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Sensitive Fluorescence Detection of Etimicin Based on Derivatives of Formaldehyde and Acetylacetone

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Abstract A novel fluorescence method for the determination of etimicin is described. Etimicin reacts with acetylacetone and formaldehyde in pH 4.0 Britton-Robinson (B.R.) buffer solution to from a fluorescent substance [I]. Emission spectra of [I] and the reagent blank were overlapped, so the arithmetic emission spectra of the fluorescent substance were obtained by subtracted form the spectra of [I] to the spectra of the reagent blank using the Fluorescence Data Software. There is a linear relationship between the intensity of the arithmetic emission spectra and the concentration of etimicin. Effects of pH, amount of acetylacetone-formaldehyde, and heating time on the determination of etimicin have been examined. Etimicin can be determined over the concentration range of 1.0 to 10.0 $\mu g mL^{-1}$ with a correlation coefficient of 0.9991. The relative standard deviation (RSD) for 11 repetitive determinations of 5.0 $\mu g mL^{-1}$ etimicin is 0.22 %. The utility of this method was demonstrated by determining etimicin in commercial samples.

Keywords Etimicin · Fluorescence · Detection · Acetylacetone formaldehyde

Introduction

Etimicin (ETM), which is mainly used as the sulfate, a new semi-synthetic, the 1-N-ethyl derivative of gentamycin C_{1a}, is active against both Gram-positive and Gramnegative bacteria [1, 2]. The oto- and nephro-toxicity of etimicin are substantially lower than those of other aminoglycisides antibiotics and even lower than netilmicin. Nevertheless, etimicin still has a narrow therapeutic range and it must be careful to monitor the levels in the blood [3].

Several methods have been reported for the quantitative determination of etimicin, including evaporative light-scattering detection (ELSD) [4], microbiological assay [5], UV [6], electrochemiluminescence (ECL) [7], Liquid Chromatography with pulsed amperometric detection [3, 8], high-performance liquid chromatography with pre-column derivatization with detection of UV [9, 10] and reversed-phase liquid chromatography with 1-fluoro-2,4-dinitrobenzene[11] in which derivatived etimicin was used as internal standard. A volatile mobile phase is required for ELSD detection and it is low sensitive in detection [3]. The linear relationship of UV detection was good in range of 0.05–0.25 $mg mL^{-1}$ (r=0.999) [6], 0.1–1.0 $mg mL^{-1}$ (r= 0.9999) [9] and 0.04–0.20 $mg mL^{-1}$ (r=0.999) [10] respectively. Zhu and coworker developed an analysis method of etimicin sulfate by liquid chromatography with pulsed amperometric detection. Linear calibration curves were obtained in the rage of 0.005 and 0.125 $mg mL^{-1}$ [3]. Wang et al. have conducted a similar study in that they report determination etimicin sulfate by using liquid chromatography with pulsed amperometric detection. The detection limits was 1.0 $\mu g mL^{-1}$. Analytical signal of the etimicin was linear in the concentration range of 0.019–0.15 $mg mL^{-1}$ [8]. The determination method using liquid chromatography with pulsed amperometric detection allows for sensitive, rapid detection and determines etimicin without derivatization. However, pulsed amperometric detection suffers from some stability problems and some experience is required to obtain a good reproducibility [3]. We have developed a sensitive ECL method to detection etimicin [7]. This method showed a linear response to etimicin in the range of 8.0 to 160.0 $ng mL^{-1}$ with a detection limit of 6.7 $ng mL^{-1}$. However, the reproducibility with a coefficient of variation of 5.1 % (n=7) should be improved. It is necessary to develop

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a rapid and inexpensive method for sensitive and selective determination of etimicin.

This paper reports a fluorimetic method for estimating the concentration of etimicin, which is based on the Hantzsch reaction [12], i.e. acetylacetone and formaldehyde react with the amino group of etimicin molecule to form a fluorescent substance [I]. λ_{ex}max of the fluorescent is at 413 nm; the λ_{em} max value in a pH 4.0 Britton-Robinson (B.R.) buffer solution is at 480 nm. In the same wavelength, the emission spectra of the fluorescent substance and the reagent blank are overlapped, so the arithmetic emission spectra of the fluorescent substance can be obtained by subtracted form the emission spectra the fluorescent substance to the emission spectra of the reagent blank. There is a linear relationship between the intensity of the arithmetic fluorescence emission spectrum and the concentration of etimicin. This method is able to detect the quantitative of etimicin. The principal advantages of the method are that it is rapid, sensitive, and possesses good reproducibility. To our best knowledge, this paper, it describes the first application of fluorimetic method to the determination of etimicin.

Experimental

Reagents

Etimicin was obtained from Jiangsu Institute For Drug Control, Nanjing (China). Acetylacetone was obtained from Kelong Huaxue Shiji (Chendu, China) and formaldehyde (37 %) (Sinopharm Chemical Reagent Co,. Ltd, China) was used as received. All reagents were of analytical-reagent grade, unless stated otherwise. Double distilled water was used in all experiments.

Buffer Solution

The Britton-Robinson (B.R.) buffer was prepared by titrating a stock solution containing 0.02 mol/l acetic acid, 0.02 mol/l phosphoric acid, 0.02 mol/l boric acid with 0.02 mol/l sodium hydroxide to the desired pH value.

Acetylacetone - formaldehyde Solution

A total of 80 μl of acetylacetone and 170 μl formaldehyde (37 %) solution were added to 1.0 ml B.R. buffer, diluted to 3.0 ml with B.R. buffer and mixed well, the solution was prepared fresh daily.

Apparatus

Spectrofluorimetric measurements were made on Hitachi F-4600 luminescence spectrometer equipped with a xenon discharge lamp and 1.0 cm quartz cuvettes (Hitachi, Japan). Both the operation and data processing were controlled by the Fluorescence Data Software. The excitation and emission wavelengths were set at 413 nm and 480 nm respectively. A pH meter (Model pHS-3C, Shanghai Leici instruments Factory, China) was used for pH adjustment.

Procedures

A certain portion of 1.00 mgmL^{-1} of etimicin solution was transferred into a 10.0 ml standard flask, 3.0 ml of acetylacetone-formaldehyde solution were added, and diluted to approximately 6.0 ml with water. The mixture was shaken and heated for 30 min in a boiling water bath and then cooled in ice water at once, diluted to the mark with water and mixed well. All fluorescence measurements were made at scan rate of 1200 nmmin^{-1} using 5 nm excitation and emission windows. The excitation and emission spectra of [I] and the blank reagent, were recorded on the luminescence spectrometer, and the arithmetic fluorescence emission spectrum was obtained using the Fluorescence Data Software. The peak height of arithmetic emission spectrum was estimated at 480 nm on the longitudinal coordinate axis (absolute value).

Results and Discussion

Excitation, Emission and Arithmetic Emission Spectra

As can be seen (Fig. 1), the λ_{ex}max and λ_{em}max of [I] are 413 (Fig. 1a) and 480 nm (Fig. 1b), respectively, and the λ_{em}max of reagent blank is at 475 nm (λ_{ex}=413) (Fig. 1c).

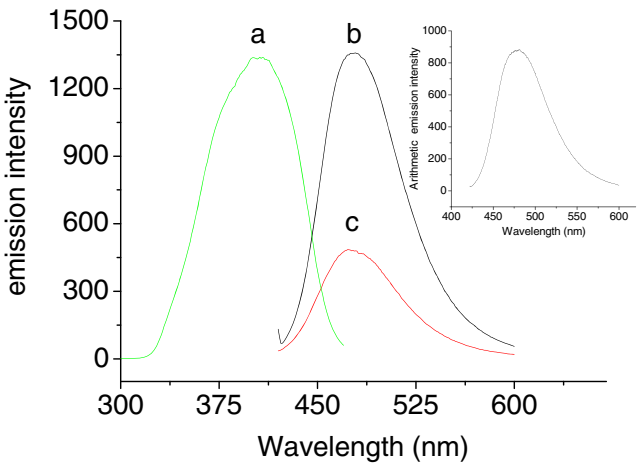

Fig. 1 Excitation and emission of [I] and reagent blank. Excitation spectrum (**a**) (λ_{em}=480 nm) and emission spectrum (**b**) (λ_{ex}=413 nm) of [I]. Emission spectrum (**c**) (λ_{ex}=413 nm) of reagent blank. *Inset*: arithmetic emission fluorescence spectrum of [I]. Concentration of etimicin, 5.0 μgmL^{-1}; pH 4.0 B.R. buffer solution

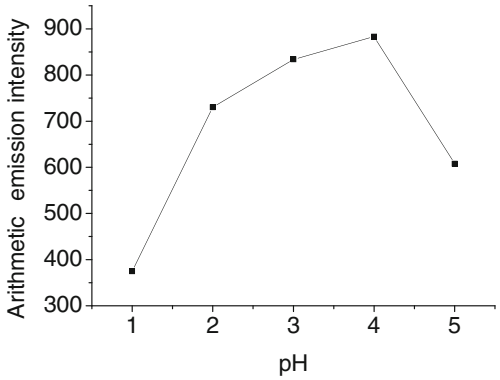

Fig. 2 Effect of pH of the buffer solution on arithmetic emission intensity. Etimicin, 5.0 $\mu g mL^{-1}$; B.R. buffer solution

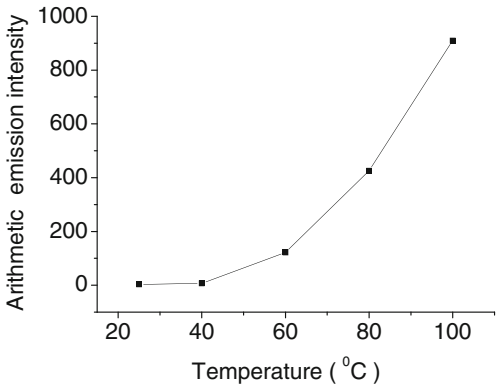

Fig. 4 Effect of the heating temperature on arithmetic emission intensity. Etimicin, 5.0 $\mu g mL^{-1}$; pH 4.0 B.R. buffer solution

Obviously, the spectra of [I] and the reagent blank are overlapped. Therefore, the arithmetic modified spectra (Fig. 1 insert) was obtained by subtracted form the fluorescent substance to the reagent blank (b-c) using the Fluorescence Data Software in order to remove the interference of reagent blank.

Optimization of Arithmetic Emission Intensity

Effect of pH

According to the procedure, the mixture solution of 5.0 $\mu g mL^{-1}$of etimicin was prepared in different pH B.R. buffer solution and the arithmetic fluorescence emission intensity was measured at λ_{em}max 480 nm with excitation at 413 nm against a reagent blank prepared with the same reagent concentration but no etimicin. The arithmetic emission intensity of [I] was effect by pH of the solution and the result is presented in Fig. 2. It was found that the values of arithmetic emission intensity increased with increase of the pH in the range of 1.0–4.0, however, decreased with its further increase. Therefore, pH of the solution was justed to 4.0 by B.R. solution for further study.

Effect of Amount of Acetylacetone-formaldehyde

It was found that addition of 3.0 mL of acetylacetone-formaldehyde solution was sufficient for determining etimicin (Fig. 3). It was possibly related to the background emission intensity increased with increase of acetylacetone-formaldehyde amount. Therefore, 3.0 mL of acetylacetone-formaldehyde solution is recommended.

Effect of the Heating Temperature

Figure 4 shows the relation between the arithmetic emission intensity of [I] and the heating temperature of the reaction solution. According to the procedure, the mixture solution of 5.0 $\mu g mL^{-1}$of etimicin and reagent blank were heated in water bath of different temperature. Obviously, etimicin does not react with acetylacetone and formaldehyde at room temperature. The arithmetic emission intensity increased with the increasing heating temperature. Thus, it was decided to supply the boiling water bath.

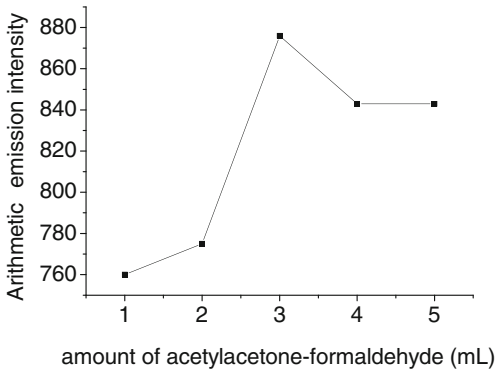

Fig. 3 Effect of the amount of acetylacetone-formaldehyde on arithmetic emission intensity. Etimicin, 5.0 $\mu g mL^{-1}$; pH 4.0 B.R. buffer solution

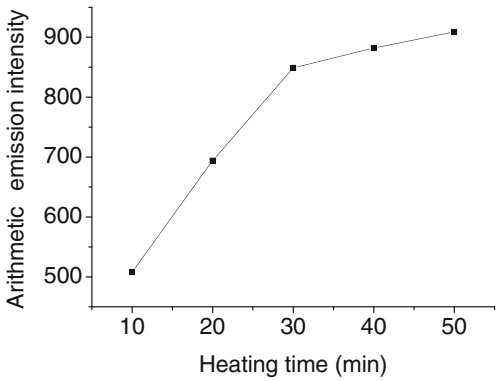

Fig. 5 Effect of the heating time on arithmetic emission intensity. Etimicin, 5.0 $\mu g mL^{-1}$; pH 4.0 B.R. buffer solution

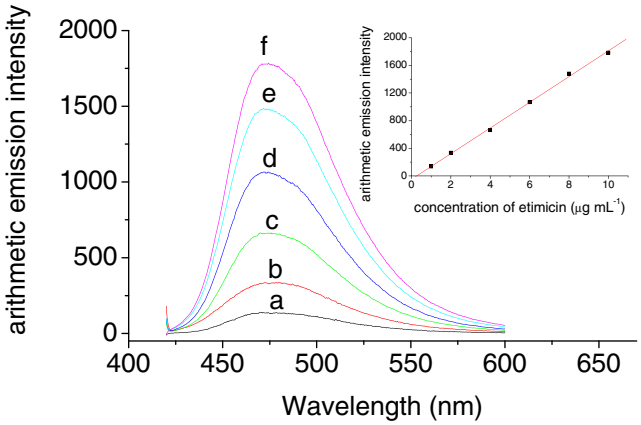

Fig. 6 Typical arithmetic emission signals of [I]. Concentrations of etimicin were 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 $\mu g mL^{-1}$(**a**–**f**, respectively). pH 4.0 B.R. buffer solution. *Insert*: plots of arithmetic emission intensity vs etimicin comcentration

Formation and Stability of [I]

The result of the mixture solution of 5.0 $\mu g mL^{-1}$of etimicin was heated for different times in a boiling water bath is shown in Fig. 5. When heated for above 30 min, the arithmetic emission intensity increased gently. To insure accuracy of the result, heating time (30 min) should be controlled strictly. The sample solution has to be put in ice water at once to stop reaction when the heating time reached at 30 min. Therefore, heating 30 min was selected as optimum. The relative fluorescence intensity of [I] remained stable for at least 12 h at room temperature.

Analytical Characteristics of Etimicin

Under the optimum conditions mentioned above, the calibration curve was obtained for etimicin determination by plotting the arithmetic emission signal versus etimicin concentration, which gave a linear range from 1.0 to 10.0 $\mu g mL^{-1}$ with a correlation coefficient of 0.9991(shown in Fig. 6). Linear regression equation of calibration graph is $Y = 185.86x - 49.13$. x means concentration of etimicin: $\mu g mL^{-1}$, Y means arithmetic peak height in the linear regression equation calibration graph. The relative standard deviation for 11 repetitive determinations of 5.0 $\mu g mL^{-1}$ etimicin was 0.22 %, showing a good reproducibility.

Table 1 Tolerance to different substances in the determination of 5.0 $\mu g mL^{-1}$ etimicin

Species added	Maximum tolerable mole ratio[a]
Na^+, K^+, Cl^-	1000
urea, glucose, dextrin, starch, fructose	500
SO_4^{2-},NO_3^-, EDTA,	100
Ba^{2+}, Zn^{2+}, Mg^{2+},	10
Al^{3+}, Cu^{2+}	1

[a] 1000 is the greatest ratio tested

Table 2 Determination of etimicin in injections

Sample	Labeled value	Proposed method[a]
Injection 1	50 $mg ml^{-1}$	49.6±0.18 $mg ml^{-1}$
Injection 2	50 $mg ml^{-1}$	51.0±0.22 $mg ml^{-1}$

[a] Average of three determinations

Interferences

In order to assess the possible analytical applications of the fluorescence method described, the influences of different metal ions and some excipients used in pharmaceutical preparations on the arithmetic emission intensity were investigated by determining the solutions containing 5.0 $\mu g mL^{-1}$ etimicin and foreign species with continuously increasing concentration up to 1000 ratio. When the effect of each foreign species on the peak height was less than 5.0 %, it was thought not to interfere with the determination of etimicin. The obtained results in Table 1 showed that under the optimized conditions, some ions and the studied excipients in the tablets did not interfere with the determination of etimicin. Therefore, this method can be suggested for the determination of etimicin in pharmaceutical preparations.

Determination of Etimicin in Injection Samples

Injection solutions of etimicin were diluted appropriately with water prior to measurement so that the concentrations of etimicin were in its linear response range. These solutions were used as sample solutions. Following the procedure, the sample solutions were measured. The results are shown in Table 2. The *t*-test assumes that there was no significant difference between the labeled value and the measurement results at confidence level of 95 %. The results suggest that the proposed method can be satisfactorily used for the determination of etimicin in real samples.

Table 3 Results of etimicin determinations in urine

Human urinee sample	Added ($\mu g mL^{-1}$)	Found[a] ($\mu g mL^{-1}$)	RSD (n=3) (%)	Recovery (%)
No. 1	4.00	4.09	1.1	102.2
	8.00	8.13	0.8	101.6
No. 2	5.00	4.98	1.2	99.6
	8.00	8. 18	0.5	102.3

[a] Average of three determinations

Determination of Etimicin in Human Urine

When the suggested method was used for the determination of etimicin in urine, a 1.0 mL of sample was mixed with 0.5 mL of acetonitrile and centrifuged for 5 min at 3000 r min^{-1}. Then the supernatant was fetched and the rest acetonitrile was blow-dried under a gentle stream of nitrogen gas. Finally, the prepared sample was diluted with distilled water to 100.0 mL and used for the fluorescence determination of etimicin. The recovery for etimicin added to the different concentration of sample solutions is shown in Table 3, the recovery is from 99.6 to 102.3 %.

Conclusions

The results presented in this paper clearly demonstrate that etimicin can be determined by fluorimetric method. Etimicin can be determined at $\mu g mL^{-1}$ level. In all the cases, recoveries of 99.6 to 102.3 % were obtained. This method is simple, highly sensitive and possesses good reproducibility, and can be satisfactorily used in the determination of etimicin in practical sample.

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