Direct cefepime determination in human milk using solid mercury amalgam electrode manufactured with silver nanoparticles

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Keywords: Cefepime β-lactams Human milk Electroanalysis Silver solid amalgam electrode

ARTICLE INFO

Article history:
Received 28 March 2012
Received in revised form 5 June 2012
Accepted 19 June 2012
Available online 27 June 2012

1. Introduction

According Vyskocil and Barek, in a review paper [1], during the 1950s and 1960s, DC polarography (DCP) was one of the five most frequently used analytical techniques. However, with the progress of spectrometric and separation techniques – employed in the determination of a variety of organic compounds – DCP lost its importance. Resurgence of electroanalytical techniques was based on new possibilities in trace analysis from use of square-wave voltammetry (SWV) and differential pulse voltammetry (DPV) at hanging mercury drop electrode (HMDE) [1]. However, the toxicity of mercury limits the usage of the mercury electrodes in the analytical practice and excludes them from the out-of-laboratory applications [2].

The search for new simple electrochemical sensors for analysis and study of biomolecules represents an inseparable part of the development of bioelectrochemical methods and instrumentation. Recently, new types of metal solid amalgam electrodes (MeSAEs) for common voltammetric applications were suggested [3–6]. Silver solid amalgam electrodes (AgSAEs), introduced by Mikkelsen and Schroder and independently by Yosypchuk and Novotny, represent an intermediate between the mercury electrode and usual solid electrodes; it combines advantages of both [7]. In this context, the p-MeSAE is closely connected to electrodes based on amalgamated metals (Ag, Au, Pt, Ir, Cu). MeSAE can be easily polished using emery paper followed by wet alumina. They contain a relatively high content of metal (in the case of AgSAE it is 20% of Ag or even more) and they can be easily prepared by thorough mixing of corresponding aliquots of mercury and metal powder [8]. In this contribution, we show the scope and limitations of AgSAE on the example of voltammetric determination of cefepime antibiotic that belongs to the family of cephalosporins.

Cephalosporins are among the safest and the most effective broad-spectrum bactericidal antimicrobial agents and therefore, they are the most frequently prescribed class of antibiotics [9]. These are classified into four generations [10,11] based on their resistance towards β-lactamase degradation.

Cefepime, [2-aminothiazol-4-yl]-2-[Z]-[methoxy-iminoacetamido]-3-[methyl-1-pyrrolidino] methyl-ceph3-em4-carboxylic acid is a new injectable fourth-generation β-lactam cephalosporin with a positively charged quaternised N methyl-pyrollidine substitution at the 3 position of the cephem nucleus [12]. The drug is considered a broad spectrum and shows an excellent activity against Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter cloacae, Staphylococcus aureus and Estreptococos spp. [13], reaching a wide distribution into most body tissues and fluids and is excreted in human milk and urine [14,15], which at least 85% is excreted unchanged in human urine. This drug is mainly used for the treatment of lower respiratory infections, febrile neutropenic events, urinary tract infections skin soft tissue infections,
complicated intra-abdominal infections, gynecological infections, and sepsis; it also can be used for children with bacterial meningitis [16,17]. It was reported that both in elective and non-elective situations, use of prophylactic antibiotics prior to caesarean section significantly reduced the incidence of postpartum infectious morbidities [18]. Used above the therapeutic levels, these drugs can cause a wide variety of adverse effects and their fast analysis could have a significant impact in treatment and recovery of the patients.

Currently, several analytical methods for the quantitation of cefepime have been reported due to extensive use of drug in medical routine. The analytical methods most often used to determine of cefepime in biological materials from human origin are liquid chromatography with on-line microdialysis [19], high performance liquid chromatography [20] and spectrophotometry [21]. These methods give satisfactory sensitivity and selectivity but are rather time consuming and have complicated analysis and sample preparation steps.

There is a need for fast, straightforward methods for analysis of antibiotics and other residuals in human milk, and electroanalytical techniques have great potential to meet this demand, yielding results in a matter of minutes without the need for preliminary sample treatment and ensuring sensitivity comparable to HPLC methods. In our group, several papers have been published using the electroanalytical technique for the quantitative determination of organic compounds in different matrices, such as, milk [22,23] and bovine serum [24].

Surveying the literature revealed that there are two electrochemical methods for determination of cefepime, both related to Palacios and coworkers [25,26]. This literature is related to the adsorptive stripping voltammetric determination of cefepime at the mercury electrode in human urine and cerebrospinal fluid, and differential pulse polarographic determination in serum. However, nothing has been published concerning electrochemical determination of cefepime at silver solid amalgam electrode in human milk samples. Thus, the aim of this paper is the development of a fast and simple electroanalytical method for the direct determination of cefepime in human milk sample without any tedious pretreatment steps.

2. Experimental

2.1. Equipments

Electrochemical experiments were performed with an µAutolab TYPE II device (Eco Chemie) controlled by General Purpose Electrochemical System (GPES) software (Eco Chemie BV). A three-electrode system was employed, which was composed of a silver solid amalgam electrodes (AgSAEs) (area: 0.52 mm²), an Ag/AgCl as reference electrode and a platinum wire as auxiliary electrode. A ProStar chromatographic system (Varian) equipped with a ProStar 210 ternary pump and a ProStar 325 UV–Vis detector (Varian, Melbourne, Australia) was employed. Sample injection was performed through a Rheodyne injector valve (Varian, Cotati, CA, USA) with a 20-µL sample loop. The chromatographic analysis was performed using an OmniSpher C18 (250 mm × 4.6 mm, 5 µm) column (Varian, USA) maintained at 25 °C. Before use, the mobile phase was degassed and vacuum-filtered through 0.45-µm nylon membranes (Alltech, Belgium), and then pumped at a flow rate of 1.0 mL min⁻¹. Detection of analyte was performed at 254 nm.

Deionized water was produced by Milli-Q plus system (Millipore, USA).

2.2. Reagents and solutions

A sample of cefepime was kindly provided by Bristol-Myers-Squibb, Brazil and was used as received. All other chemicals were of suprapur or analytical reagent grade. Stock solutions of cefepime (1.0 × 10⁻³ mol L⁻¹) were prepared daily by dissolution of the solid substance in deionized water. The supporting electrolyte used was Britton–Robinson (BR) buffer solution, prepared by mixing a solution 0.04 mol L⁻¹ in orthophosphoric acid, 0.04 mol L⁻¹ in acetic acid and 0.04 mol L⁻¹ in boric acid with appropriate volumes of 0.2 mol L⁻¹ sodium hydroxide. Milk was obtained from healthy volunteers and kept at ~50 °C in sterile containers.

Mobile phase used was composed of two solvents: (A) aqueous solution of dibasic potassium hydrogen phosphate (10 mmol L⁻¹) at pH 7.0 adjusted with concentrated phosphoric acid and (B) methanol.

2.3. Preparation and activation of the electrode

The amalgamation was carried out after adjustment of the process previously published in the literature [8]. The silver solid amalgam electrodes (AgSAEs) were prepared by packaging silver powder in a thin glass capillary of internal diameter of about 0.5–1 cm. The electrical contact was made through a platinum wire (Ø 0.1 mm) inserted into the silver powder (particle size: 150 nm, purity: 99.9%, Sigma–Aldrich). The metal powder was then amalgamated by liquid mercury. Subsequently, the disc amalgamated resulting solid was polished manually with aqueous slurry of alumina powder (diameter of 0.01 mm). The surface was electrochemically activated in 0.2 mol L⁻¹ of KCl supporting solution by applying a potential of −2.2 V, while the solution was stirred. The AgSAE performance was tested registering voltammograms in the BR 0.04 mol L⁻¹ before and after addition the target analyte. For the AgSAE surface regeneration, was applied a potential of −2.2 V during 30 s while the electrode surface was stirred. The electrochemical activation was repeated after the amalgamation, before starting the work – or after pause longer than hours – or when occurs lose of electrode performance. This process removed possible oxides and/or reactants or products adsorbed on the electrode surface. Additionally, the charge transfer resistance became smaller and more suitable for analytical purposes [27].

2.4. Voltammetric procedure

Ten milliliter of the BR buffer solution was deoxygenated in the cell with nitrogen gas. After that, an aliquot of standard-electroactive-species solution (30 μL) was added to the electrochemical cell and the voltammograms were recorded. The experimental and voltammetric parameters were studied following a systematic analysis that affect the response, such as the pH of the buffer solution, the frequency (f) related to the total pulse duration, the pulse amplitude (a) and the step potential or scan increment (ΔEs). These parameters were optimized in relation to the maximum value of the peak current and the voltammetric resolution (half-peak width), which enhanced analytical applicability. All measurements were performed in triplicate and average was used to plot the calibration curve.

2.5. Chromatographic conditions

The chromatographic condition for the cefepime detection in human milk was performed after a simple adjustment of the previous work reported by Farthing and coworkers [28]. Thus, the HPLC system was used in a gradient elution mode with reversed-phase column OmniSpher C18 (250 mm × 4.6 mm, 5 µm, Varian, USA). The injection volume was 20 μL and the mobile phase flow rate was set at 1.0 mL min⁻¹. The mobile phase gradient profile was performed using, respectively, water (containing 10 mmol L⁻¹ of K₂HPO₄) and methanol according to the following proportions, 80:20 (v/v) between 0
and 3 min; decreasing to the 10:90 (v/v) and held for 2 min and reestablishing the initial condition (80:20, v/v) at 7 min. The mobile phase was degassed using an ultrasound system for 20 min.

Milk human samples were prepared by pipetting 150 μL of milk and 150 μL of acetonitrile into a polypropylene bullet centrifuge tube. Milk proteins were precipitated by vortexing for 1.0 min using a centrifuge (Fanen, Excelsa®, 280R, BRA). The vortexed samples were centrifuged (at 10,000 g) for 10 min at room temperature. The clear supernatant was transferred to syringe HPLC (Hamilton, Milford MA, USA) and injected into a loop of 20 μL for analysis.

2.6. Analysis of spiked milk samples

Milk samples of healthy individuals (after having obtained their written consent) were stored in refrigerator (−50 °C). After gentle, thawing, an aliquot volume of milk sample was spiked with cefepime – in concentration range between 3.06 × 10^{-6} and 6.06 × 10^{-6} mol L^{-1} – and left stirred for 5.0 min. These serial dilutions (samples were mixed with buffer solution at a ratio of about 1:10) were analyzed in the voltammetric cell containing BR buffer 0.04 mol L^{-1} (pH 2.5) as supporting electrolyte.

3. Results and discussion

3.1. Voltammetric studies of cefepime

Fig. 1 shows representative square-wave voltammograms for electrochemical reduction of 1.0 × 10^{-5} mol L^{-1} of the cefepime on the AgSAE in BR buffer pH 2.5. The voltammetric response, towards the negative sweep direction, is characterized to the presence of two well-defined peaks in BR buffer medium. The first one at peak potential (E_0) = −0.28 V (peak 1C) and the second at around E_0 = −0.45 V (peak 2C) vs Ag/AgCl (KCl 3.0 mol L^{-1}). This response is similar to that seen in previous works published using mercury electrode [25], but the reduction waves (peaks 1C and 2C) were displaced towards positive potentials (200 mV and 450 mV, respectively) when used the AgSAE. The less cathodic wave (peak 1C) is due to the reduction of the methoxymine group to methanol and the aminic cephalosporin derivative involving a complex process [29]. For analytical interest, we focused our study mainly on the reduction peak (named peak 2C).

The nature and acidity of the supporting buffer are some of the most important factors which strongly influence the stability of the analyte and its cathodic reduction as well as the adsorption processes [30]. Among the various investigated buffers (Britton–Robinson, acetate and phosphate) the best voltammetric signal in terms of sensitivity (peak height) and resolution (peak shape) have been available using Britton–Robinson buffer. The dependence of the pH on the peak current (i_p) for the buffer solution is shown in Fig. 2 (curve A). An increase in the peak current (i_p) (peak 2C) can be observed for pH values from 2.0 and 5.0, whereas the peak practically disappears at pH values greater than 5.0. This behavior confirms the presence of chemical reactions involving protons in the reduction process [31]. Since the pH is a variable that tends to strongly influence the shape of voltammograms, the best results with respect to enhancement, shape, and repeatability of the peak current were obtained with Britton–Robinson buffer (0.04 mol L^{-1}) at pH 2.5, thus constituting the most convenient pH value for further analytical purposes.

At the same time, the influence of pH on peak potential (E_0) can be seen in Fig. 2 (curve B). The shape and the position of the peak current were strongly pH dependent. The peak potential (peak 2C) is shifted to more negative values with increasing pH, indicating that protonation of the reactive part of the molecule is involved in the overall electrode reaction mechanism [32]. The E_0 exhibited also a linear relation with pH, which is expressed by the following equations: E_0 (V) = −0.25–0.089 × pH (r = 0.997) for 2.0 ≤ pH ≤ 3.0 and E_0 (V) = −0.25–0.077 pH (r = 0.995) for 3.5 ≤ pH ≤ 5.0, indicating cefepime reduction involves a protonated form in the investigated pH range.

The dependence of the peak current (peak 2C) on the accumulation potential (E_{acc}) was evaluated over the range of −0.45 to 0 V vs Ag/AgCl for electrochemical detection of 5.0 × 10^{-6} mol L^{-1} cefepime at accumulation time of 20 s (Fig. 3). The voltammetric response was highly influenced by the accumulation potential. The peak intensity was maximal when the accumulation potential was set between 0 and −0.15 V, reaching a maximum value at −0.05 V. At more negative values, a decrease in peak current was observed, indicating that the drug is no longer adsorbed on the AgSAE electrode. Therefore, the optimal accumulation potential was fixed at −0.05 V vs Ag/AgCl for all further experimental measurements.

The effect of accumulation time (from 0 to 120 s) on peak current intensity (Fig. 4) was investigated using square-wave...
volammetric technique for electrochemical reduction of $5.0 \times 10^{-6}$ mol L$^{-1}$ of cefepime. The peak current intensity decreased noticeably when increasing the accumulation time, indicating that long accumulation times are not favorable for the reduction process of cefepime on the electrode surface amalgam. Accumulation time shorter than 20 s—i.e. shorter than the time required for full saturation of the AgSAE electrode surface—was selected to evaluate the best working condition for the proposed methodology.

The detection of cefepime ($5.0 \times 10^{-6}$ mol L$^{-1}$) in BR buffer (pH 2.5) following preconcentration at $E_{acc} = -0.05$ V during 20 s was optimized after changing the pulse-amplitude ($\alpha$), scan increment ($\Delta E$), and frequency ($f$) within the range 10–100 mV, 2.0–16 mV, and 10–150 Hz, respectively. The peak current increased with the increase of pulse-amplitude; however a 50 mV value was chosen since at higher values there was peak distortion. At a constant pulse-amplitude ($\alpha$) of 50 mV and a frequency of 60 Hz, the peak current intensity increased linearly over the range of 2.0 and 12 mV for scan increment ($\Delta E$) study. Although current values rose linearly in this scan increment range, peak distortion was observed beyond 8.0 mV, resulting in poor definition of the peak current shape. Accordingly, a scan increment of 5.0 mV was preferable in the current study.

A variation in the frequency of application of pulse potential ($f$) usually exerts a marked effect on the response of SWV. Based on this aspect, the effects of frequency on the reduction process of cefepime on the AgSAE were evaluated for values from 10 to 150 Hz. We observed that an increase in the $f$ values was accompanied by an increase in the peak current (peak 2C). Additionally, no linear relationship was observed between the peak current and the frequency. Moreover, the peak potential ($E_p$) shifted to a more positive value (around 25 mV) with the increase in frequency. Therefore, 60 Hz was chosen as an optimal.

Several instrumental parameters, specifically those that directly affect the voltammetric response, were optimized for stirring rate and rest period. The peak current was significantly affected when varying the rest period because it was found that 25 s was sufficient for the formation of a uniform concentration of the reactant onto the AgSAE. Thus, a Britton–Robinson buffer (pH 2.5), preconcentration time ($t_{acc}$) = 20 s, accumulation potential ($E_{acc}$) = 5.0 mV, and pulse amplitude ($\alpha$) = 50 mV were used throughout the development of electroanalytical method.

3.2. Analytical curves

Calibration curves were constructed using the average peak currents obtained from recorded voltammograms at cefepime concentrations between $3.0 \times 10^{-7}$ and $2.1 \times 10^{-6}$ mol L$^{-1}$. The peak current increased linearly over the entire concentration range investigated (data not showed), according to the equation: $I_p$ (µA) = $-1.90 + 4.82 \times 10^6 C$ (mol L$^{-1}$), with $r = 0.997$ ($n = 7$). The limits of detection (LOD) and quantification (LOQ) were calculated using the statistic treatment 3 $\times$ SD/$b$ and 10 $\times$ SD/$b$, respectively, where SD is the standard deviation of then measurement peak current for the blank (measured at the potential of cefepime) and $m$ the slope of the analytical curve [33]. LOD and LOQ were $8.50 \times 10^{-8}$ and $2.80 \times 10^{-8}$ mol L$^{-1}$, respectively.

The satisfactory detectability of adsorptive voltammetry was accompanied by suitable repeatability. Evaluation of this analytical performance was based on 10 repeated measurements of the electrochemical signals of cefepime in a $6.0 \times 10^{-7}$ mol L$^{-1}$ pure solution and in the presence of milk. The precisions of the relative standard deviation were 0.83% and 2.80%, respectively.

3.3. Determination of cefepime in spiked milk samples

Drugs identification was performed according to peaks potential by comparison with standard solutions and using the standard addition method. Milk components differ across species, but can contain significant amounts of saturated casein, lipids, carbohydrate, and calcium, as well as vitamin C, all of which may interfere with the electrochemical detection of cefepime. In order to avoid a matrix effect, the standard addition method was applied, allowing cefepime to be determined in human milk samples without previous extraction. Blank sample of milk was spiked with cefepime to concentrations of $3.06 \times 10^{-7}$ and $6.06 \times 10^{-7}$ mol L$^{-1}$. The spiked sample was submitted to the procedures described in Section 2.6 and the concentration of the cefepime was measured (Table 1). Fig. 4 shows representative voltammograms for the milk sample spiked to a final concentration of cefepime ($3.06 \times 10^{-7}$ mol L$^{-1}$) upon successive additions of the standard compound. According to Fig. 4, there are no reduction peaks of compounds present in milk sample where the analytical peak appears (peak 2C). Consequently, the voltammetric signal increases after additions of standard solution, as shown in Fig. 4 (lines b–g), revealing an absence of matrix effects.
The recovery values of cefepime based on the average of four replicate measurements on two levels of concentrations $3.06 \times 10^{-7}$ and $6.06 \times 10^{-7}$ mol L$^{-1}$ was found as 98.00% and 100.5%, respectively (Table 1). These results demonstrate that the proposed method can be successfully employed for cefepime determination in human milk in a fast, simple manner, without any sample pretreatment.

3.4. Analysis of human milk sample using HPLC method

To achieve the chromatographic studies human milk samples were spiked at levels of $3.03 \times 10^{-7}$ and $6.07 \times 10^{-7}$ mol L$^{-1}$ with cefepime antibiotic. Such samples were then used for verification of the proposed electroanalytical method. A small difference was found between the mean recoveries involving the electroanalytical and the chromatographic methods. The average recovery was 92.40% and 97.20%, indicating that the extraction of the analyte in the chromatographic method may have contributed to the somewhat lower values in recoveries when compared with the proposed method (Table 1). Fig. 5 illustrates the chromatographic profiles obtained for spiked milk sample containing $3.03 \times 10^{-7}$ mol L$^{-1}$ of cefepime (Fig. 5, chromatogram A) and $3.03 \times 10^{-7}$ mol L$^{-1}$ of cefepime in absence of matrix (Fig. 5, chromatogram B). Thus, the HPLC method was satisfactorily for the determination of cefepime in human milk samples.

The obtained results, between both methods, were compared using the Student’s t test and F test. The results summarized in Table 1 reveal that the experimental values did not exceed the theoretical values, since the results were close to the labeled value, demonstrating that the proposed method is an acceptable alternative for analytical determination of cefepime in target sample.

### 4. Conclusions

The electrochemical reduction of cefepime onto AgSAE was studied using square-wave adsorptive cathodic stripping voltammetry in a fast and simple procedure. The methodology was applied for assay of human milk without sample pretreatment or time-consuming extraction steps prior to analysis of the drug. The comparison between the analytical data obtained for the determination of cefepime antibiotic using electroanalytical method and HPLC/UV–Vis led to very coherent results, indicating that the proposed electroanalytical method is an important tool to detect cefepime in small concentrations. Since all procedures were performed without the use of any organic solvents or hazardous chemicals which were detrimental to the environment and had a low consumption of reagents.

Finally, this work demonstrated that the AgSAE can be considered an environmentally friendly tool and a interesting alternative for the analytical determination of cefepime in human milk sample. In addition, the use of the standard addition method allowed high sample dilution, minimizing matrix effects.

### Acknowledgements

The authors gratefully acknowledge the financial support provided by CNPq, CAPES, FUNDECT and the generous gift by Bristol-Myers-Squibbl for the cefepime drug.

### References
