O) stock solution were used for electrolyte solution preparation.

2.2. Instrumentation

Voltammetric experiments were carried out with a computer-controlled Palm Sens potentiostat with PS Trace software. All the experiments were performed in a three-electrode cell using a homemade Ag/AgCl (NaCl saturated) electrode as reference, a platinum wire, and the proposed composite sensor. Flow injection analysis (FIA) experiments were performed using an Ag/AgCl (NaCl saturated) electrode and platinum wire as reference and counter electrodes, respectively. The working electrode was the proposed composite sensor. The electrochemical cell was mounted in a wall jet configuration. Different volumes of sample and a peristaltic pump (Gilson, France) with different flows were used to optimise the developed method. The supporting electrolyte for FIA experiments consisted of phosphate buffer (pH 8.0). Experiments were performed at room temperature unless otherwise indicated.

The experiments involving separation were conducted in a capillary electrophoresis (CE) system (HP3d CE, Agilent Technologies, Palo Alto, USA) equipped with a diode array detector (DAD) set at 262 nm, a temperature control device maintained at 25°C, and software (HP ChemStation, rev A.06.01) for equipment control and data acquisition. Samples and standards were injected hydrodynamically (30 mbar for 5 s) and the electrophoretic system was operated under normal polarity and constant voltage of +25 kV. For all experiments, a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) 48.5 cm (40 cm effective length) ×75 mm inner diameter ×375 mm outer diameter was used. When a new capillary was used, it was pre-treated by a pressure flush with 1.00 mol L−1 NaOH solution (30 min), deionized water (5 min) and electrolyte solution (10 min). In between runs, the capillary was flushed with 0.20 mol L−1 NaOH solution (2 min), deionized water (2 min) and fresh electrolyte solution (3 min).

2.3. Composite electrode preparation

Polyurethane resin was prepared by mixing 0.85 parts of prepolymer A-249 and 1.0 part of polyol B-471 (Poliquil, Brazil). Suitable amounts of graphite powder, 1–2 mm diameter (Aldrich, USA) were added in order to reach 60% in mass. This mixture was homogenized in a mortar for 15 minutes and then extruded as 3.0 mm diameter rods [19]. The rods were left to cure for 24 hours and then cut into 1 cm sections. Contacts were made using silver epoxy glue and copper wire, and the
Flow injection analysis of ethambutol in synthetic urine using a graphite-polyurethane composite electrode as an amperometric detector

2.4. Amperometric determination of ethambutol and comparison method

Ethambutol content in synthetic urine was determined using the proposed FIA amperometric method. The results obtained by the proposed method were compared with those obtained using the capillary zone electrophoresis (CZE) method based on ETB complexation with copper(II) [9]. The samples were diluted in 0.5 mmol L⁻¹ CuSO₄ and 60.00 mmol L⁻¹ HAc/NaAc buffer before CZE analysis. Quantification was performed by external calibration at the wavelength of maximum absorbance at 262 nm.

2.5. Samples

The use of a graphite-paraffin composite sensor for the detection/quantification of antituberculosis drugs has been previously described in the literature by our group [18]. Ethambutol excretion occurs by the renal system; however, 50% of the dose is excreted without metabolism. The synthetic urine was used in order to evaluate the performance of the proposed procedure for biological analysis without the risk of contamination during the assay. The sample solutions were prepared in the laboratory as described by Semaan and Cavalheiro [20] and analysed by the proposed method. Synthetic urine samples were spiked in order to achieve concentrations close to 1 mmol L⁻¹ ethambutol, which were added from standards.

2.6. Statistical analysis

Statistical tests such as for normality (Shapiro-Wilk) were performed in SPSS 8.0 for Windows software. Homoscedasticity, lack of fit (analysis of variance - ANOVA), 3² experimental design calculations and response surface methodology were performed in Microsoft Office® Excel software.

3. Results and discussion

3.1. Electrochemical behaviour of ETB and interferents on a graphite-polyurethane composite electrode

Initially, in order to verify if ETB was electroactive at the electrode surface, voltammetric studies involving the electrochemical behaviour at the phosphate buffer solution (pH = 8.0) using the composite electrode prepared were performed. It was observed that as various concentrations of ETB were added, an increase in current signal values occurred at potentials higher than +0.9 V (Fig. 1).

As the study involved the development of an electroanalytical method for determination of ETB in urine, the next step was to check for possible interferences in the analysis, since the working potential would exceed +0.9 V. For this, compounds occurring physiologically in certain pathological conditions in human urine were evaluated. Urea, glucose, ethanol, lactate and uric acid at concentrations of 0.5, 5 and 50 mmol L⁻¹ (except for uric acid due to the great difficulty in solubilizing uric acid in water) were studied. For this, square wave curves are recorded and the measurements are obtained at 1.2 V for the relation between electrode response for ETB and interferents.

Based on these results (Table 1), it was still possible to measure ETB in the presence of these possible interferents since it was observed that the electrode response for ETB was seven to ten times higher than the signal current, taking into account the interferents evaluated, except for uric acid (C = 5 mmol L⁻¹). With that, the next step was to use the composite electrode as an amperometric detector in a flow injection system.

3.2. Optimisation for FIA determinations

Preliminarily, a 2³ full experimental design [21] involving flow, volume of the sample and potential as factors with triplicates at the central point was performed (not shown). In the present case the current was the response considered, since the objective was to maximize the sensitivity. In this screening experiment, it was verified
that the potential and volume of the sample were significant within the experimental levels considered. Therefore, in a second step, a $3^2$ experimental design with quadruplicates at the central point was performed in order to better study the behaviour of the system. Table 2 shows the experimental design matrix with factors, levels and responses. The solutions were propelled using a peristaltic pump at 2.0 mL min$^{-1}$, since the results obtained from the preliminary study performed using the $2^3$ full experimental design had shown this factor was not significant. Moreover, maintaining the peristaltic pump at 2.0 mL min$^{-1}$ is desirable because it will result in high throughput.

The fit model obtained for the employed experimental design for FIA factors optimization is described in Table 3. With this model it is possible to observe that only the $X_2 X_2$ and $X_1 X_2$ coefficients were significant in the 95% confidence interval, because the p-values calculated were lower than 0.05. Since the results found did not evidence lack of fit at the 95% confidence interval, once p-value calculated was equal to 0.36, i.e., higher than 0.05, the representative surface response for the model can be presented (Fig. 2). For the response surface analysis it was concluded that for any sample volume within the range studied, the response current is high since the potential is maintained at a high level. Thus, in this case, a volume of 150 mL and a potential of 1.2 V were selected as the optimum conditions.

The calibration system was evaluated within a range from 0.50 to 1.10 mmol L$^{-1}$ of ETB dissolved in synthetic urine solution (Fig. 3). The fit model obtained was $I(\mu A) = 0.442 + 26.59[ETB](\text{mmol L}^{-1})$, with $r^2 = 0.9981$. The reproducibility for successive injections of a 0.7 mmol L$^{-1}$ ETB standard solution was 5.1% ($n=30$), demonstrating the good performance of the developed amperometric sensor. The detection and quantification limits were
estimated as 0.0634 and 0.317 mmol L⁻¹, respectively, and the analytical frequency was calculated as approximately 161 determinations h⁻¹.

### 3.3. Ethambutol determinations in synthetic urine samples by FIA and CZE

After optimisation of these experimental parameters, the composite sensor was used to determine ETB in synthetic urine samples. With the purpose of obtaining quantification models by FIA and by CZE, external calibration curves with ETB standard dissolved in synthetic urine solution, through four levels for FIA (0.5, 0.7, 0.9 and 1.1 in authentic replicates, n = 6, see Fig. 3 and Table 4) and five levels for CZE (0.3, 0.5, 0.7, 0.9 and 1.1 in authentic replicates, n = 3, see Table 5) were randomly performed. Statistical assumptions such as homoscedasticity (Cochran’s test), lack of fit through a priori test hypothesis (ANOVA) as shown by Eq. 1 [22], and normality test (Shapiro-Wilk) in residues were carried out for FIA and CZE within 95% confidence interval.

$$F_{calc} = \frac{S_{y,s}^2}{S_y^2} = \frac{\sum_{i=1}^{p} m_i (\bar{y}_i - \hat{y})^2 / (p - 2)}{\sum_{j=1}^{m} \sum_{k=1}^{m_j} (y_{ij} - \hat{y})^2 / (m - p)}$$

The fit model obtained for FIA was $I (\mu A) = 2.10 + 24.66 [ETB] (mmol L⁻¹)$, with $R^2 = 0.987$ and for CZE was $\text{Area} = 13.55[ETB] (mmol L⁻¹) - 0.0019$, with $R^2 = 0.9778$. The calculated values for the Cochran’s test (0.45 for FIA and 0.43 for CZE) are both lower than the tabulated ones (0.59 for FIA and 0.68 for CZE), indicating that the variances are homoscedastic. The p-values obtained for ANOVA were 0.67 and 0.16 for FIA and CZE, respectively. Finally, the p-values for residues normality were 0.86 and 0.59 for FIA and CZE, respectively. As the calculated p-values obtained to ANOVA and residues normality were higher than 0.05, the statistical diagnosis can be considered satisfactory within the considered confidence interval ($\alpha = 0.05$). Once the statistical assumptions were not violated, we could use the fit models obtained, to quantify ETB in samples by both methodologies. Besides, the regression significance values (F significance, $F_{sig}$) were statistically acceptable, once the $F_{sig}$ calculated values were 1642 and 572 for FIA and CZE, respectively.

In order to compare quantitative results from FIA and CZE methods in six synthetic urine samples, in two concentration levels (0.70 and 0.90 mmol L⁻¹), a paired sample t-test was performed (Table 6). The normality assumption for the differences between both methodologies results was not violated. Therefore, the

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**Table 4.** Signal replicate values used for FIA regression model implementation.

| [ETB] (mmol L⁻¹) | 1st   | 2nd   | 3rd   | 4th   | 5th   | 6th   |
|-----------------|-------|-------|-------|-------|-------|-------|
| 0.5             | 14.90 | 15.63 | 15.28 | 13.75 | 13.77 | 13.70 |
| 0.7             | 19.24 | 19.09 | 19.00 | 19.39 | 19.26 | 19.08 |
| 0.9             | 24.61 | 24.49 | 23.52 | 24.95 | 24.78 | 24.32 |
| 1.1             | 29.05 | 28.27 | 27.95 | 30.06 | 30.10 | 29.69 |

**Table 5.** Signal replicate values used for CZE regression model implementation.

| [ETB] (mmol L⁻¹) | 1st   | 2nd   | 3rd   |
|-----------------|-------|-------|-------|
| 0.3             | 4.09  | 3.64  | 4.27  |
| 0.5             | 7.57  | 7.20  | 6.59  |
| 0.7             | 9.93  | 8.82  | 9.41  |
| 0.9             | 12.49 | 10.88 | 11.43 |
| 1.1             | 15.41 | 15.71 | 14.81 |

**Table 6.** ETB content expressed in mmol L⁻¹ for synthetic urine samples obtained from FIA and CE.

| Samples | Added | FIA | CE |
|---------|-------|-----|-----|
| 1       | 0.70  | 0.68| 0.67|
| 2       | 0.70  | 0.67| 0.71|
| 3       | 0.70  | 0.68| 0.64|
| 4       | 0.90  | 0.87| 0.81|
| 5       | 0.90  | 0.87| 0.86|
| 6       | 0.90  | 0.86| 0.88|
t-test results obtained showed no evidence of significant difference between methods within the 95% confidence interval, since the p-value obtained, i.e., 0.637, is higher than α = 0.05. Thus, it is possible to conclude that the amperometric methodology presented adequate accuracy.

Fig. 4 shows electropherograms obtained for urine samples before (a) and after (b) addition of ETB. It is important to stress that the negative peak from a matrix effect did not interfere with the analyte peak.

4. Conclusions

In this report the analytical performance of the new amperometric detector described has been demonstrated by measuring ethambutol in a flow assembly system, with close agreement between the results from the sensor and those found by using a CZE comparative method. The sensitivity, stability and detection limit of the electrode are sufficient for monitoring ethambutol in synthetic urine samples, confirming the suitability of this analytical method for investigations in clinical analysis.

Acknowledgements

This work was supported by FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and Propesq-UFJF (Pró-reitoria de Pesquisa da Universidade Federal de Juiz de Fora).

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