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Short communication

Electrochemical study of imipenem's primary metabolite at the mercury electrode Voltammetric determination in urine

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Abstract

Imipenem shows a fast chemical conversion to the more stable imin form (identical to that from biochemical dehydropeptidase degradation) in aqueous solutions that shows a wave at lower cathodic potential than the imipenem one.

The aim of this work is the study of the electrochemical behaviour of the primary metabolite of imipenem (M1) and the proposal of electrochemical methods for the determination of M1 in human urine samples. Electrochemical studies were realized in phosphate buffer solutions over pH range 2.0–8.0 using differential pulse polarography, dc-tast polarography, cyclic voltammetry and linear sweep voltammetry (staircase). In acidic media, a non-reversible diffusion-controlled reduction involving two electrons and two protons occurs and the mechanism for the reduction was suggested.

A differential pulse polarographic method for the determination of M1 in the concentration range 10^{-6} to 10^{-4} M with a detection limit of 4.5×10^{-7} M was proposed. Also, a method based on controlled adsorptive pre-concentration of M1 on the hanging mercury drop electrode (HMDE) followed by linear sweep voltammetry allows its determination in the concentration range 2×10^{-9} to 4×10^{-8} M with a detection limit of 1.05×10^{-9} M. The proposed methods have been used for the direct determination of M1 in spiked human urine and real human-derived urine with good results and should be appropiate for monitoring purposes.

Keywords: Imipenem metabolite; Differential pulse polarography; Linear sweep voltammetry; Adsorptive stripping voltammetry; Urine

1. Introduction

Imipenem [5*R*-[5α,6α(*R**)]]-6-(1-hydroxyethyl)-3-[[2-[(iminomethyl)amino]ethyl]thio]-7-oxo-1-azabicyclo[3.2.0] hept-2-ene-2-carboxylic acid (Fig. 1) is the *N*-formimidoyl derivative of thienamycin, the first member of a new class of beta-lactam antibiotics, the carbapenems, having a broad-spectrum activity against aerobic and anerobic bacteria. Because imipenem is hydrolysed by the renal brush border enzyme dehydropeptidase-I (DHP-I) [1], it is co-administered in clinical usage with cilastatin, a specific and highly active dehydropeptidase inhibitor, which improves the plasma concentration and markedly increases the urinary excretion of the unmetabolyzed drug [2].

The renal metabolism occurs by the cleavage of beta-lactam ring giving imipenemoic acid, the primary metabolite of imipenem (M1) (Fig. 1) [3]. A previous work has demonstrated that the enzymatically degraded antibiotic structure is the same to that obtained by deliberate chemical hydrolysis or by degradation on storage in aqueous solution [4]. A stability study of imipenem in aqueous solution was realized [5] and revealed that rate determining beta-lactam ring opening preceded the formation of several products whose structures were not clearly established.

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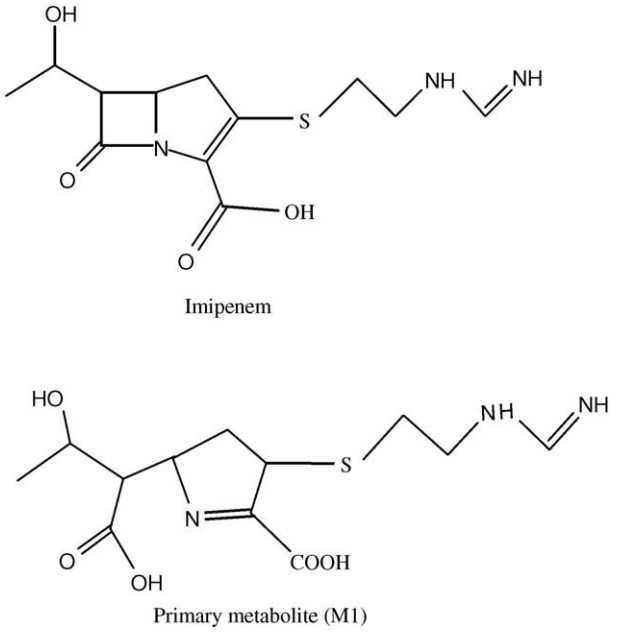

Fig. 1. Structures for imipenem and its primary metabolite (M1).

An excellent work on the imipenem's degradation products using ^{1}H RMN and ^{13}C RMN has been published [4] and the conversion mechanisms (chemical and biochemical) were proposed (Fig. 2) with a tautomeric equilibrium of M1.

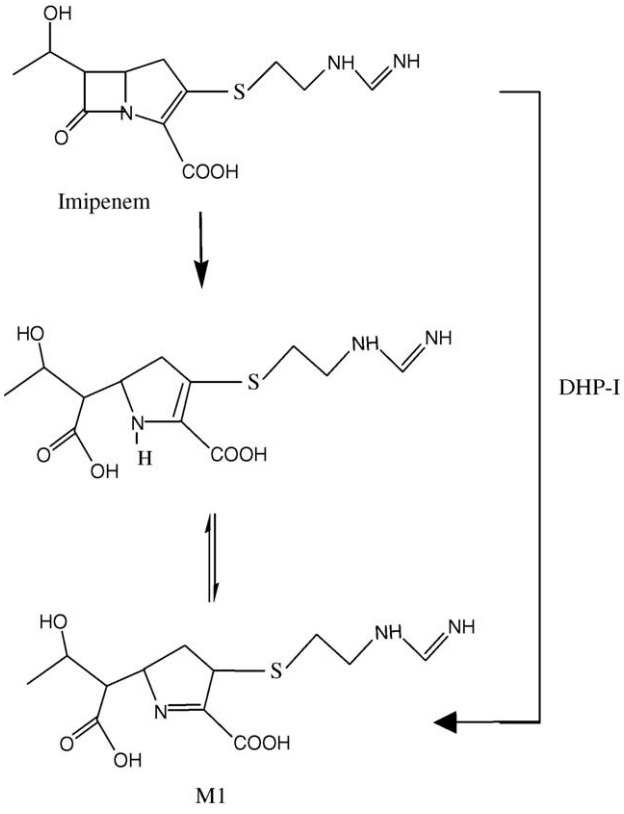

Fig. 2. Chemical and metabolic transformations of imipenem including M1 tautomeric rearrangement.

Numerous other minor products were detected by HPLC in the decomposition mixtures derived from imipenem. However, Carlucci et al. [6] have reported that the only metabolite from real urine samples was the parent drug, thienamycin. In the present work, authors would not have detected this compound but the M1 like the above-referred authors.

The degradation of imipenem's primary metabolite (M1) to a not-well-defined product that absorbs strongly at 308 nm has also been studied under different conditions [7]. This work also constitutes the unique reference found for the M1 determination (an ion-pair, reverse phase HPLC assay using postcolumn chemical rearrangement and UV detection); authors mention that M1 has no electrochemical properties to monitor. However, Bersier et al. [8] also described the fast chemical conversion of imipenem solutions to the imin form which is also electroactive and proposed the polarographic techniques as potential methods to determine carbapenem antibiotics.

Nowadays, no electrochemical procedures are available for the determination of imipenem or its primary metabolite (M1).

We have realized electrochemical studies of imipenem in order to propose determination procedures, but the fast conversion to M1 and the interference of the stabilizing substances commonly used for imipenem HPLC determinations avoided the proposal of any electrochemical procedure for its determination.

In this work, the electrochemical behaviour of the primary metabolite of imipenem (M1) has been studied, the reduction mechanism has been suggested and differential pulse polarographic and controlled adsorptive pre-concentration on the hanging mercury drop electrode followed by linear sweep voltammetry methods have been proposed and applied to the determination of M1 in human urine.

2. Materials and methods

2.1. Chemicals and reagents

Imipenem was kindly provided by Merck Sharp & Dohme, Spain. All the chemicals were of analytical-reagent grade and purchased from Merck (Darmstadt, Germany). High purity water was obtained from Millipore (Milford, MA, USA) Milli-Q Plus system. Working aqueous standard imipenem 10^{-3} M solution was freshly prepared daily and after its preparation, 2 h of storage at room temperature is necessary in order to warrant their complete conversion to M1. To adjust the pH of the solution, phosphate 0.1 M pH 2 and pH 7 buffers were used; suitable aliquots of 1 M H_3PO_4 and NaOH were added to adjust the pH at other values when necessary.

2.2. Apparatus

An ECO-Chemie PGSTAT (Eco Chemie B.V., Utrecht, The Netherlands) potentiostat–galvanostat was used in com-

bination with a Metrohm VA-663 polarographic stand fitted with a Hyundai 486/100 PC provided with the appropriate GPES (General Purpose Electrochemical Software) Version 4.2.

A three-electrode combination was used, consisting of a saturated KCl–Ag/AgCl reference electrode, a dropping-mercury electrode (DME) or hanging mercury drop electrode (HMDE) as working electrode and a platinum wire as auxiliary electrode. The temperature was fixed by using a double-walled polarographic cell and a Tetron (Barcelona, Spain) 3000543 thermo-regulator. Controlled-potential microcoulometry plots were used for evaluating the results. A Crison (Barcelona, Spain) Model 2002 pH-meter with a combined glass-calomel electrode was also used.

A HPLC equipment (Merck-Hitachi) consisted of a L-7100 pump, a Rheodyne (Cotati, CA, USA) model 7725i injection valve with a 20 μl sample loop, a 25 cm × 4 mm LichroCART RP-18 5 μμm column (Tecknokroma, Barcelona, Spain) and a model L-7455 diode array detector controlled by a Merck-Hitachi D-7000 interface equipped with a HPLC System Manager® software.

2.3. Determination of M1 by differential pulse polarography (DPP)

An aliquot containing 7.48–748.35 μg of M1 was placed into a 25 ml calibrated flask and 5 ml of buffer solution, pH 2, was added. The solution was diluted to the mark with water and mixed well. The solution was transferred into a polarographic cell. After deoxygenation for 10 min with a stream of pure nitrogen, the differential pulse polarogram was recorded from 0 to –1.0 V (pulse amplitude 50 mV, scan rate 5 mV s^{-1}). A calibration plot obtained with known concentrations of M1 was used to convert peak height into sample concentrations.

2.4. Determination of M1 by cathodic adsorptive stripping voltammetry (CAdSV)

An aliquot containing 0.30–11.97 μg of M1 was placed into a 25 ml calibrated flask, 5 ml of buffer solution, pH 7, was added and diluted to the mark with water. The solution was transferred into a polarographic cell and, after deoxygenation for 10 min with a stream of pure nitrogen, was measured from 0 to –1.0 V by linear sweep voltammetry (scan rate 300 mV s^{-1}) after a pre-concentration step consisting in the application of a potential of 0.0 V for 120 s and an equilibration time of 15 s.

2.5. Determination of M1 in human urine by DPP

Up to 2 ml of untreated urine containing between 374 μg and 37.4 mg of M1 was placed into a 25 ml volumetric flask and diluted with water to the mark. A 0.5 ml of this solution with 5 ml of pH 2 buffer solution were diluted with water to 25 ml into a volumetric flask. The polarograms were recorded according to the above recommended procedure. The voltammograms of samples without M1 do not show any signal that can interfere with the direct determination, so external calibration can be used.

2.6. Determination of M1 in human urine by CAdSV

Up to 1 ml of untreated urine containing between 15 and 300 μg of M1 was diluted with water to 25 ml in a volumetric flask; a 25 μl aliquot of this solution was transferred into a 25 ml calibrated flask, 5 ml of phosphate buffer pH 7.0 was added and diluted to the mark with water. The samples were measured according to the above described procedure. The voltammograms of samples without M1 do not show any signal that can interfere with the direct determination, so external calibration can be used.

2.7. Clinical study protocol

A dose of 1000 mg imipenem and cilastatin was administered to two volunteers. Urine samples were collected after 2 h from the injection and immediately submitted to the above described procedures in order to determine their M1 contents.

3. Results and discussion

3.1. Stability of imipenem and M1 aqueous solutions

In aqueous 10^{-3} M solutions, we have tested that imipenem changes completely to M1 in 2 h and remains stable for at least 24 h at room temperature. More diluted imipenem solutions at low pH values show a more fast conversion. For this reason, the stock solution were stored at least 2 h before been used with the aim to study the M1 form.

3.2. Reduction waves for M1

M1 is reduced on the DME, in acidic media, producing one cathodic wave with a half-wave potential ($E_{1/2}$) around –0.28 V (Fig. 3).The effect of pH on the dc-tast polarogram was investigated by recording the current–voltage curves at a drop time of 1 s and 5 mV s^{-1} scan rate for a 10^{-4} M aqueous M1 solution. A cathodic wave in the pH range 2.0–8.0 was observed. In Fig. 4, the plot of $E_{1/2}$ versus pH is shown; as can be seen, when the pH increases, the half peak potential shifted towards more negative values. The study of the influence of pH on the limit current (Fig. 5) was carried out with the aim to determine if protons are directly involved in the reduction process of M1. The limit current reaches a maximum around pH 2.0, so a pH of 2.0 was chosen for the determination of the primary metabolite of imipenem.

3.3. Effect of operating parameters

The drop time was ranged from 0.5 to 2 s and an increase in the intensity of the peak with the drop time was observed.

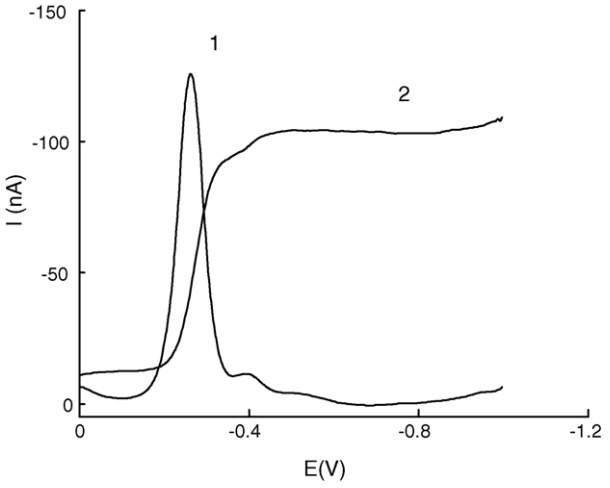

Fig. 3. Polarograms of 10^{-4} M M1 aqueous solution at pH 2.0 (1: DPP, 2: dc-tast).

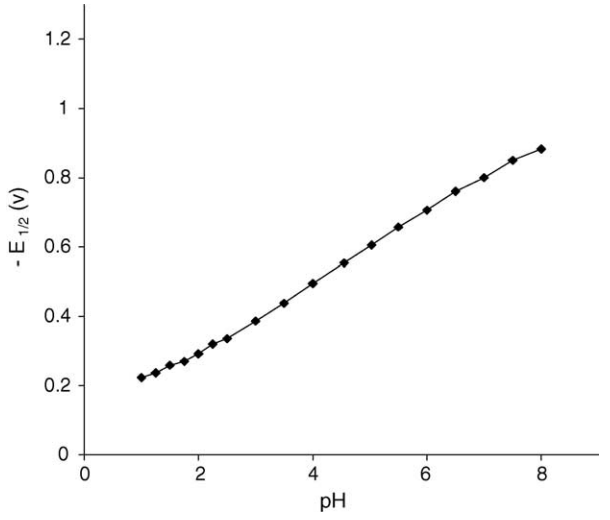

Fig. 4. Effect of pH on $E_{1/2}$ for dc-tast polarography of 10^{-4} M M1 aqueous solution.

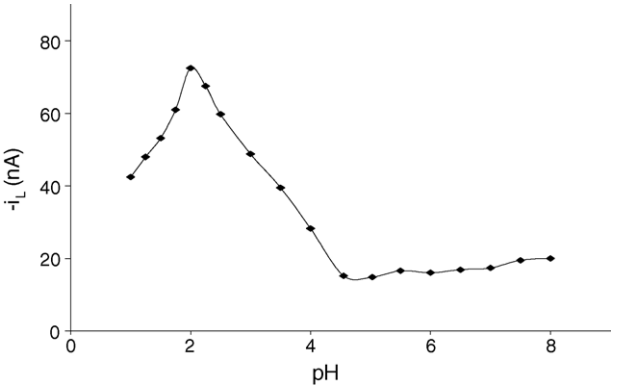

Fig. 5. Effect of pH on the limit current for dc-tast polarography of 10^{-4} M M1 aqueous solution.

Table 1
Variation of peak intensity as a function of modulation amplitude

$-\Delta E$ (mV)	$-i_p$ (nA)	$-E_p$ (V)
10	16.25	0.312
20	39.19	0.307
30	64.83	0.297
40	93	0.287
50	120	0.282
60	147.9	0.272
70	174.5	0.267
80	192.6	0.262
90	203.4	0.252
100	205.8	0.242
110	204	0.231

Plotting the intensity of the peak obtained by DPP versus $t^{2/3}$, a linear relationship was observed with a correlation coefficient of 0.993.

The peak height increases linearly when pulse amplitude ranges from 10 to 80 mV; however, the peak potential was displaced towards more positive values (Table 1). The effect of the temperature on the electrochemical behaviour of M1 was tested by DPP and dc-tast polarography between 20 and 60 °C; in both the instances, a linear relationship between temperature and the current intensity was observed. For DPP, a temperature coefficient of 1.29% was obtained. For dc-tast polarography, the value of 1.016% for the temperature coefficient confirms that the processes are controlled mainly by diffusion; however, the variation of i_L with the pH suggests, likewise, a ligth kinetic contribution due to the acid–base characteristics of M1 [9].

3.4. Number of electrons transferred, reversibility of the system and mechanism of reduction

Controlled-potential microcoulometry was used to determine *n*, the number of electrons transferred in the reduction process. This study was done at two pH values, 3.0 and 5.0, giving two electrons ($n = 2$), indicating one double bond reduction hypothesis.

The plot of $\log[i/(i_d - i)]$ versus potential (i_d = diffusion current) for dc-tast polarography shows a linear relationship with a slope of 53.3 ± 1.9 mV; $E_{3/4}$–$E_{1/4}$ are between 40 and 60 mV over the pH range studied. Both data indicate that the reduction of M1 is not a reversible process.

In linear sweep cyclic voltammetry, M1 yields one well-defined reduction wave but the oxidation wave is not observed (Fig. 6) which is a characteristic of the irreversible process. The reduction wave is displaced to more negative potentials when the scan rate (ν) increases. Plotting i_p versus $\nu^{1/2}$, a linear relationship (correlation coefficient 0.998) is observed which indicates that the reduction of M1 is a diffusion-controlled electrode processes.

The electron transfer coefficient (αn_a) and the number of protons (*p*) corresponding to the rate-determining step in the reduction process were calculated at different pH values. In the pH range 3–7, αn_a was found to be

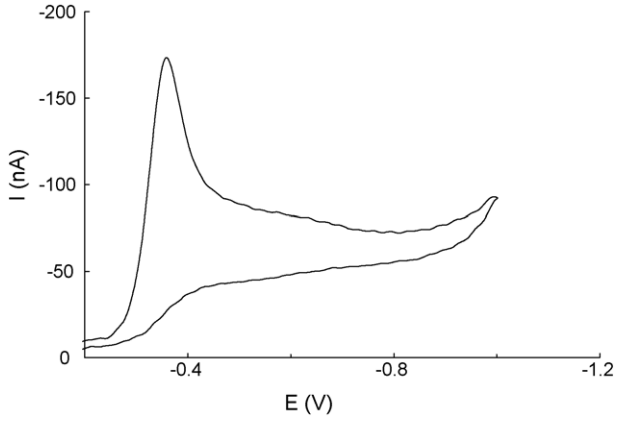

Fig. 6. Current vs. potential for linear sweep cyclic voltametry of 10^{-4} M M1 aqueous solution (deposition time 30 s).

1.15 ± 0.01 and $p = 2.12$, value obtained from the expression $\Delta E_{1/2}/\Delta\text{pH} = 0.059p/\alpha n_a$.

From the results obtained and on the basis of M1 which shows a wave at lower cathodic potential than the imipenem one, the mechanism of reduction for the metabolite can be assigned to the direct reduction of the double bond C=N of the imin group, which involves two electrons and two protons (Fig. 7).

3.5. DPP: variation of peak intensity with the concentration of M1

A linear relationship between the peak height and the concentration of M1 was obtained in the range from 10^{-6} to 10^{-4} M. The measurement of 12 solutions with concentration values within the range for triplicate allows the next expression:

$$-\Delta i_{max} = (0.928 \pm 0.337) + (0.935 \pm 0.007)C \quad (r = 0.9997)$$

where $-\Delta i_{max}$ is given in nA and C in μmol l^{-1}.

According to the Analytical Methods Committee [10], the detection limit (LOD) is the concentration of M1 corresponding to a signal equal to the blank mean (y_B) plus three times the standard deviation (S.D.) of the blank (s_B); from 11 blank measurements, a detection limit of 4.5×10^{-7} M was obtained. From the calibration straight line, it is also possible to estimate the quantitation limit as the concentration corresponding to the ratio between 10 times the S.D. of the intercept and the slope of the calibration line [10], this leads to a quantitation limit of 10^{-6} M. In order to check the precision of the method [11,12], 10^{-6}, 10^{-5} and 10^{-4} M solutions of M1 were prepared and measured in quadruplicated obtaining a repeatibility (intra-assay precision) of 3.1, 2.6 and 2.8% for the relative standard deviation (R.S.D.), respectively; intermediate precision obtained measuring within a week 10^{-5} M solutions was 3.2%.

The parameters for using differential pulse polarographic as a suitable method to determine M1 are given in Table 2.

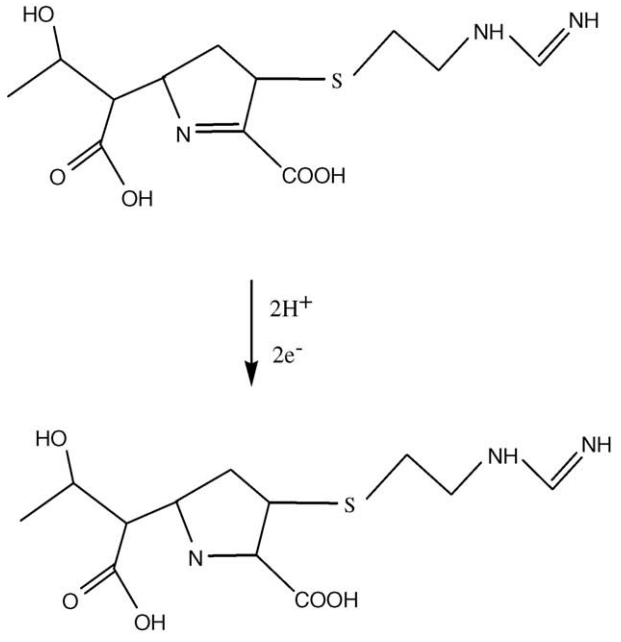

Fig. 7. Proposed reduction mechanism of M1 on hanging mercury drop electrode.

Table 2
Optimization of variables for analytical purposes (DPP)

Initial potential (V)	0
Modulation amplitude (mV)	50
Scan rate (mV s^{-1})	5
Scan direction	Negative
Current range (nA)	1–500
Drop time (s)	1
Buffer concentration (mol l^{-1})	0.1
pH	2
Purge time (min)	10

3.6. Cathodic adsorptive stripping voltammetry of M1 (CAdSV)

The electrochemical studies with HMDE, using linear sweep voltammetry (recorded in the negative direction from 0.0 to –1.0 V), carried out indicates that an adsorption process occurs on the mercury electrode surface which can be used as an effective pre-concentration step prior to voltammetric measurement. An exhaustive study of the dependence of adsorptive peak currents on accumulation potential and accumulation time, equilibration time, scan rate and pH was performed using 10^{-8} M M1 solutions. Voltammograms were recorded at different pH values and a maximum intensity for pH 7 was obtained (Table 3); at this pH, M1 yields a well-defined peak at around – 0.38 V.

The influence of the deposition potential on the peak height shows that M1 gives a strong increase of adsorption at potentials higher than –0.1 V; a deposition potential of 0 V was used as the accumulation potential for all the measure-

Table 3
Effect of pH on the peak height (i_p) by linear sweep voltammetry of 10^{-8} M M1 with a scan rate of 0.3 V s^{-1}

pH	$-i_p$ (nA)	$-E_p$ (V)
6	12.23	0.378
6.3	19.31	0.381
6.5	25.17	0.383
6.8	38.45	0.386
7	55.45	0.391
7.5	36.58	0.394
8	5.23	0.403

ments. The influence of accumulation time on the peak current was studied in the 10^{-10} to 4×10^{-8} M range. The current versus deposition time plots appears in Fig. 8. An initial linear relationship was observed up to around 240 s. The influence of the calibration time on the peak height was studied in the range 10–40 s and no dependence between both the magnitudes was found, a 15 s equilibration time was chosen for all the measurements.

The effect of scan rate (ν) on the peak current and peak potential was evaluated for the adsorbed M1. The plots of log i_p versus log ν (Fig. 9) and $-E_p$ versus log ν (Fig. 10) show linear relationships with correlation coefficients of 0.997 and 0.996, respectively.

A linear relationship of adsorption holds between the peak current and the concentration of M1 in the range from 10^{-9} to 4×10^{-8} M with a good precision and accuracy. The triplicate measurements of eight solutions with concentration values within this range follow the expression:

$$-\Delta i_{max} = (36.84 \pm 0.28) + (1.68 \pm 0.0.017)C$$
$$(r = 0.9991)$$

where $-\Delta i_{max}$ is given in nA and C in 10^{-9} M.

Using the expressions previously described in the above section, the LOD calculated was 1.05×10^{-9} M and the quantitation limit was 3.62×10^{-9} M. In order to check the precision of the method [11,12], 5×10^{-9}, 10^{-8} and 4×10^{-8} M solutions of M1 were prepared and measured in quadruplicate, with 120 s deposition time, obtaining a repeatibility (intra-assay precision) of 3.9, 4.2 and 4.6% for the R.S.D., respectively; intermediate precision obtained measuring within a week 10^{-8} M solutions was 5.3%.

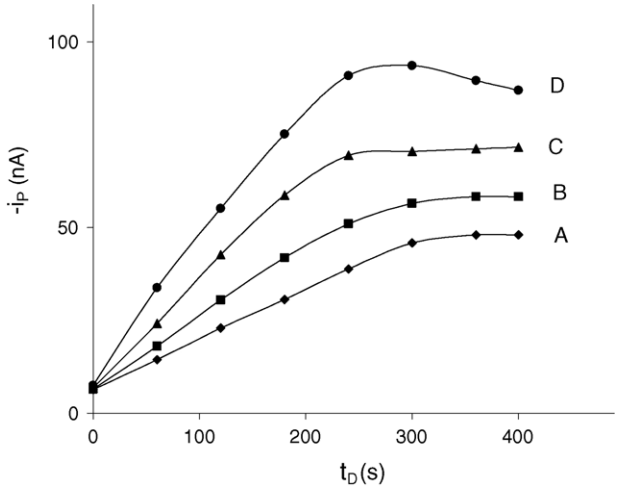

Fig. 8. Peak current vs. deposition time for different concentrations of M1 solutions at pH 7.0. Scan rate 0.3 V s^{-1} (A) 10^{-10} M; (B) 10^{-9} M; (C) 6×10^{-9} M; and (D) 10^{-8} M).

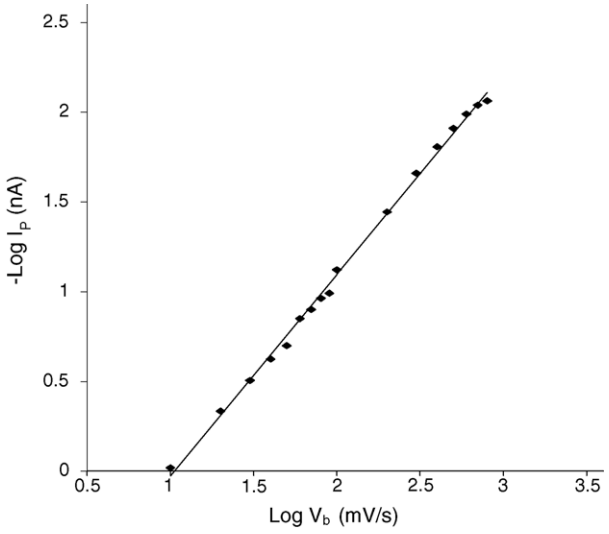

Fig. 9. Peak current vs. scan rate (10^{-8} M M1 at pH 7.0).

3.7. Analysis of urine samples

Previously to the M1 determinations in urine samples, due to its 1:1 co-administration, the possible interference of cilastatin was checked. Both DPP and CAdSV procedures were checked with cilastatin amounts up to 10-fold the corresponding M1 amounts and no interference were observed.

The possible interference of imipenem must be considered in two ways. Imipenem shows a more cathodic wave, as can

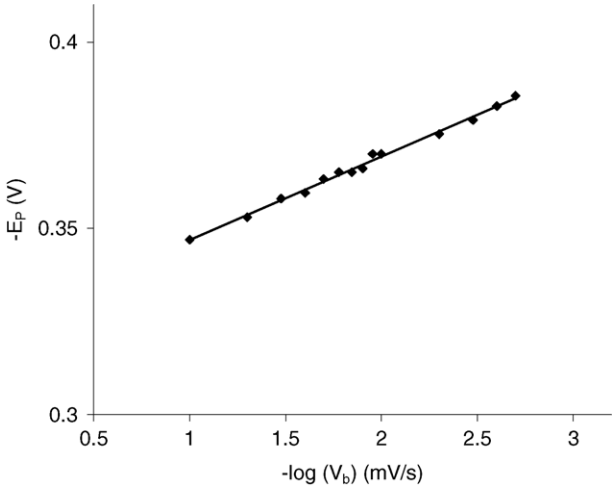

Fig. 10. Peak potential vs. scan rate (10^{-8} M M1 at pH 7.0).

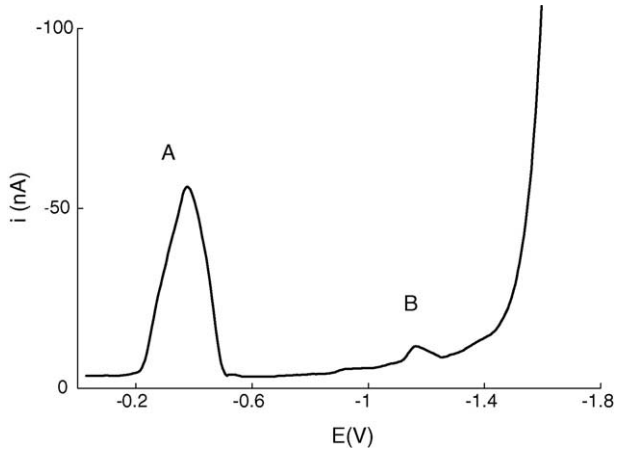

Fig. 11. Voltammograms corresponding to the reduction of an aqueous mixture of (A) M1 and (B) imipenem.

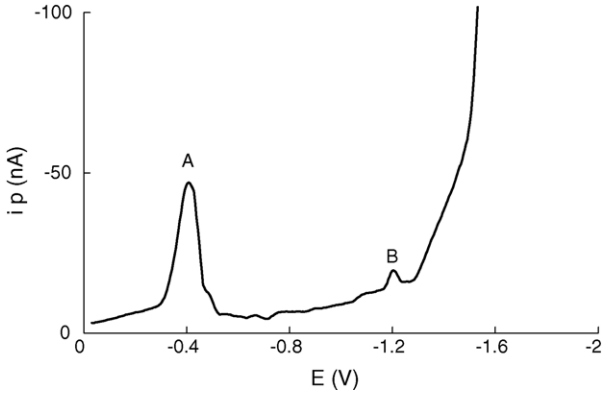

Fig. 12. Voltammograms corresponding to the reduction of real human urine containing (A) M1 and (B) imipenem.

Table 4
M1 recoveries from spiked urine samples

Spiked sample (μg/ml)	DPP		CAdSV	
	Content[a] (μg/ml)	*t*-test	Content[a] (μg/ml)	*t*-test
200	205 ± 6	1.44	203 ± 8	0.65
300	295 ± 9	0.96	305 ± 10	0.87
400	385 ± 13	1.99	391 ± 16	0.87
		2.78[b]		2.78[b]

[a] Average of three determinations ± S.D.
[b] Critical values for t ($p = 0.05$).

be seen in Fig. 11, which does not interfere with the M1 reduction wave. On the other hand, imipenem shows a major stability in urine than in aqueous solutions which permits the M1 determination without imipenem interference due to its hydrolysis. Stability studies of imipenem in urine shows that no significative hydrolysis occurs within 15 h at room temperature.

Urine blank samples were spiked with M1 to obtain 200, 300 and 400 μg/ml concentrations and these spiked samples were submitted, in triplicate, to the proposed procedures [11,12] and the M1 concentrations determined. The recoveries obtained are shown in Table 4. The accuracy of the proposed methods was statistically proved according to the *t*-test for the comparison of an experimental mean with a known value [13]. As can be seen, the recoveries obtained show an adequate accuracy for both the methods.

3.8. Human study

Real human urine samples obtained from two volunteers, as has been previously described in Section 2.7, were submitted to the described procedures and measured by DPP and CAdSV. The results obtained are shown in Table 5.

Fig. 12 shows the reduction waves for (A) M1 and (B) imipenem; as can be seen, like in aqueous solutions, the more cathodic wave for imipenem does not interfere in the M1 determination.

Urine samples were also measured by a HPLC procedure [7] as another accuracy determination for the proposed methods [11,12]. Table 5 shows the results obtained. As can be seen, good agreement was found between the HPLC and the two proposed methods, statistically proved according to the *t*-test for comparison of two experimental means and two-tailed *F*-test, indicates that the two proposed methods are accurate (null hypothesis accepted) [13].

The detection of M1 as the major metabolite of imipenem is according to previous works [4,5,7], widely described in Section 1.

Table 5
M1 contents for the analyzed real urine samples for both the proposed methods and compared with another HPLC method (for the statistical data treatment see text)

Sample	DDP			CAdSV			HPLC
	Content[a] (μg/ml)	*F*-test	*t*-test	Content[a] (μg/ml)	*F*-test	*t*-test	Content[a] (μg/ml)
1	370 ± 11	3.36	0.07	363 ± 14	5.44	0.13	375 ± 6
2	183 ± 8	1.78	0.32	173 ± 7	1.36	0.09	170 ± 6
		39.00[b]	2.78[b]		39.00[b]	2.78[b]	

[a] Average of three determinations ± S.D.
[b] Critical values for F and t ($p = 0.05$).

4. Conclusions

The electrochemical reduction of the primary metabolite of imipenem (M1) under the conditions described in this work is an irreversible process controlled by diffusion. The proposed reduction mechanism involves two electrons transferred. Also, an adsorption process of the metabolite occurs on the mercury electrode surface which can be used as an effective pre-concentration step prior to the voltammetric measurement.

The results obtained show that the proposed methods may be useful to determine M1 in human urine at the levels obtained after the administration of normal clinical doses and they would be the methods of choice for monitoring this substance in patients.

The proposed procedures can be alternative methods to the chromatographic one for the analysis of M1 and they are easy, inexpensive and also they do not require the use of organic solvents. Also, the CAdSV procedure alows the determination of concentrations lower than the chromatographic procedure.

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