Arabian Journal of Chemistry (2020) 13, 335–345

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Flow injection analysis system with electrochemical detection for the simultaneous determination of nanomolar levels of acetaminophen and codeine

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Received 6 February 2017; accepted 16 April 2017
Available online 26 April 2017

KEYWORDS

Paracetamol;
Codeine;
Flow injection analysis;
Boron-doped diamond;
Multiple pulse amperometry

Abstract A simple, rapid and low-cost electroanalytical method is proposed for the determination of acetaminophen (ACP) and codeine (COD) at nanomolar levels in pharmaceutical and biological samples. The analytical procedure is based on a flow injection analysis system coupled to electrochemical detection, which was multiple pulse amperometry (FIA-MPA). Boron-doped diamond was used as the working electrode for electrochemical detection. The electrode was subjected to a cathodic pretreatment and was selected in this work due its good electrochemical performance. By applying the FIA-MPA method, after a number of optimization assays, the analgesics were simultaneously determined at excellent linear concentration ranges. The analytical curves ranged from 80 nmol L^{-1} to 100 μmol L^{-1} for ACP and from 50 nmol L^{-1} to 10 μmol L^{-1} for COD, and the obtained limits of detection were 30 nmol L^{-1} and 35 nmol L^{-1} for ACP and COD, respectively. The practical applicability of the electroanalytical method was evaluated from the ACP and COD determination in two sample matrices: commercial pharmaceutical samples and biological fluids. In the case of pharmaceutical formulation samples, the obtained results were statistically similar to those obtained using a reference chromatographic method. In addition, these drugs were simultaneously quantified in biological fluid samples of urine and human serum with excellent recovery percentages.

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Peer review under responsibility of King Saud University.

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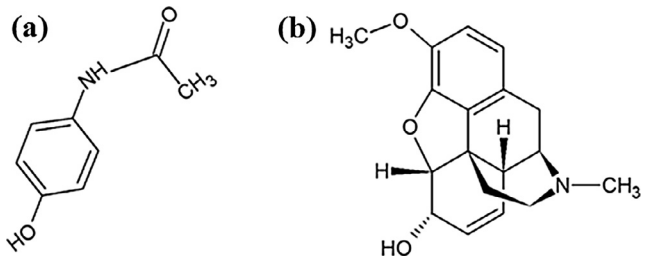
1. Introduction

Acetaminophen (ACP, N-acetyl-p-aminophenol, Scheme 1), more popularly known as paracetamol, is an analgesic antipyretic drug widely used for the treatment of pain and fever. Due to the similar action to aspirin, ACP is an appropriated alternative drug for patients allergic to aspirin, and it is the major ingredient in numerous pharmaceutical formulations. Despite ACP causing fewer side effects than

http://dx.doi.org/10.1016/j.arabjc.2017.04.012
1878-5352



Scheme 1 Molecular structure of (a) ACP and (b) COD.

aspirin in therapeutic doses, ACP can cause skin rashes and occasionally other allergic reactions (Clissold, 1986). After oral administration, paracetamol is rapidly absorbed, distributed and largely excreted in urine in the following chemical forms: 45–55% as glucuronide conjugates, 15–55% as cysteine, 20–30% as sulfates and mercapturic acid conjugates, and 1–5% remains unchanged (Filik et al., 2006).

Codeine (COD, 7,8-didehydro-4,5a-epoxy-3-methoxy-17-methyl morphinan-6a-ol, Scheme 1) is a opiate alkaloid that could be obtained from natural resources as poppies (Thorn et al., 2009) or synthetically from morphine by a methylation reaction. It has important effects on the central nervous system and is used as an effective analgesic, in addition to its good antitussive properties (Bjune et al., 1996; Irwin et al., 1993; McDonald and Lambert, 2005; Popa et al., 1998; Srinivasan et al., 1996; Zhou et al., 2002). COD and its metabolites are mainly eliminated via urine, with 4.9–8.2% remaining unchanged (Cone et al., 1991). Narcotic effects can be associated with COD use, and hence, addiction can occur (Backmund et al., 2001; Moriya et al., 1999; Panda et al., 2004; Seymour et al., 2001; Sproule et al., 1999). The uncontrolled use of products containing COD is strongly associated with serious adverse effects or even death (Hau et al., 2004; Klinder et al., 1999; Seymour et al., 2001). This drug is found as a single agent or in combination with other drugs, such as acetylsalicylic acid, ibuprofen, phenacetin, diclofenac, caffeine and ACP, for the reduction in pain and pyrexia.

The assumption that the mechanism of the ACP analgesic action is peripheral provides the justification for its combination with centrally acting analgesics, such as COD (Beaver, 1984; Mattia and Ferrari, 2014). From this combination, there is an increase in the analgesic efficacy, with rapid pain relief and no significant side effects (Mattia and Ferrari, 2014; Straube et al., 2014; Zhang and Po, 1996). However, their uncontrolled or improper use can cause serious health complications, and its overdose can be toxic to organisms or even lead to death (Hakkinen et al., 2012). Due to these factors, the development of sensitive, selective, rapid and simple analytical methods to provide the simultaneous determination of these compounds in pharmaceutical products and biological fluids is of utmost importance to prevent undesirable effects or determine intoxication by these compounds. In this sense, chromatographic techniques were reported and are the most common analytical methods explored for the simultaneous determination of these drugs (Al-kaysi and Salem, 1986; Badea et al., 2013; Ramos-Martos et al., 2001; Schmidt, 2006). Besides chromatographic methods, there are some analytical approaches exploring UV-vis spectrophotometry (Hanaee, 1997), chemiluminescence (Mokhtari et al., 2016) and thermogravimetric analysis (TGA) combined with chemometric (Khanmohammadi et al., 2012) for the simultaneous determination of ACP and COD. On the other hand, electroanalytical methods have shown significant advantages in the simultaneous determination of different drugs in matrice samples of pharmaceutical products and biological fluids (Deroco et al., 2014; Figueiredo-Filho et al., 2014; Silva et al., 2014). These advantages include the simplicity of the method, low costs and relatively short analysis time compared to chromatographic methods.

In the scenario of electroanalytical methods dedicated to simultaneous determination of drugs, an excited and intelligent strategy is the coupling of a flow injection analysis system with an electrochemical detector based on multiple pulse amperometry technique (FIA-MPA). This approach combines the advantages of FIA systems, such as low cost of system components, high analytical frequency, reduced consumption of reagents and samples and waste generation with the remarkable analytical features of MPA technique, including high sensitive, lower effects of working electrode contamination and negligent capacitive current (ideal for the detection of low concentrations) (Felix and Angnes, 2010; Lourencao et al., 2017; Pio dos Santos et al., 2011). This experimental design has been successfully applied for the simultaneous determination of multiple samples using a single working electrode, where the aliquots of standards or samples are directly injected onto the working electrode surface positioned in a wall-jet configuration (Gimenes et al., 2013; Lima et al., 2013; Santos et al., 2015b; Silva et al., 2011). In addition to simultaneous determination, this method is of low cost and allows for better selectivity, high sensitivity and high analytical frequency (Quintino and Angnes, 2004; Wang and Taha, 1991).

The electrodic material to be used as a working electrode in FIA-MPA procedures needs to present a number of intrinsic features, including, in particular, chemical and physical stability and good repeatability of response. Considering these aspects, boron-doped diamond (BDD) electrodes are very suitable for use in FIA-MPA procedures, because they are carbon-based materials with a number of advantages, such as low corrosion in aggressive media, extreme electrochemical stability in alkaline and acidic media, a very low and stable background current, a wide working potential window, low adsorption and long-term stability of response (Hupert et al., 2003; Kawarada, 1996; Rao and Fujishima, 2000; Salazar-Banda et al., 2006; Silva et al., 2016).

In the present work, we describe the advantages of using a FIA-MPA system combined with a cathodically pretreated BDD electrode for the simultaneous determination of ACP and COD in pharmaceutical commercial products and biological fluids at trace levels of nanomolar concentrations. The developed method is compared and contrasted with others described in the literature (Afkhami et al., 2014; Babaei et al., 2012; Ensafi et al., 2015; Mashhadizadeh and Rasouli, 2014; Pournaghi-Azar et al., 2010; Pournaghi-Azar and Saadatirad, 2010; Santos et al., 2015a), in addition to being independently compared with a chromatographic method.

2. Experimental

2.1. Reagents and solutions

ACP standard was purchased from the Sigma-Aldrich company (São Paulo, SP, Brazil) and the codeine-phosphate (COD) standard was acquired from FAGRON (São Paulo, SP, Brazil) commercial source. Additional chemical reagents of analytical grade were used and applied during the experiments as received. The solutions were prepared using ultrapure water (resistivity $>18\ M\Omega\ cm$) obtained from a Millipore Milli-Q system (Billerica, USA). The 0.2 mol L^{-1} phosphate buffer (pH 4.0) solution used as supporting electrolyte was prepared using an adequate volume and mass of H_3PO_4 and KH_2PO_4. In the case of Britton–Robinson (BR) buffer solutions with different values of pH, a stock solution containing 0.04 mol L^{-1} H_3PO_4, 0.04 mol L^{-1} CH_3COOH and 0.04 mol L^{-1} H_3BO_3 was prepared, and the desired pH was adjusted with the addition of 0.1 mol L^{-1} NaOH solution in the previous mixture using a pH meter for the pH control. Stock solutions of ACP and COD, both at 0.1 mol L^{-1}, were prepared directly in ultrapure water. The pharmaceutical formulations (tablets) containing ACP and COD were acquired in local drug stores.

2.2. Preparation of samples

Before electroanalytical measurements, the pharmaceutical formulation samples (tablets) were subjected to a sample preparation procedure. Thus, firstly, ten tablets of each sample were weighed and macerated to a powder in a mortar and pestle. Next, an adequate amount of powder (mass equivalent to one tablet) was added to a 10 mL of ultrapure water and the mixture submitted to sonication for 15 min in an ultrasonic bath for complete dissolution of the active principles. Then, the non-solubilized excipient compounds were separated from the solution by filtration using a filter paper. The final stock solution of each sample was subsequently diluted in a suitable electrolyte volume (0.05 mol L^{-1} H_2SO_4) for subsequent injection in the FIA-MPA system.

The synthetic biological fluids of urine were prepared as previously reported by Laube et al. (2001) with the addition of the following reagents: 4.0×10^{-2} mol L^{-1} KCl, 9.8×10^{-2} mol L^{-1} NaCl, 3.0×10^{-2} mol L^{-1} KH_2PO_4, 2.0×10^{-2} mol L^{-1} $CaCl_2$, 3.6×10^{-2} mol L^{-1} NH_4Cl and 3.6×10^{-2} mol L^{-1} urea in a 50 mL volumetric flask. Then, the flask volume was completed with ultrapure water. In the case of synthetic human serum, this sample was prepared as previously reported by Parham and Zargar (Parham and Zargar, 2001) using the following reagents: 8.0×10^{-6} mol L^{-1} methionine, 4.0×10^{-3} mol L^{-1} NaCl, 4.0×10^{-4} mol L^{-1} $NaHCO_3$, 2.6×10^{-6} mol L^{-1} cysteine, 7.0×10^{-6} mol L^{-1} glycine, 4.2×10^{-6} mol L^{-1} tryptophan, 4.0×10^{-6} mol L^{-1} tyrosine, 6.0×10^{-6} mol L^{-1} serine, 8.0×10^{-6} mol L^{-1} phenylalanine, 1.0×10^{-5} mol L^{-1} lysine, 7.0×10^{-6} mol L^{-1} histidine, 4.4×10^{-5} mol L^{-1} aspartic acid, 1.0×10^{-5} mol L^{-1} arginine and 2.0×10^{-5} mol L^{-1} alanine in 50 mL volumetric flask. Then, the flask volume was completed with ultrapure water. Posteriorly, appropriate aliquots of the standard solutions of ACP and COD were carefully added to the synthetic biological fluid samples and, 1 mL of this sample was added in the supporting electrolyte to obtain simultaneous concentrations of 2.0 μmol L^{-1} ACP and 0.60 μmol L^{-1} COD, and 50 μmol L^{-1} ACP and 5.0 μmol L^{-1} COD.

2.3. Apparatus and reference method

All electrochemical measurements were recorded using an Autolab PGSTAT-30 (EcoChemie) potentiostat/galvanostat managed by the GPES 4.9 software. For the assays involving voltammetric measurements as well as for the BDD electrode pretreatment, it was used a three-electrode cell system composed of an Ag/AgCl (3.0 mol L^{-1} KCl) reference electrode, a platinum auxiliary electrode and a BDD working electrode. The BDD electrode consisted of a BDD film with a doping level of 8000 ppm (B/C) (NeoCoat SA, La Chaux-de-Fonds, Switzerland) (Gandini et al., 1999). For the MPA measurements, it was adopted an electrochemical detector system constructed according to work of Richter et al. (Richter et al., 2003). In this way, the auxiliary electrode was a stainless steel tube and as reference electrode was applied a miniaturized Ag/AgCl (3.0 mol L^{-1} KCl) prepared using a micropipette tip. The working electrode was a pretreated BDD electrode. The FIA system was composed of a polyethylene tubing of 1.0 mm i.d. and for the flow rate control was employed a peristaltic pump. Fig. 1 shows a general scheme of the FIA system.

The pharmaceutical formulation samples containing ACP and COD were also analyzed by a reference method, which was high performance liquid chromatography (HPLC) (adapted from Ramos-Martos et al. (Ramos-Martos et al., 2001)). The details about adapted HPLC assays could be consulted in ref. (Santos et al., 2015a, 2015b).

2.4. Analytical procedure

The BDD electrode (0.69 cm^2 exposed area) was anodically (AP–BDD) or cathodically (CP–BDD) pretreated in 0.5 mol L^{-1} H_2SO_4 by applying 0.04 A cm^{-2} or −0.04 A cm^{-2} for 30 s or 180 s, respectively. The electrochemical response of the target molecules was evaluated on the BDD subjected to both pretreatments. Experimental conditions were optimized taking into account the best supporting electrolyte (composition and pH). The applied potentials for the MPA measurements were selected through the analysis of the respective hydrodynamic voltammograms constructed from the limiting current registered at each applied potential pulse. Moreover, the flow rate, sample injection volume and pulse time were also systematically optimized. After the exhaustive optimization steps, the analytical curves were simultaneously constructed from successive injections of supporting electrolyte solutions containing ACP and COD in the FIA-MPA system. To predict the limits of detection (LOD) for ACP and COD analytes was adopted the signal (S)-to-noise (N) ratio concept, in which S/N ratio of 3–1 was fixed. At the experimental level, the noise for the COD case was taken as the sum of the baselines registered in the two applied potentials.

By conducting intra-day (n = 10) and inter-day (n = 3) repeatability studies the precision of the developed method was verified. The ACP and COD concentrations in each pharmaceutical formulation sample were simultaneously determined in triplicate using the analytical curves constructed under the optimum conditions. Also, the possible effects of some excipients found in these samples were evaluated. In order to evaluate whether ACP and COD can be analyzed in biological samples, the synthetic urine and human serum samples were spiked with known concentrations of ACP and COD and analyzed in terms of recovery percentage of the spiking concentration.

3. Results and discussion

3.1. Electrochemical behavior of ACP and COD

As reported by Santos et al. (2015a), the electrochemical behavior of the ACP and COD molecules was investigated by cyclic voltammetry using the AP–BDD and CP–BDD electrodes. The electrochemical response of both compounds was recorded in a 0.2 mol L^{-1} phosphate buffer (pH 4.0) solution as supporting electrolyte. The cyclic voltammograms obtained in the absence and the simultaneous presence of ACP and COD (both at 0.1 mmol L^{-1}) using the two types of pretreated BDD electrodes are shown in Fig. 2(a). From these, it was verified that the analyte molecules exhibited well defined oxidation peaks. In addition, no reduction peaks were observed for ACP and COD during the cathodic potential scanning, suggesting an irreversible oxidation process for both molecules. Based on previous studies, possible electrooxidation

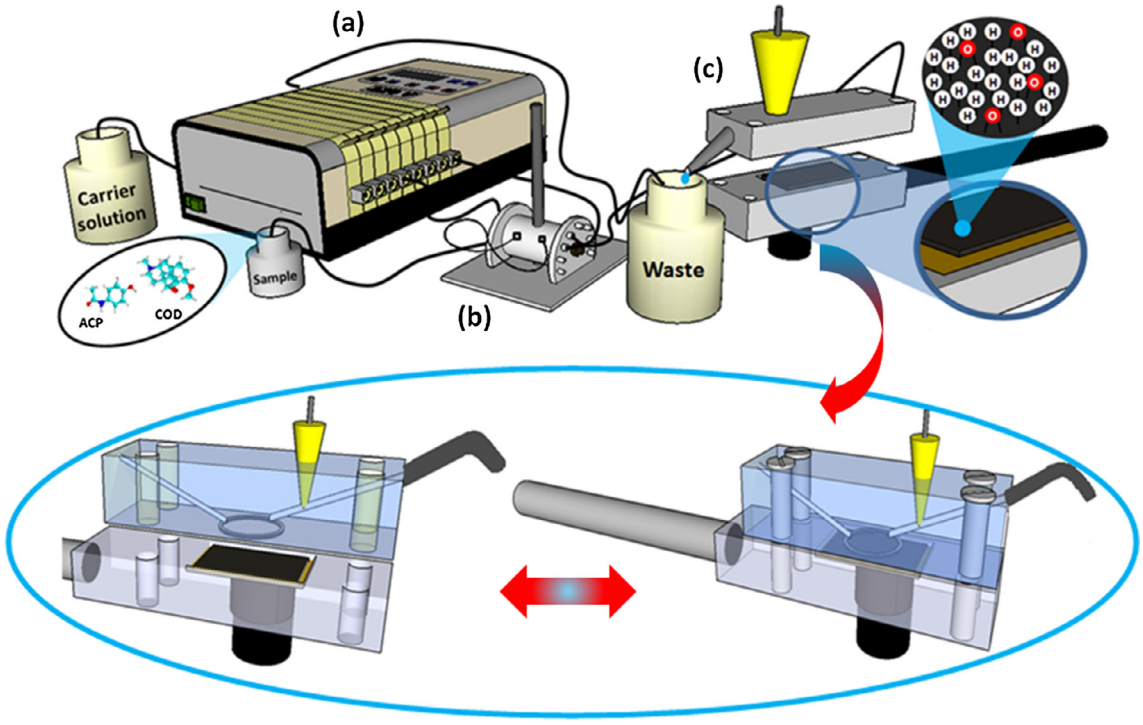


Figure 1 Schematic representation of the flow injection analysis (FIA) system employed: (a) peristaltic pump, (b) manual injector and (c) electrochemical detector with an Ag/AgCl (3.0 mol L^{-1} KCl) electrode as the reference electrode, a stainless steel tube as the auxiliary electrode and a CP–BDD electrode as the working electrode.

reactions of ACP and COD are shown in Scheme 2. Therefore, ACP molecules suffered irreversible electrooxidation involving the loss of two electrons and two protons forming N-acetyl-p-quinoneimine species (Kalambate et al., 2015; Silva et al., 2017), while the COD electrooxidation could be related to the oxidation of the 6-hydroxy group with loss of two electrons and one proton in the studied pH condition (Garrido et al., 2002; Silva et al., 2017). In addition, the effect of the electrochemical BDD on the simultaneous determination of ACP and COD could also be evaluated from the results of Fig. 2 (a). The cathodic pretreatment of the BDD electrode surface provided a remarkable enhancement of the anodic peak current for both analytes, thus indicating that a predominantly hydrogen-terminated surface can substantiality improve the electrochemical activity of the BDD electrode for the ACP and COD oxidation processes. Moreover, a greater separation between the oxidation peak potentials for the two analgesics was verified when the CP–BDD was used. In addition to the cyclic voltammetry results, Fig. 2(b) and (c) shows the square-wave voltammograms obtained individually for ACP and COD, confirming, again, the best electrochemical performance of CP-BDD toward the irreversible oxidation of these molecules. Taking into account the previous results, all subsequent experiments were carried out using the CP–BDD electrode.

Next, the influence of cyclic voltammetry scan rate on the ACP and COD anodic response was accessed by ranging this parameter from 20 to 500 mV s^{-1} in sequenced measurements. The obtained cyclic voltammograms are shown in Fig. 3 (a) and (b). In the inset (i) of Fig. 3(a) and (b), the plots of $\log I_p$ versus $\log v$ are arranged. The linear relationship verified between $\log I_p$ and $\log v$ presented slopes of 0.45 and 0.60 for ACP and COD, respectively, indicating the diffusional control of the electron transfer kinetic on the CP-BDD surface, since the obtained slope values were close to theoretical value of 0.5 (Bard and Faulkner, 2001; Brett and Brett, 1994). This finding was confirmed by the perfect linearity between I_p and $v^{1/2}$ (square root of the scan rate, v) for both cases (please see the inset (ii) of Fig. 3(a) and (b)), as expected by the Randles-Ševčik equation Eq. (1) for diffusion controlled electrodic processes (Bard and Faulkner, 2001; Brett and Brett, 1994).

$$I_p = \pm(2.99 \times 10^5)\alpha^{1/2}n^{3/2}D^{1/2}Cv^{1/2} \quad (1)$$

where I_p is the anodic or cathodic peak current, α is the charge transfer coefficient, n is the number of electrons, D is the diffusion coefficient and C is the electroactive specie concentration (in mol cm^{-3}).

3.2. Optimization of FIA–MPA system conditions

After defining the optimum BDD pretreatment in the study of the electrochemical response of the ACP and COD target molecules, a number of assays were conducted for optimization of the FIA–MPA system conditions. The first evaluated parameter was the supporting electrolyte composition. In this study, the following supporting electrolyte compositions were tested: 0.04 mol L^{-1} BR buffer (pH ranging from 2.0 to 12.0) and 0.1 mol L^{-1} H_2SO_4. The best analytical signal (higher intensity) was obtained using 0.1 mol L^{-1} H_2SO_4. Thus, the effect of H_2SO_4 concentration was investigated in a range from 0.01 to 0.50 mol L^{-1}. By doing this, the highest intensity of the

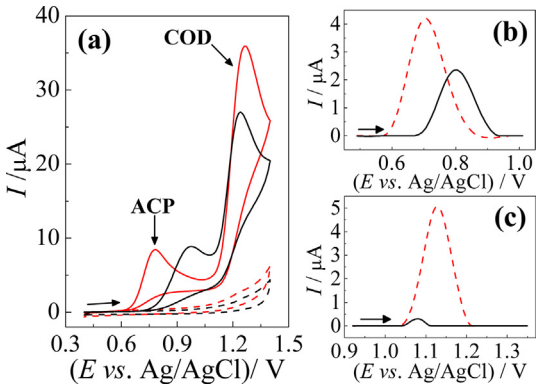
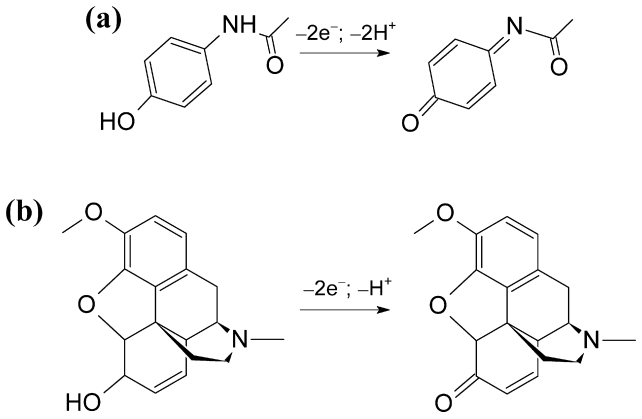


Figure 2 (a) Cyclic voltammograms obtained for a 0.2 mol L^{-1} phosphate buffer (pH 4.0) solution containing 0.1 mmol L^{-1} ACP and 0.1 mmol L^{-1} COD using a (–) CP–BDD electrode and an (–) AP–BDD electrode. v = 50 mV s^{-1}. Square-wave voltammogram obtained for a 0.2 mol L^{-1} phosphate buffer (pH 4.0) solution containing (b) 10.0 μmol L^{-1} ACP and **(c)** 10.0 μmol L^{-1} COD using a (–) CP–BDD electrode and an (–) AP–BDD electrode. Square-wave voltammetry parameters: f = 70 Hz, a = 50 mV ΔE_s = 4 mV s^{-1}.



Scheme 2 Possible electrooxidation reaction for (a) ACP and (b) COD.

analytical signal was verified using the 0.05 mol L^{-1} H_2SO_4 solution as the supporting electrolyte. Consequently, the 0.05 mol L^{-1} H_2SO_4 solution was selected as the supporting electrolyte for further assays.

Then, it was found the best applied potentials for the simultaneous amperometric determination of ACP and COD. In this evaluation, ten sequential potential pulses (600–1600 mV) were applied on the CP–BDD electrode with successive injections of supporting electrolyte solutions containing ACP or COD at 10 μmol L^{-1}. The obtained amperograms are shown in Fig. 4(a). From the peak currents registered at each applied potential pulse shown in the amperograms of Fig. 4(a), the respective hydrodynamic voltammograms were constructed, and these are presented in Fig. 4(b). As can be accessed from the hydrodynamic voltammograms, the ACP analyte begins to oxidize at 750 mV, and the peak current increased until 950 mV; the peak current then remained nearly constant for higher values of applied potential pulse. However, the oxidation of the COD molecule begins at 1100 mV, and, similar to the ACP case, the peak current increased significantly until 1400 mV and remained nearly constant for higher values of applied potential pulse. Based on these data from the hydrodynamic voltammograms, potential pulses of 950 mV and 1400 mV were selected for ACP and COD, respectively. At these values of potential pulses, the ACP molecule is determined without COD interference, because COD did not suffer oxidation at 950 mV. However, at 1400 mV, both compounds (ACP and COD) were oxidized and, therefore, the use of a correction factor (CF) was necessary to obtain the real current of COD in the simultaneous determinations. The CF was obtained after the final optimization of the FIA–MPA system, which included the investigation of the influence of flow rate, injected sample volume and potential pulse time, as discussed below.

In Fig. S1(a) (supplementary material), there is a plot of peak current versus different values of flow rate. The flow rate was studied in a range from 1.1 to 5.5 mL min^{-1}, and from the plot of Fig. S1(a), it was possible to verify that the current signal increased significantly with a flow rate up to 3.8 mL min^{-1}, and remained almost constant thereafter and, thus, a value of 3.8 mL min^{-1} was selected as the optimum flow rate. The analytical signal (current) increased at higher flow rates due to the increase in mass transport by time unit in the electrode surface, because of the diminishment of the Nernst diffusion layer at high flow rate values. However, after a certain flow rate value, the thickness of the Nernst diffusion layer does not decrease significantly anymore and the current tends to become constant, even with a new increase in the flow rate (Santos et al., 2011). The effect of the injected sample volume to the FIA–MPA system was studied in the range of 50–500 μL. As can be seen from Fig. S1(b), the current signal increased until 350 μL, and then did not undergo any significant oscillation. Thus, an injected sample volume of 350 μL was selected for further experiments. The analytical signal increases with increasing injection volume due to the decrease in the dilution effect along the path between the injector and the electrochemical cell. However, after a certain value of injected sample volume a maximum current signal is obtained, once the dilution that occurs at the ends of the sample zone no longer reaches the central region of the sample (Santos et al., 2011). Finally, the potential pulse time was analyzed, which ranged from 100 to 250 ms for the ACP and COD analgesics simultaneously, and the selected values were 200 and 100 ms for ACP and COD, respectively. The use of higher potential pulse times has not resulted in significative increment of analytical signal due to the constant flow rate (Silva et al., 2011). Under the optimum FIA–MPA parameters, a high analytical frequency of 90 samples per hour was obtained.

3.3. Analytical parameters for the simultaneous ACP and COD determination

The possibility of simultaneously determining the ACP and COD analgesics by FIA–MPA was confirmed from amperograms recorded with the application of two potential pulses, 950 mV by 200 ms and 1400 mV by 100 ms, for triplicate injections of supporting electrolyte solutions containing 10.0 μmol L^{-1} ACP, 10.0 μmol L^{-1} COD or a mixture of ACP and COD at 10.0 μmol L^{-1}, respectively. As can be

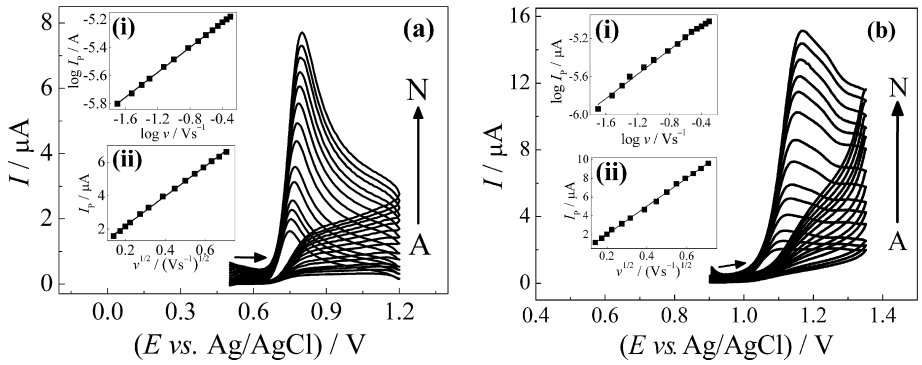


Figure 3 Cyclic voltammograms obtained for a 0.2 mol L^{-1} phosphate buffer (pH 4.0) solution containing (a) ACP 10.0 μmol L^{-1} and (b) COD 10.0 μmol L^{-1} using a CP–BDD electrode at different scan rates: 20 (A); 30 (B); 40 (C); 50 (D); 75 (E); 100 (F); 150 (G); 200 (H); 250 (I); 300 (J); 350 (K); 400 (L); 450 (M); and 500 (N). Insets: (i) $\log I_p$ *vs.* $\log v$ curves and (ii) I_p *vs.* $v^{1/2}$ curves.

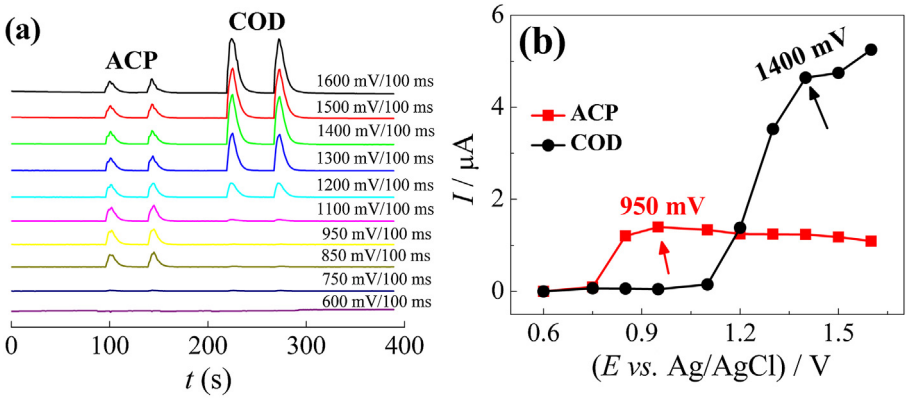


Figure 4 (a) Amperogramms obtained in FIA–MPA system after single injections of supporting electrolyte solutions containing ACP 10.0 μmol L^{-1} or COD 10.0 μmol L^{-1}. (b) Hydrodynamic voltammograms obtained for ACP and COD by plotting the values of peak current as a function of the corresponding applied potential pulses. Supporting electrolyte: 0.05 mol L^{-1} H_2SO_4; injection volume: 350.0 μL and flow rate: 3.8 mL min^{-1}.

observed in Fig. 5, at 950 mV, only ACP was oxidized. However, both analytes suffered electrooxidation at a potential of 1400 mV. Also, it was possible to confirm that the oxidation current detected for ACP at 950 mV was the same in the presence or absence of COD, and that the oxidation current at 1400 mV is the sum of the analytical signal of both analytes at this potential (ACP + COD). Therefore, no interference or any interaction effects between the compounds occurred with the optimum conditions, making it possible for simultaneous determination of these analgesics by FIA-MPA.

The previously mentioned CF was determined taking into account the peak currents registered in the multiple pulse amperograms presented in Fig. 5 The CF is necessary in order to get the exact oxidation current value of ACP at the potential of 1400 mV, because the ACP oxidation currents are not the same at the two potential pulses (950 mV and 1400 mV). Thus, the CF was obtained by the comparison of the ACP peak currents obtained at 950 and 1400 mV (Eq. (2)). The CF value was constant (1.04 ± 0.02) when solutions containing different concentrations of ACP were injected in the FIA–MPA system. The concentration of COD in the simultaneous determination was able to be determined from the subtraction of the value of ACP oxidation current at 950 mV (I_{ACP} (950 mV)) × CF from

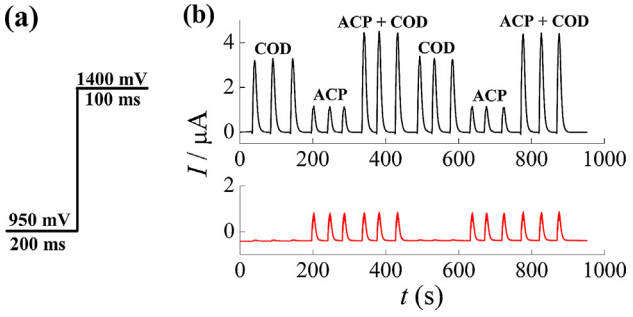


Figure 5 (a) MPA waveform applied to the CP–BDD electrode *vs.* potential pulse time. (b) FIA–MPA response for injections in triplicate of supporting electrolyte solutions containing only ACP (10.0 μmol L^{-1}), only COD (10.0 μmol L^{-1}) or ACP + COD (both at 10.0 μmol L^{-1}). Supporting electrolyte: 0.05 mol L^{-1} H_2SO_4; injection volume: 350.0 μL and flow rate: 3.8 mL min^{-1}.

the total oxidation current in the potential of 1400 mV (I_{total}, ACP + COD), as shown in Eq. (3). Doing this, only the COD oxidation current value (I_{COD}) was obtained. Therefore, by using this predicted CF value, it was possible to quantify

both analytes simultaneously, without interference after a simple signal subtraction.

$$CF = \frac{I_{ACP}(1400\ \text{mV})}{I_{ACP}(950\ \text{mV})} \quad (2)$$

$$I_{COD} = I_{total(1400\ \text{mV})} - (CF \times I_{ACP(950\ \text{mV})}) \quad (3)$$

In Fig. 6(a), the multiple pulse amperograms obtained for injections of standard solutions containing ACP and COD at different concentration levels, as well as for injections of pharmaceutical samples, are presented. The standard solutions were added with increasing ACP and COD concentrations, and after the injection of the pretreated commercial pharmaceutical samples, the standard solutions were re-injected with decreasing concentrations, in order to verify the response repeatability of the MPA–FIA system. The analytical curves obtained for ACP and COD from the current signal registered in the simultaneous presence of these analytes are shown in Fig. 6(b) and (c). The current increased linearly from 80 nmol L^{-1} to 100 μmol L^{-1} for ACP and from 50 nmol L^{-1} to 10 μmol L^{-1} for COD, according to the following linear regression equations, Eqs. (4) and (5)

$$\textbf{ACP}: \Delta I_p\ (\mu\text{A}) = 0.024 + 1.0 \times 10^5 C_{ACP}\ (\text{mol L}^{-1})(r = 0.999) \quad (4)$$

$$\textbf{COD}: \Delta I_p\ (\mu\text{A}) = -0.013 + 2.9 \times 10^5 C_{COD}\ (\text{mol L}^{-1})(r = 0.999) \quad (5)$$

The LOD values obtained were 30 nmol L^{-1} for ACP and 35 nmol L^{-1} for COD. The LOD for ACP was calculated using three times signal/noise at 950 mV (3 S/N_{950mV}) and for COD was calculated in the same way, but taking into account the sum of noise at 950 mV and 1400 mV (3 $S/(N_{950\,mV} + N_{1400\,mV})$).

The analytical features found for the ACP and COD simultaneous determination using the proposed FIA–MPA method and other reported electroanalytical methods are presented in Table 1 (Afkhami et al., 2014; Babaei et al., 2012; Ensafi et al., 2015; Pournaghi-Azar and Saadatirad, 2010; Santos et al., 2015a). Performing a systematic comparison, it is clear that the proposed method provided important analytical advantages regarding the detection and quantification of both analytes at very low concentration levels (in the nanomolar range), with only one exception (Afkhami et al., 2014). However, we should point out that the electroanalytical method designed in this work explored for the first time the coupling of a FIA system with electrochemical detection for the ACP and COD simultaneous determination, and it provided simplicity and speed of analysis, with an analytical frequency significantly higher than those involving voltammetric

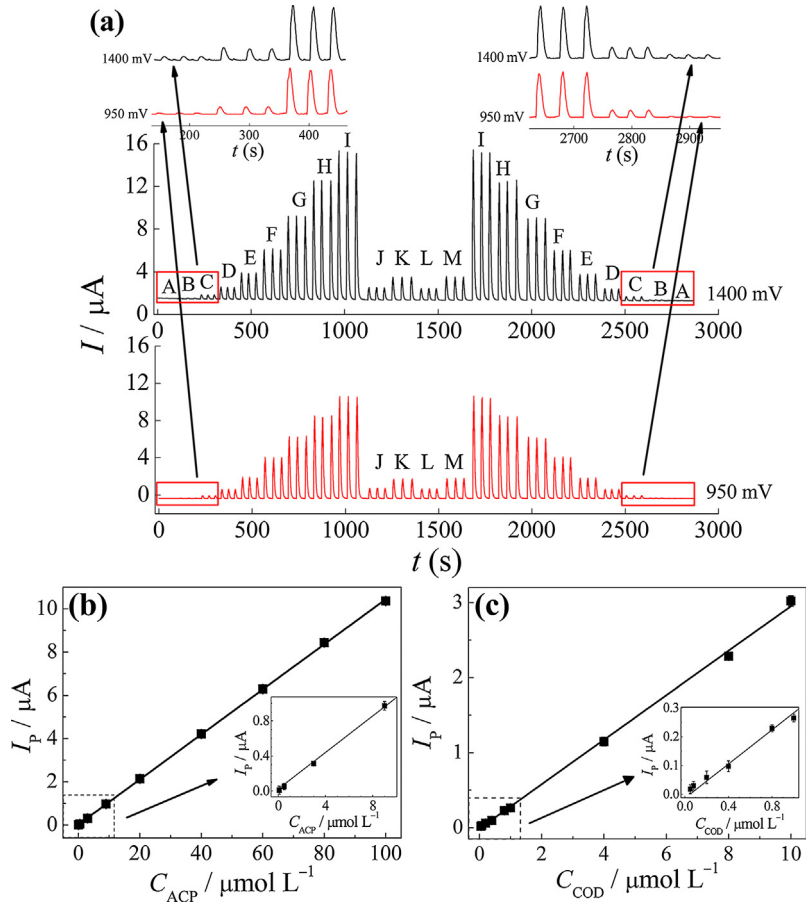


Figure 6 (a) FIA–MPA amperogramms obtained after injections of supporting electrolyte solutions containing ACP (A–I: 0.080, 0.50, 3.0, 9.0, 20, 40, 60, 80 and 100 μmol L^{-1}) and COD (A–I: 0.050, 0.080, 0.20, 0.40, 0.80, 1.0, 4.0, 8.0 and 10 μmol L^{-1}) simultaneously or four appropriately diluted pharmaceutical samples (J–M). (b) Analytical curve obtained for ACP and **(c)** COD. Supporting electrolyte: 0.05 mol L^{-1} H_2SO_4; injection volume: 350.0 μL and flow rate: 3.8 mL min^{-1}.

Table 1 Comparison between electroanalytical methods for the simultaneous determination of ACP and COD.

Electrode	Method	Analytical parameter				Biological fluids			Pharmaceutical samples	Ref.
		Linear range (μmol L^{-1})		Limit of detection (μmol L^{-1})						
		ACP	COD	ACP	COD	Urine	Serum	Plasma		
Aluminum electrode modified by thin layer of palladium	DPV	100–3000	100–3000	5	5	No	No	No	Yes	Pournaghi-Azar and Saadatirad (2010)
Glassy carbon electrode modified with multi-walled carbon nanotubes	DPV	5–400	5–240	0.19	0.20	Yes	Yes	No	Yes	Babaei et al. (2012)
Porous silicon/palladium nanostructure	DPV	1.0–700	1.0–700	0.4	0.3	Yes	No	Yes	Yes	Ensafi et al. (2015)
Boron-doped diamond electrode	SWV	0.2–95.8	0.4–9.6	0.018	0.014	Yes	Yes	No	Yes	Santos et al. (2015a)
Graphene/$CoFe_2O_4$ nanocomposite	SWV	0.03–12	0.03–12	0.025	0.011	Yes	No	Yes	Yes	Afkhami et al. (2014)
Boron-doped diamond electrode	FIA– MPA	0.08–100.0	0.05–10.0	0.030	0.035	Yes	Yes	No	Yes	This work

determinations. This reveals that the use of FIA–MPA using a CP–BDD electrode showed excellent analytical performance in terms of wide linear range and very low detection limit, over other methods reported in the literature.

3.4. Repeatability and interference studies

The repeatability of the FIA–MPA procedure was tested through intra-day and inter-day analyses. For intra-day repeatability, ten successive measurements were performed for three solutions of different ACP and COD concentrations (9.0, 40 and 100 μmol L^{-1} for ACP and 0.40, 1.0 and 10 μmol L^{-1}), as can be seen in Fig. S2. The peak current values presented relative standard deviations (RSDs) of 0.90%, 1.21% and 1.16% for ACP and 1.46%, 0.76% and 1.80% for COD, respectively. For the inter-day repeatability study, the analytical signal obtained for the same three solutions was monitored during three different days, thereby obtaining RSD values of 2.57%, 3.03% and 2.93% for ACP and 4.11%, 3.28% and 3.97% for COD, respectively. Thus, it was demonstrated that the proposed analytical method using a CP–BDD electrode and the FIA system coupled with MPA exhibits excellent precision measurements.

The potential interference of some excipient compounds typically found in ACP and COD pharmaceutical formulations was studied. In this study, the following excipients were considered: magnesium stearate, cellulose, sodium docusate, silicon dioxide, starch, sodium bisulfite, benzoic acid and sodium benzoate. Thus, to evaluate possible effects of these excipient compounds on the amperometric response, a set of amperograms were collected for ACP and COD solutions (both at 10 μmol L^{-1}) in the absence and presence of each substance at a proportion of 1:10 (analyte:excipient) (Fig. S3). Comparing the analytical signals registered for ACP and COD in the absence and presence of the excipients, RSDs in the range of 2.5% to +5.7% were verified, as shown in Table S1. Analysis of the RSD values allows us to conclude that the proposed method is selective for ACP and COD simultaneous determination.

3.5. Applications

In this step, it was performed the practical application of the proposed method. Initially, the FIA–MPA method was applied for the determination of ACP and COD in commercial pharmaceutical samples. In order to have a comparative set of results, these samples were also analyzed by a reference method (HPLC). The results collected by both the methods are presented in Table 2, and as can be seen, the relative errors ranged from −8.5% to +9.8%, demonstrating the agreement of the methods. Moreover, the similarity of the results was checked by a statistical test, in this case, the paired *t*-test. Taking into account a confidence level of 95%, it was obtained values of $t_{experimental}$ below to the $t_{critical}$ value (3.2) in both cases (ACP: 3.1 and COD: 0.69). This fact proved that the results generated by both analytical methods were statistically equivalent in the considered confidence level. Thereby, the potentiality of the proposed electroanalytical method for the simultaneous ACP and COD determination in commercial pharmaceutical products became clear.

Table 2 Comparison of the results obtained by the proposed FIA-MPA method and a HPLC method for the simultaneous determination of ACP and COD in commercial pharmaceutical formulation samples (n = 3).

Sample	ACP			COD		
	HPLC	FIA-MPA	Error (%)[a]	HPLC	FIA-MPA	Error (%)[a]
J	576 ± 12	527 ± 7	−8.5	29.0 ± 0.4	27.8 ± 0.2	−4.1
K	525 ± 1	522 ± 6	−0.57	6.1 ± 0.1	6.7 ± 0.7	9.8
L	553 ± 8	507 ± 2	−8.3	26.6 ± 0.7	28.1 ± 0.5	5.6
M	541 ± 4	510 ± 3	−5.7	5.7 ± 0.1	5.9 ± 0.7	3.5

[a] Error (%) = [(FIA–MPA value − HPLC value)/HPLC value)] × 100.

Table 3 Simultaneous determination of ACP and COD in synthetic biological fluid samples (n = 3).

Sample	ACP			COD		
	Added (μmol L^{-1})	Found (μmol L^{-1})	Recovery (%)[b]	Added (μmol L^{-1})	(μmol L^{-1})	Recovery (%)[b]
Urine	2.00	1.84 ± 0.04	92.0	0.60	0.64 ± 0.04	107
	50.0	49.1 ± 0.6	98.2	5.0	5.1 ± 0.3	102
H. S.[a]	2.00	1.85 ± 0.04	92.5	0.60	0.65 ± 0.02	108
	50.0	49.7 ± 0.3	99.4	5.0	4.9 ± 0.4	98

[a] H.S. = Human serum.
[b] Recovery (%): (Found value/Added value) × 100.

Finally, the versatility of the FIA–MPA method in terms of application was complemented for the ACP and COD analysis in synthetic urine and human serum biological samples. In a previous analysis of these samples in the absence of the analytes (blank analysis), no peaks of potential interferents were detected. Thus, the biological samples were spiked with the appropriate amounts of both analytes and directly analyzed. The multiple pulse amperograms obtained for the construction of the analytical curves applied for the biological samples analyses, as well as the respective ACP and COD analytical curves, are shown in Fig. S4. The results of recovery percentages achieved for the analysis of these samples are given in Table 3. As can be seen, the recovery percentages were within the excellent range of 92.0–108%. From these results, one may conclude that the developed procedure did not suffer from interference from the biological synthetic sample matrices, which suggests the viability of the proposed procedure for analysis conducted in biological matrices environments. In addition, it was demonstrated the robustness of the proposed method from the application in the simultaneous determination of ACP and COD in a number of varied matrice samples.

4. Conclusions

We reported a set of results that sustained the proposal that the combination of a FIA–MPA system with a BDD electrode ensure the development of high performance electroanalytical methods, in this case, for the simultaneous determination of ACP and COD. Applying the FIA–MPA system excellent detectability, sensitivity and selectivity was achieved. The optimization of the experimental parameters yielded linear analytical curves in the range of 80 nmol L^{-1} to 0.1 mmol L^{-1} for ACP and 50 nmol L^{-1} to 10 μmol L^{-1} for COD, with limits of detection at nanomolar concentrations: 30 nmol L^{-1} for ACP and 35 nmol L^{-1} for COD, respectively. Moreover, an analytical frequency of 90 samples per hour was obtained. In addition, it was recorded exciting results for the practical applicability of the proposed FIA-MPA method toward the simultaneous determination of ACP and COD in three different matrice samples. The method described here is a potential alternative for the simultaneous determination of ACP and COD in different matrice samples.

Acknowledgments

This work was supported by the Brazilian funding agencies CNPq – Brazil [grant numbers 444150/2014-5 and 160658/2013-6], CAPES – Brazil, and FAPESP – Brazil.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.arabjc.2017.04.012.

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