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Flow injection analysis of doxycycline or chlortetracycline in pharmaceutical formulations with pulsed amperometric detection

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Abstract

A flow injection with pulsed amperometric detection for determination of doxycycline or chlortetracycline in pharmaceutical formulations is described. Doxycycline or chlortetracycline were studied at a gold rotating disk electrode with cyclic voltammetry as a function of pH of supporting electrolyte solution. The optimized PAD waveform parameters were obtained with a flow injection system. The optimized pulsed conditions of doxycycline were 1150 mV (versus Ag/AgCl reference electrode) detection potential (E_{det}) for 220 ms (150 ms delay time and 70 ms integration time), 1500 mV (versus Ag/AgCl reference electrode) oxidation potential (E_{oxd}) for 70 ms oxidation time (t_{oxd}) and 250 mV (versus Ag/AgCl reference electrode) reduction potentail (E_{red}) for 400 ms reactivation time (t_{red}). The optimized pulsed conditions of chlortetracycline were 1050 mV (versus Ag/AgCl reference electrode) detection potential (E_{det}) for 300 ms (200 ms delay time and 100 ms integration time), 1300 mV (versus Ag/AgCl reference electrode) oxidation potential (E_{oxd}) for 70 ms oxidation time (t_{oxd}) and 250 mV (versus Ag/AgCl reference electrode) reduction potentail (E_{red}) for 400 ms reactivation time (t_{red}). The optimized PAD waveform was applied to the determination of doxycycline hydrochloride and chlortetracycline hydrochloride standard solution and in pharmaceutical formulations. The linear dynamic ranges of doxycycline hydrochloride and chlortetracycline hydrochloride were 1 μM–0.1 mM. The sensitivity of this method was found to be 23 μA/mM for doxycycline hydrochloride and 33.76 μA/mM for chlortetracycline hydrochloride. The detection limit for both compounds is 1 μM. The doxycycline hydrochloride and chlortetracycline hydrochloride content in commercially available tablet dosage forms by the proposed method was comparable to those specified by the manufacturer.

Keywords: Doxycycline; Chlortetracycline; Pulsed amperometric detection; Flow injection analysis

1. Introduction

Tetracyclines are well-known antibiotics used routinely in human and veterinary medicine for prevention and control of disease [1,2]. Chlortetracycline and doxycycline are antibiotics commonly used in food-producing animals because of their wide antibacterial spectrum, high potency and low cost. These raise the possibility that a residue of tetracyclines may remain in animal tissues intended for human consumption [3]. Moreover, they are important antibiotics widely used to control bacterial infections in human. Therefore, it is necessary to develop an assay method with high sensitivity and accuracy for monitoring the chlortetracycline and doxycycline. Many methods for determination of these compounds have been reported. These include microbiological assay [4,5]. These procedures are subject to problems such as high pH dependence, low sensitivity, low stability, as well as being time consuming. Recently, these antibiotics have been analyzed by HPLC with UV [6,7], fluorescence [8], MS [9,10] or electrochemical detection [11]. The peaks of these compounds tend to tail and exhibit low efficiency due to interaction with the residual silanol groups on silica-based packing materials. Also techniques such as spectrophotomety [12,13], chemiluminescence [14–18], spectrofluorimetry [19,20] or electrochemical methods [21,22] have been employed. However, one of the important limitations of

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method based on spectrophotometer, chemiluminescence or spectrofluorimetry is the fact that these compounds are inactive species for direct detection. Therefore, the derivatization procedure is normally required that make the methods are tedious, expensive, and long time analysis. The high sensitivity and selectivity of electrochemical detection are desired for antibiotics determination. Electrochemical techniques are alternatives, which can be cheap, fast and simple. The working electrode, mercury, is extensively used for determination tetracyclines [23,24]. This electrode has some problems such as the toxicity and limited stability of responses. In recent years, the flow injection system has received much attention and some analytical applications have been reported. A flow injection system was introduced to conventional analytical instrument to improve sample throughput and sensitivity that are the requirement to develop the assay method in pharmaceutical industry. Thus, use of the flow injection system coupled to the mercury electrode is complicated. Moreover, problems associated with easily oxidized mercury electrode have to be considered. Voltammetry and amperometry are the techniques that offer the high sensitivity. Their disadvantage is deposition of the detection product or solution impurities on the electrode surface. Therefore, pulsed amperometric detection with alternated anodic and cathodic polarization to clean and reactivate the electrode surface, has been introduced to overcome this problem. PAD offers the possibility to clean and reactivate the electrode surface effectively after measuring cycle without mechanical polishing [25–28]. In the simplest implementation of PAD, the potential of the working electrode is stepped between the potentials for detection, E_{det}, cleaning, E_{oxd} and reactivation, E_{red}. All three steps of PAD require the following: (a) the oxidation of analyte during the detection step; (b) the oxidative desorption of adsorbed detection products or solution impurities at the cleaning step; (c) the cathodically dissolved of inert oxide product during reactivation step [29]. Pulsed amperometric detection has been used for the sensitive detection of numerous compounds [30–34]. It also has been successful for the determination of tetracycline in pharmaceutical formulation [35]. The goal of this work is extended the use of the PAD waveform for the determination of doxycycline or chlortetracycline in pharmaceutical formulations. In this research also employed the flow injection system with PAD to reduce time analysis and to obtain low detection limit. The present method has been proved to be simple, rapid, sensitive and suitable for automatic analysis.

2. Experimental

2.1. Chemicals

All the chemicals were analytical grade, and the water used was deionized water. Phosphate solutions (for pH 2–4.5) were prepared from 0.1 M potassium dihydrogen phosphate (Merck) and adjusted to the desired pH using 85% phosphoric acid (J.T. Baker). For the phosphate solutions (pH 5–10) were adjusted by 0.1 M sodium hydroxide. Standard doxycycline hydrochloride and chlortetracycline hydrochloride (Sigma-Aldrich) solutions were freshly prepared in 0.1 M potassium dihydrogen phosphate solution prior to use.

Stock standards of doxycycline (0.5 mM) and chlortetracycline (0.5 mM) were prepared by accurately weighing the hydrochloride of doxycycline or chlortetracycline into volumetric flasks and dissolved with 0.1 M potassium dihydrogen phosphate solution. To prepare solutions for the standard addition method, 2.5 ml of sample (prepared as described in Section 2.2) solution was pipetted in a set of five 10 ml volumetric flasks. Then, the 0, 1.0, 2.0, 3.0 and 4.0 ml of a stock solution of standards were added in sample solutions and the volume was adjusted by 0.1 M potassium dihydrogen phosphate solution.

2.2. Sample preparation

Doxycycline hydrochloride capsules (100 mg Medochemie, USA) and chlortetracycline hydrochloride capsules (250 mg, F. E. Sillic, Thailand) were used in this study.

A mass powder of ten capsules of doxycycline hydrochloride (Medomycin, 100 mg) or chlortetracycline hydrochloride (Aureomycin, 250 mg) were transferred to each 1000 ml volumetric flask then dissolved in 0.1 M potassium dihydrogen phosphate solution (pH 2 for doxycycline and pH 2.5 for chlortetracycline). Both of sample solutions were filtrated through a 0.45 μm Nylon membrane syringe filter. The filtered solutions were further diluted with 0.1 M potassium dihydrogen phosphate solution to obtain a final concentration of 240.45 and 257.65 $\mu g\,ml^{-1}$ (0.5 mM), respectively.

2.3. Electrode

The gold rotationg disk electrode (Au RDE, Metrohm, Switzerland) and gold disk electrode (Bioanalytical System, West Lafayete IN, USA) were pretreated by polishing with 0.05 μm of alumina/water slurries on a felt pad, followed by rinsing with ultrapure water prior to use.

2.4. Rotating disk voltammetry

Electrochemical measurements were carried out in a single compartment three-electrode glass cell. The rotation speed was held at 250 rpm. A Ag/AgCl electrode and a platinum electrode were used as the reference and auxiliary electrode, respectively. Cyclic voltammetry was performed with an Autolab Potentiostat 100 (Metrohm, Switzerland).

2.5. Flow injection analysis with pulsed amperometric detection

The flow injection analysis system consisted of a thin-layer flow-through electrochemical cell (Bioanalytical

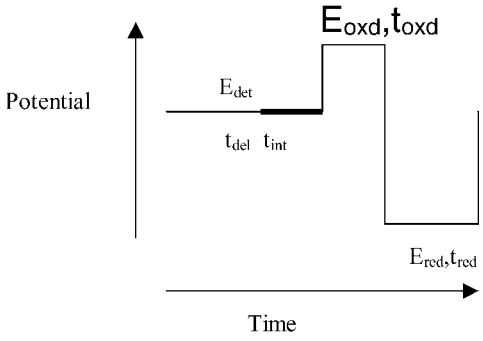

Fig. 1. Typical PAD waveform.

System, Inc.), an injector port (Rheodyne 7125) with a 20 μl sample loop, a peristaltic pump (BIO-RAD) and an electrochemical detector (PG 100). The carrier solution, 0.1 M potassium dihydrogen phosphate, was regulated at a flow rate of 1.0 ml min^{-1}. The thin-layer flow, through electrochemical cell consisted of a silicone rubber gasket as a spacer, a gold disk electrode as the working electrode, a Ag/AgCl electrode as the reference electrode and a stainless steel tube as the auxiliary electrode and the outlet from the flow cell. The experiments were performed in a faraday cage to reduce the electrical noise. The used PAD waveform to obtaine the FI-PAD response was depicted in Fig. 1.

The FI-PAD response was monitored for independent variation of all potential and time parameters. The electrode was condition in a solution of 0.1 M potassium dihydrogen phosphate solution and pumped through the flow system at a constant flow rate of 1.0 ml min^{-1} with the selected PAD waveform until a stable baseline was established. The sample was then injected into the flow injection system via an injection valve equipped with a fixed sample loop of 20 μl and the resulting peaks were recorded.

3. Results and discussion

3.1. pH dependence study

In our initial experiments, the electrochemical behaviors of both doxycycline or chlortetracycline were studied at Au RDE in 0.1 M potassium dihydrogen phosphate solution of pH 2–10. It was found that the best-resolved anodic signals for oxidation of doxycycline or chlortetracycline were obtained at pH 2 and pH 2.5, respectively. Therefore, these pHs were used for the rest of the experiments.

3.2. Cyclic voltammetry

The cyclic voltammetric *(I–E)* responses are shown in Fig. 2 for the Au RDE in 0.1 M potassium dihydrogen phosphate solution with and without 1 mM doxycycline (Fig. 2a) and 1 mM chlortetracycline (Fig. 2b). The background response for the supporting electrolyte exhibits an anodic wave

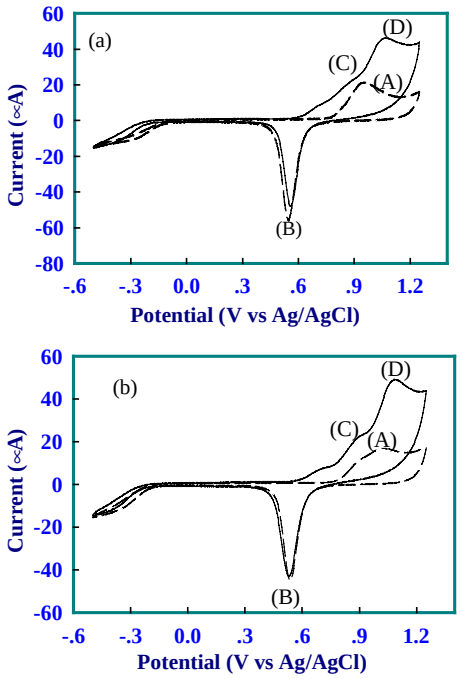

Fig. 2. Cyclic voltammetric response for (a) 1 mM doxycycline (pH 2) and (b) 1 mM chlortetracycline (pH 2.5) at the Au RDE (0.07 cm^2). Condition: 250 rpm rotation speed: 50 mV s^{-1} scan rate. The background cyclic voltammograms are also shown (dash line).

on the positive scan in the region of ca. +0.8 to +1.2 V versus Ag/AgCl (wave A). This background response corresponds to charging of the interfacial double layer and formation of a small amount of surface oxide. The cathodic peak obtained on the negative scan in the region of ca. +0.7 to +0.4 V versus Ag/AgCl (wave B) corresponds to dissolution of the surface oxide formed on the positive scan. In the presence of doxycycline or chlortetracycline, the two-step anodic signal for oxidation of them were observed on the positive scan beginning at ca. 0.6 V versus Ag/AgCl. The first and secon steps were occurred in the region ca. +0.6 to +0.9 V versus Ag/AgCl (wave C) and +0.95 to 1.15 V versus Ag/AgCl (wave D), respectively. The anodic responses for doxycycline or chlortetracycline on the positive scan were sharply inhibited by the onset of the surface oxide formation at potential greater than ca. +1.2 V versus Ag/AgCl. The decrease of signal on the subsequent negative scan in the region of ca. +1.25 to +0.8 V versus Ag/AgCl indicates the reduction of activity for the oxide covered gold surface.

3.3. PAD waveform optimization

The PAD waveform used in this experiment is described in Fig. 1. E_{det} is the detection potential applied for the time period t_{det} ($t_{det} = t_{del} + t_{int}$), and the electrode current is sampled by electronic integration over the time period t_{int} following a delay of t_{del} to allow the charging current to decrease to a negligible value. A positive cleaning potential (E_{oxd}) that removes the oxidizable contaminant on the elec-

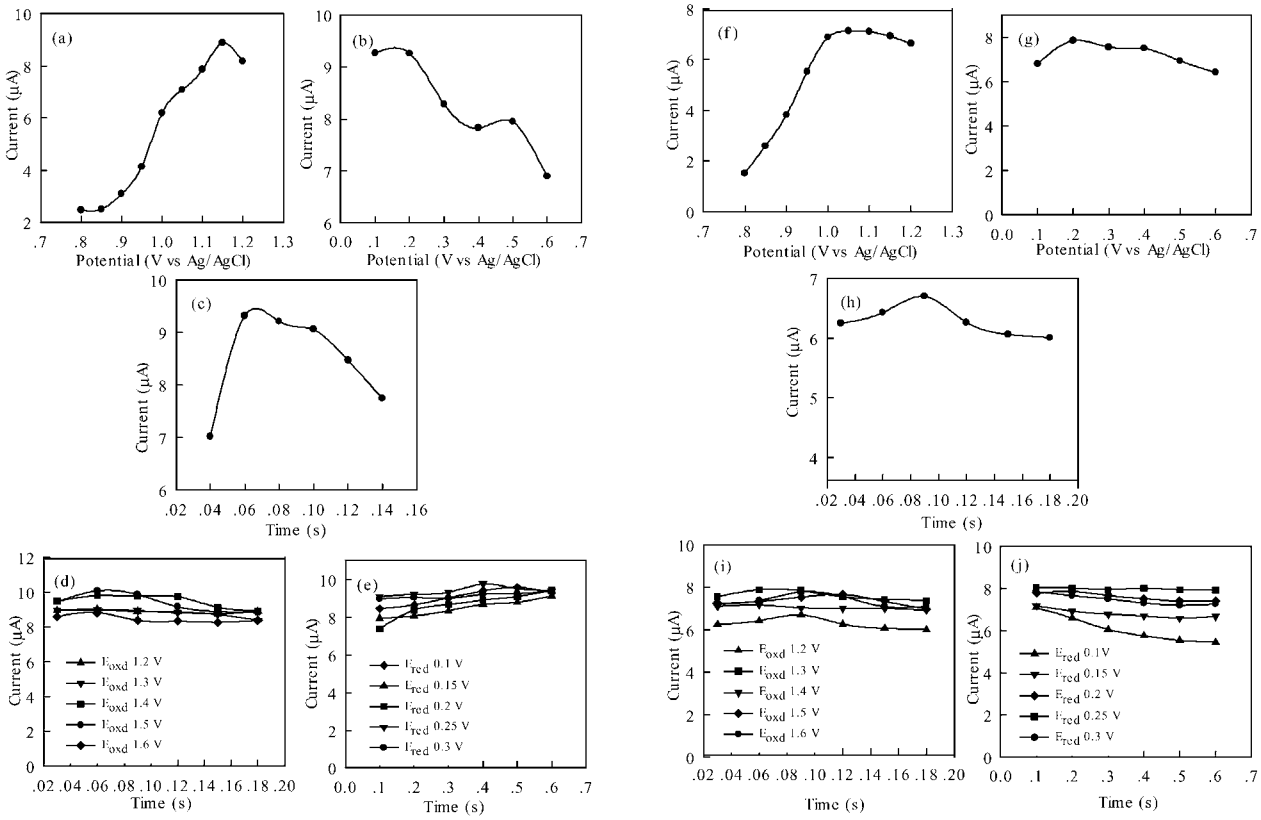

Fig. 3. FI-PAD response as a function of: (a) E_{det}; (b) t_{del}; (c) t_{int}; (d) E_{oxd} and t_{oxd}; and (e) E_{red} and t_{red}, for chlortetracycline and (f) E_{det}; (g) t_{del}; (h) t_{int}; (i) E_{oxd} and t_{oxd}; and (j) E_{red} and t_{red} for doxycycline in 0.1 M potassium dihydrogen orthophosphate solution (pH 2) at the Au RDE (0.07 cm^2).

trode surface is applied for the time period t_{oxd} following E_{det}. A negative reactivating potential (E_{red}) that dissolves the inert oxide product on the electrode surface is applied for the time period t_{red} following E_{oxd}. The optimization of each waveform parameter carried out in the FI system was studied while the other parameters were held constant. The average peak currents for each parameter were plotted versus the varied parameter. Observations of each parameter are discussed later.

3.4. Optimization of detection step (E_{det}, t_{int} and t_{del})

Fig. 3a and f show the FI-PAD response variations for 0.5 mM doxycycline and 0.5 mM chlortetracycline respectively according to E_{det} variation in the range +0.8 to +1.2 V versus Ag/AgCl in intervals of 0.5 V. The potential range used for E_{det} optimization was chosen from the potential region in the cyclic voltammogram (Fig. 2) that the oxidation of each doxycycline or chlortetracycline occurred. The optimum detection potential for doxycycline was obtained at $E_{det} = 1.15$ V versus Ag/AgCl and for chlortetracycline was obtained at $E_{det} = 1.05$ V versus Ag/AgCl.

Fig. 3b and g show the FI-PAD response variations for 0.5 mM doxycycline and 0.5 mM chlortetracycline with t_{del} variation from 100 to 500 ms. The t_{del} optimal values of doxycycline and chlotetracycline were 150 ms and 200 ms, respectively.

Fig. 3c and h show the FI-PAD response variations for 0.5 mM doxycycline and 0.5 mM chlortetracycline with variation of t_{int} from 40 to 140 ms. The optimal values of t_{int} for doxycycline was obtained 100 ms and chlortetracycline was obtained 70 ms.

3.5. Optimization of oxidation step (E_{oxd} and t_{oxd})

A clean electrode surface is progressively fouled by the detection products during application of E_{oxd} and to avoid this problem, t_{oxd} was applied to clean the electrode surface. Fig. 3d and i show the FI-PAD response variations for 0.5 mM doxycycline and 0.5 mM chlortetracycline as a result of the variation of t_{oxd} from 30 to 180 ms at intervals of 30 ms for difference E_{oxd} values in the range +1.2 to +1.6 V versus Ag/AgCl in interval 0.1 V. The optimal values of doxycycline was obtained $E_{oxd} = 1.3$ V versus Ag/AgCl and $t_{oxd} = 70$ ms. For chlortetracycline, the $E_{oxd} = 1.5$ V versus Ag/AgCl and $t_{oxd} = 70$ ms was recommended as optimal.

3.6. Optimization of reduction step (E_{red} and t_{red})

The formation of surface oxide at the electrode surface, which reduced the electrode surface activity, occurred during the oxidation step. Therefore, it is necessary that the values of E_{red} and t_{red} are chosen to achieve complete re-

Table 1
The optimal PAD waveform parameters of doxycycline and chlortetracycline

Parameter	Doxycycline	Chlortetracycline
E_{det} (V vs. Ag/AgCl)	1.05	1.15
t_{del} (ms)	200	150
t_{int} (ms)	100	70
E_{oxd} (V vs. Ag/AgCl)	1.3	1.5
t_{oxd} (ms)	7.0	70
E_{red} (V vs. Ag/AgCl)	0.25	0.25
t_{red} (ms)	400	400

ductive dissolution of the surface oxide. Fig. 3e and j show the FI-PAD response variations for 0.5 mM doxycycline and 0.5 mM chlortetracycline with variation of t_{red} from 100 to 600 ms at intervals of 100 ms for difference E_{red} values in the range +0.1to +0.5 V versus Ag/AgCl in interval 0.1 V. For doxycycline or chlortetracycline, the optimal values of reduction step were obtained the value of $E_{red} = 0.25$ V versus Ag/AgCl and $t_{red} = 400$ ms. To conclude, the potentials and times for the optimization are shown in Table 1.

3.7. Linear range, detection limit and repeatability

From a series of repetitive 20 μl injections of doxycycline or chlortetracycline in 0.1 M potassium dihydrogen phosphate solution under the optimum pH conditions and the optimized PAD waveform parameters described above provided well-defined signals as shown in Fig. 4. The current signal increased with increase in concentration. The calibration curves for doxycycline or chlortetracycline were obtained from using the optimized PAD waveform parameters. The analytical performance results are shown in Table 2. The dynamic linear working range of both compounds is the same and over two orders of magnitude.

3.8. Drug analysis of pharmaceutical formulations

The proposed PAD methods for doxycycline or chlortetracycline were applied to the determination of doxycycline or chlortetracycline in pharmaceutical formulations by standard addition method. In order to evaluate, these proposed methods for the determination of doxycycline or chlortetracycline in drug capsules, the recovery, and within-day and between-day studies were carried out. The results are summarized in Table 3.

Table 2
Linear range, detection limit and repeatability of doxycycline hydrochloride and chlortetracyline hydrochloride

Parameter	Doxycycline	Chlortetracycline
Linear range	1 μM–0.1 mM	1 μM–0.1 mM
Sensitivity (μAmM^{-1})	23.4	33.6
Regression equation	$Y = 23.354X + 0.0272$	$Y = 33.785X + 0.037$
Correlation coefficient (R^2)	0.9992	0.9994
Detection limit	1 μM	1 μM
Repeatability (%RSD)	3.17	2.18

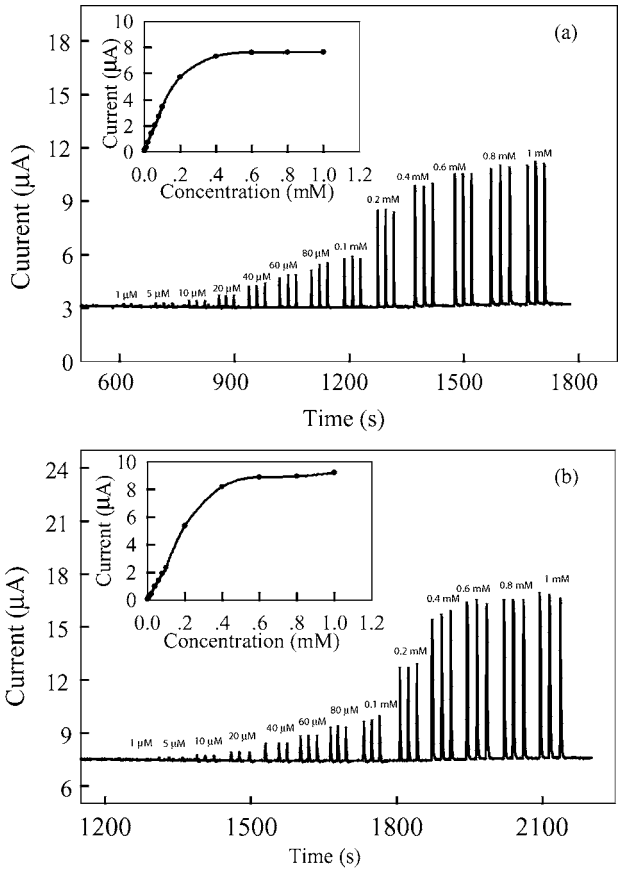

Fig. 4. FI-PAD results of (a) chlortetracycline and (b) doxycycline in 0.1 M potassium dihydrogen phosphate at gold disk electrode. The flow rate was 1 ml min^{-1}.

Table 3
Percent difference and percent recovery of doxycycline hydrochloride and chlortetracycline hydrochloride capsule samples

Parameters	Doxycycline	Chlortetracycline
% Difference	2.57 ± 1.77	7.28 ± 0.27
% Recovery of spiked standard solution	87–103	93–109

4. Conclusion

This is the first investigation of doxycycline or chlortetracycline using pulsed amperometric detection applied to a flow injection system to avoid a problem about fouling of products or interferents on the surface of a gold working electrodes. The optimized conditions, such as pH and the various potentials were investigated. The results showed that FI-PAD with optimized conditions can be used to determine doxycycline or chlortetracycline in pharmaceutical formulations. FI-PAD provided wide working concentration (0.001–0.1 mM), low detection limit (1 μM) and high re-

peatability (% RSD 3.17–2.18). The advantage of the proposed method is simple and time saving because the cleaning step occurs simultaneously during the measurement. The commercial drugs of doxycycline hydrochloride or chlortetracycline hydrochloride were also analyzed by proposed methods.

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References

[1] J.R. Walsh, L.V. Walker, J.J. Webber, J. Chromatogr. 596 (1992) 211.
[2] A.L. Cinquina, F. Longo, G. Anastasi, L. Giannetti, R. Cozzani, J. Chromatogr. A 987 (2003) 227.
[3] M. Cherlet, M. Schelkens, S. Croubels, P. De Backer, Anal. Chim. Acta 492 (2003) 199.
[4] D.L. Collins-Thompson, D.S. Wood, I.Q. Thompsom, J. Food Prot. 51 (1988) 632.
[5] E.D. Haese, H.J. Nelis, W. Reybroeck, App. Environ. Microbiol. 63 (1977) 4116.
[6] S. Skulason, E. Ingolfsson, T. Kristmundsdottir, J. Pharm. Biomed. Anal. 33 (2003) 667.
[7] B. Axisa, A.R. Naylor, P.R.F. Bell, M.M. Thompson, J. Chromatogr. B 744 (2000) 359.
[8] S. Croubels, W. Baeyens, C. Van Peteghem, Anal. Chim. Acta 303 (1995) 11.
[9] J. Zhu, D.D. Snow, D.A. Cassada, S.J. Monson, R.F. Spalding, J. Chromatogr. A 928 (2001) 177.
[10] A.K. Lykkeberg, B. Halling-Sorensen, C. Cornett, J. Tjornelund, S.H. Hansen, J. Pharm. Biomed. Anal. 34 (2004) 325.
[11] A.G. Kazemifard, D.E. Moore, J. Pharm. Biomed. Anal. 16 (1997) 689.
[12] J.L. Lopez Paz, J.M. Calatayud, J. Pharm. Biomed. Anal. 11 (1993) 1093.
[13] R. Karlicek, P. Solich, Anal. Chim. Acta 285 (1994) 9.
[14] C. Lau, J. Lu, M. Kai, Anal. Chim. Acta 503 (2004) 235.
[15] B. Li, Z. Zhang, W. Liu, Talanta 55 (2001) 1097.
[16] X. Zheng, Y. Mei, Z. Zhang, Anal. Chim. Acta 440 (2001) 143.
[17] A. Pena, L.P. Palilis, C.M. Lino, M.I. Silveira, A.C. Calokerinos, Anal. Chim. Acta 405 (2000) 51.
[18] X.R. Zhang, W.R.G. Baeyens, A. Van den Borre, G. Van der Weken, A.C. Calokerinos, S.G. Schulman, Analyst 120 (1995) 463.
[19] S. Croubels, C.V. Peteghem, W. Baeyens, Analyst 119 (1994) 2713.
[20] R. Fernandez-Gonzalez, M.S. Garcia-Falcon, J. Simal-Gandara, Anal. Chim. Acta 455 (2002) 143.
[21] C.M.C.M. Couto, J.L.F.C. Lima, M. Conceicao, B.S.M. Montenegro, S. Reis, J. Pharm. Biomed. Anal. 18 (1998) 527.
[22] J. Zhou, G.C. Gerhardt, A. Baranski, R. Cassidy, J. Chromatogr. A 839 (1999) 193.
[23] S. Sabharwal, K. Kishore, P.N. Moorthy, J. Pharm. Sci. 77 (1988) 78.
[24] T. Jochsberger, A. Cutie, J. Mills, J. Pharm. Sci. 68 (1979) 1061.
[25] C. Giuriati, S. Cavalli, A. Gorni, D. Badocco, P. Pastore, J. Chromatogr. A 1023 (2004) 105.
[26] V.P. Hanko, J.S. Rohrer, Anal. Biochem. 324 (2004) 29.
[27] A. Guzman, L. Agui, M. Pedrero, P. Yanez-Sedeno, J.M. Pingarron, J. Pharm. Biomed. Anal. 33 (2003) 923.
[28] A. Inoue, R.L. Earley, M.W. Lehmann, L.E. Welch, Talanta 46 (1998) 1507.
[29] V.P. Hanko, J.S. Rohrer, Anal. Biochem. 308 (2002) 204.
[30] P.J. Vandeberg, D.C. Johnson, Anal. Chim. Acta 290 (1994) 317.
[31] M.W. Lehmann, M.R. Fahr, L.E. Welch, Talanta 44 (1997) 1231.
[32] K. Sato, J.-Y. Jin, T. Takeuchi, T. Miwa, K. Suenami, Y. Takekoshi, S. Kanno, J. Chromatogr. A 919 (2001) 313.
[33] C. Corradini, G. Canali, E. Cogliandro, I. Nicoletti, J. Chromatogr. A 791 (1997) 343.
[34] S.B. Adeloju, S.L. Shaw, G.G. Wallace, Anal. Chim. Acta 341 (1997) 155.
[35] S. Palaharn, T. Charoenraks, N. Wangfuengkanagul, K. Grudpan, O. Chailapakul, Anal. Chim. Acta 499 (2003) 191.