CHAPTER III

EXPERIMENTAL

Chemicals and reagents

3.1.1 Tetracycline hydrochloride (Sigma)

3.1.2 Chlortetracycline hydrochloride (Sigma)

3.1.3 Doxycycline hydrochloride (Sigma)

3.1.4 Potassium dihydrogen orthophosphate (Merck)

3.1.5 Sodium hydroxide (Merck)

3.1.6 Phosphoric acid (85% J.T. Baker)

3.1.7 Standard buffer solutions of pH 4 and 7 (Metrohm)

3.1.8 Tetracycline hydrochloride capsule (TC Mycin 250 mg)

3.1.9 Chlortetracycline hydrochloride capsule (Aureomycin 250 mg)

3.1.10 Doxycycline hydrochloride capsule (Medomycin 100 mg)

Apparatus

3.2.1 Gold rotating disk electrode (Au RDE 0.07 cm^2, Metrohm) and gold disk electrode (0.07 cm^2, Bioanalytical System Inc.) pretreated by polishing with 0.05 micron of aluminum/water slurry

3.2.2 Ag/AgCl electrode

3.2.3 Platinum rod electrode (Metrohm, 6.1204.010)

3.2.4 Electrolyte vessel (Metrohm, 6.1415.150)

3.2.5 Control/Power unit for rotating disk electrode (Metrohm, 628-10)

3.2.6 Drive unit for rotating disk electrode (Metrohm, 1.628.0020)

3.2.7 Polishing set of 0.05 micron and alumina powder slurry (Metrohm)

3.2.8 Autolab Potentiostat (PGSTAT 30, Metrohm)

3.2.9 Peristaltic pump (Eyela, SMP-23)

3.2.10 Rheodyne injection valve, Model 7225 (Altech), with a 20 µl stainless steel injection loop (0.5 mm. i.d.)

3.2.11 Thin layer flow cell (Bioanalytical System Inc.)

3.2.12 Teflon cell gasket (Bioanalytical System Inc.)

3.2.13 PEEK tubing (0.25 mm. i.d.) and connecting (Upchurch)

3.2.14 Teflon tubing (1/16 inch o.d., Upchurch)

3.2.15 Cutting set (Altech)

3.2.16 0.2 µM Nylon membrane filter (Altech)

3.2.17 0.45 µM Nylon membrane syringe filter with polypropylene (PP) housing (Orange Scientific filter)

3.2.18 pH meter (Metrohm)

3.2.19 Sonicator (USA, A006651)

3.2.20 Analytical balance (Metler, AT 200)

The preparation of supporting electrolyte solution and standard solution

All solutions were prepared using deionized water obtained from a Milli-Q system (Milford, MA, USA). The preparation of buffer solutions are described below:

3.3.1 0.1 M potassium dihydrogen orthophosphate (KH_2PO_4) solution

Potassium dihydrogen phosphate 13.60 g was dissolved in 1.0 L of deionized water and then adjusted with 0.1 M sodium hydroxide or 85 % phosphoric acid to the required pH.

3.3.2 Tetracycline hydrochloride solutions

The 1 mM tetracycline hydrochloride solution was prepared by weighing 0.0481 g tetracycline hydrochloride powder and transferring into 100 ml volumetric flask. The 0.1 M KH_2PO_4 solution (pH 2) was used for diluting this aliquot to the mark. This solution was used for studying of pH dependence and investigating of the oxidation of tetracycline by rotating disk cyclic voltammetry. The 1 mM tetracycline hydrochloride solution for FIA study was also prepared using the same procedure as mentioned above. This solution was used for optimization of PAD waveform parameters for tetracycline.

3.3.3 Chlortetracycline hydrochloride solutions

The 1 mM chlortetracycline hydrochloride solution was prepared by weighing 0.0515 g chlortetracycline hydrochloride powder and transferring into 100 ml volumetric flask. The 0.1 M KH_2PO_4 solution (pH 2.5) was used for diluting this aliquot to the mark. This solution was used for studying of pH dependence and

investigating of the oxidation of chlortetracycline by rotating disk cyclic voltammetry. The 0.5 mM chlortetracycline hydrochloride solution for FIA study was also prepared by weighing 0.0258 g chlortetracycline hydrochloride powder and transferring into 100 ml volumetric flask. The 0.1 M KH_2PO_4 (pH 2.5) was used for diluting this aliquot to the mark. This solution was used for optimization of PAD waveform parameters for chlortetracycline.

3.3.4 Doxycycline hydrochloride solutions

The 1 mM doxycycline hydrochloride solution was prepared by weighing 0.0481 g doxycycline hydrochloride powder and transferring into 100 ml volumetric flask. The 0.1 M KH_2PO_4 solution (pH 2) was used for diluting this aliquot to the mark. This solution was used for studying of pH dependence and investigating the oxidation of doxycycline hydrochloride by rotating disk cyclic voltammetry. The 0.5 mM doxycycline hydrochloride solutions for FIA study were also prepared by weighing 0.0240 g doxycycline hydrochloride powder and transferring into 100 ml volumetric flask. The 0.1 M KH_2PO_4 solution (pH 2) was used for diluting this aliquot to the mark. This solution was used for optimization of PAD waveform parameters for doxycycline hydrochloride.

Procedures

3.4.1 Rotating disk voltammetry

The voltammetric measurements were performed in a glass vessel using a potentiostat. Figure 3.1 shows an electrochemical cell for cyclic votammetric experiment. A commercial rotating disk apparatus was used to rotate the electrode at the rotation speed of 250 rpm. A Ag/AgCl and platinum rod electrodes were used as reference and auxiliary electrodes, respectively. The gold rotating disk electrode (0.07 cm^2) was used as the working electrode. The drive unit was used for rotating disk voltammetric experiments.

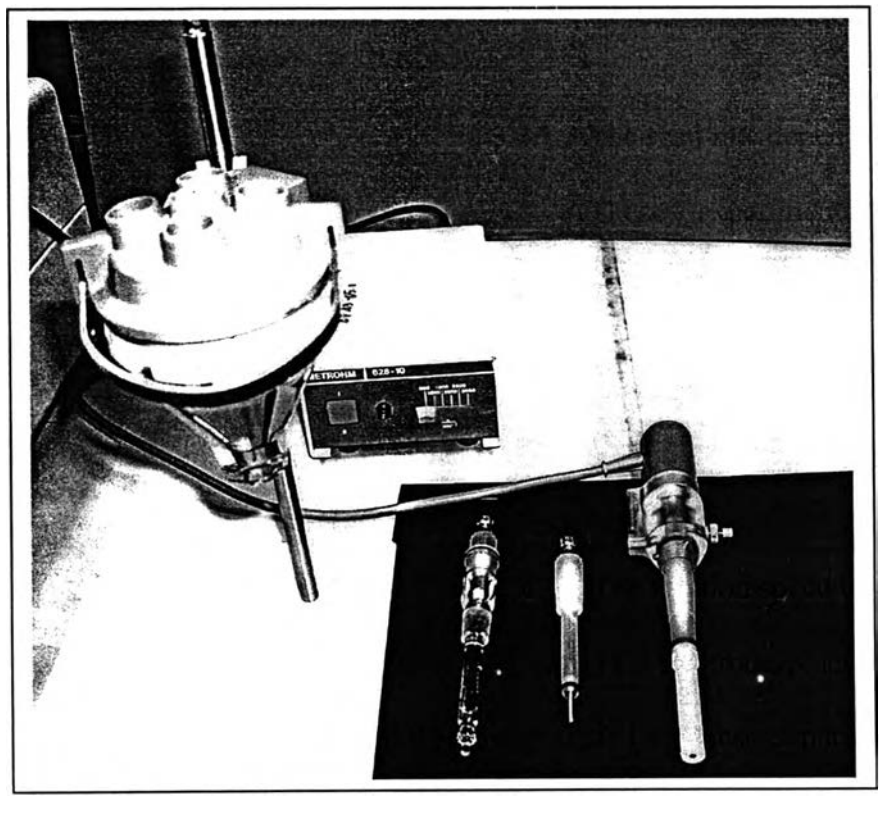

Figure 3.1 The electrochemical cell for rotating disk voltammetric study

3.4.1.1 pH dependence study

These experiments were carried out in 0.1 M potassium dihydrogen orthophosphate solution at the rotation speed of 250 r.p.m. The pH and the concentration of each analyte are shown in Table 3.1.

3.4.1.2 The electrochemical oxidation of analyte

The solutions of 0.1 M potassium dihydrogen orthophosphate at chosen pH from the previous experiment (Section 3.4.1.1) were used as the supporting electrolyte of these experiments. The 1 mM analyte solutions in that electrolyte solution were studied using the gold rotating disk electrode by the cyclic voltammetry. A scan rate of 50 mV s^{-1} and a rotation speed of 250 r.p.m. were used.

3.4.1.3 The scan rate dependence study

These experiments were performed to investigate the adsorption of the analytes at the electrode surface at various scan rates. The analyte concentration of 1 mM was used. The varied scan rates were 10, 20, 50, 100, 200, and 300 mV s^{-1}, respectively.

3.4.1.4 The rotation speed dependence study

The electrode reaction processes of each analyte at electrode surface were investigated by these experiments. These experiments were carried out in 1 mM analyte solutions. The rotation speeds of 200, 250, 300, 350, 400, 450, 500, 550 and 600 r.p.m. were used.

Table 3.1 pH and concentration of each analyte for the pH dependence studies

Analyte	Concentration	pH
1. Tetracycline hydrochloride	1 mM	2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 10
2.Chlortetracycline hydrochloride	1 mM	2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 10
3. Doxycycline hydrochloride	1 mM	2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 10

3.4.2 Flow injection with pulsed amperometric detection

The flow injection apparatus consisted of a thin-layer flow-through cell, a 20 μl sample injection loop, a peristaltic pump and an electrochemical detector. Figure 3.2 shows a thin-layer flow-through cell for the flow injection system. A thin-layer flow-through cell was utilized with a gold disk working electrode, having a diameter of 0.3 cm. An auxiliary electrode was a stainless steel tube. The flow channel was created by a teflon gasket. The reference electrode was a Ag/AgCl electrode. The flow rate was calculated by measurement of the volume delivered to a 10 ml cylinder during a known period of time and a flow rate of 1 ml min^{-1} was used.

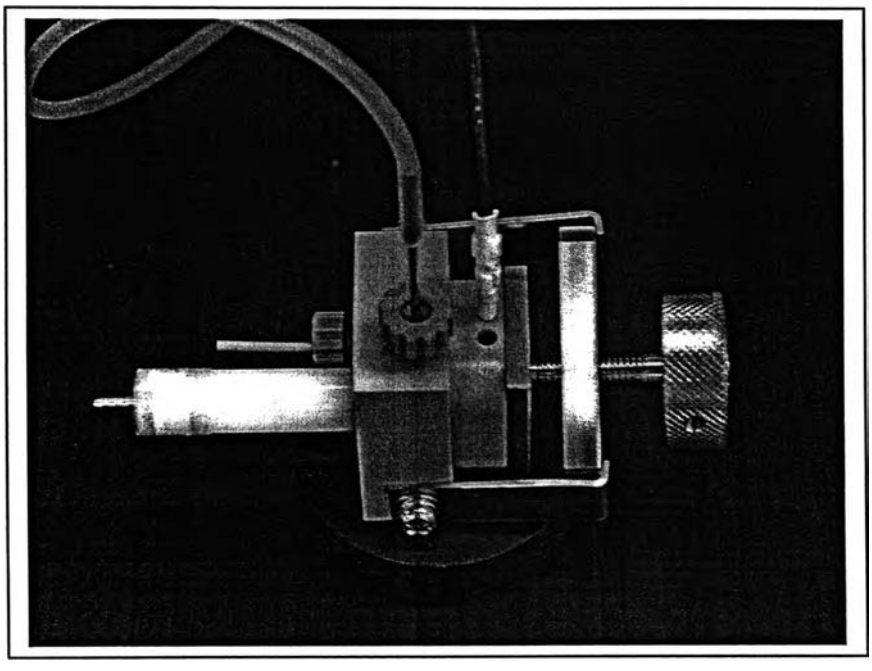

Figure 3.2 The thin-layer flow-through cell

3.4.2.1 PAD waveform optimization

The PAD waveforms were optimized for each analyte to find the optimal potential and time parameters for the waveform. Each optimal waveform parameter was obtained by injection a series of three replicates of 20 μl of 0.5 mM analyte solution. The optimization of each waveform parameter was initiated while the other parameters were held constant. The average peak currents for each parameter were plotted versus the varied parameter to obtain the optimal value.

3.4.2.2 Calibration and linear range

5 mM stock solutions of each analyte were freshly prepared and then diluted to a concentration range from 1 μM to 2 mM. The experiments were carried out by injection of three replicates of each concentration. Results were used to plot the calibration curve and find the linear range.

3.4.2.3 Detection limit

The detection limit was carried out by injection of low concentrations of analyte solutions for three replicates under the optimal PAD waveform parameters. The detection limit is defined as the concentration that provided a current response higher three times than the noise ($S/N \geq 3$).

3.4.2.4 Repeatability

The repeatability was studied by injecting of analyte solutions for five replicates. The repeatability is expressed in terms of the relative standard deviation (%RSD), using the following formula:

$$\%RSD = \frac{\text{standard deviation}}{\text{Mean}} \times 100$$

3.4.2.5 Applications

The proposed method was applied to the real samples. The real samples were drug capsules. The standard addition method and calibration curve method for finding the amount of drug in the real sample was used.

3.4.2.5.1 Tetracycline hydrochloride capsule

Tetracycline hydrochloride capsule 250 mg (Vesgo pharmaceutical, Thailand) was used in this study. The sample preparation is described below.

A mass of powder of ten capsules of tetracycline hydrochloride (TC-Mycin, 250 mg) was transferred to a 1000 ml volumetric flask and dissolved in 0.1 M KH_2PO_4 solution (pH 2), filtrated solution through a 0.45 μM Nylon membrane

syringe filter. Then, the filtrated was diluted with 0.1 M KH_2PO_4 solution (pH 2) to obtain a final concentration of 50.01 and 100.03 μg ml^{-1} (0.104 and 0.208 mM).

A stock solution of 480.9 μg ml^{-1} (1 mM) of tetracycline hydrochloride in 0.1 M KH_2PO_4 solution (pH 2) and a set of four 10 ml volumetric flasks was prepared. The final concentration of the standard solutions was 24.05, 48.10, 96.12, and 144.27 μg ml^{-1}, respectively.

3.4.2.5.2 Chlortetracycline hydrochloride capsule

Chlortetracycline hydrochloride capsule 250 mg (F. E. Sillic, Thailand) was used in this study. The sample preparation is described below.

A mass of powder of ten capsules of chlortetracycline hydrochloride (Aureomycin, 250 mg) was transferred to a 1000 ml volumetric flask and dissolved in 0.1 M KH_2PO_4 solution (pH 2.5), filtrated through a 0.45 μM Nylon membrane syringe filter. Then, the filtrated solution was further diluted with 0.1 M KH_2PO_4 solution (pH 2.5) to obtain a final concentration of 257.65 μg ml^{-1} (0.5 mM).

A stock solution of 257.65 μg ml^{-1} (0.5 mM) of chlortetracycline hydrochloride in 0.1 M KH_2PO_4 solution (pH 2.5) and a set of five 10 ml volumetric flasks were prepared. 2.5 ml of sample solution was pipetted in each flask and then 0, 1.0, 2.0, 3.0 and 4.0 ml of a stock solution of tetracycline hydrochloride was added to give final concentration of the standard solutions was 0, 25.77, 51.53, 103.06, and 206.12 μg ml^{-1}, respectively.

3.4.2.5.3 Doxycycline hydrochloride capsule

Doxycycline hydrochloride capsule 100 mg (Medline, Thailand) was used in this study. The sample preparation is described below.

A mass of powder of ten capsules of doxycycline hydrochloride (Medomycin, 100 mg) was transferred to a 1000 ml volumetric flask and dissolved in 0.1 M KH_2PO_4 solution (pH 2), filtrated through a 0.45 μM Nylon membrane syringe filter. The filtrated was then further diluted with 0.1 M KH_2PO_4 solution (pH 2) to obtain a final concentration of 240.45 μg ml^{-1} (0.5 mM).

A stock solution of 240.45 μg ml^{-1} (0.5 mM) of doxycycline hydrochloride in 0.1 M KH_2PO_4 solution (pH 2) and a set of five 10 ml volumetric flasks were prepared. 2.5 ml of sample solution was pipetted in each 10 ml flask and then 0, 1.0, 2.0, 3.0 and 4.0 ml of a stock solution of tetracycline hydrochloride was added to give final concentration of the standard solutions was 0, 24.05, 48.09, 144.27, and 192.36 μg ml^{-1}, respectively.