Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 923–933
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Flow injection and HPLC determination of furosemide using pulsed amperometric detection at microelectrodes

Alicia Guzmán, Lourdes Agüí, María Pedrero, Paloma Yáñez-Sedeño, José M. Pingarrón *

Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, 28040 Madrid, Spain

Received 12 March 2003; received in revised form 14 July 2003; accepted 15 July 2003

Abstract

The flow-injection and HPLC determination of the diuretic drug furosemide using pulsed amperometric detection (PAD) at cylindrical carbon fibre microelectrodes (CFMEs) is reported. Experimental conditions such as pH (6.5) and buffer concentration (0.05 mol l^{-1} $HPO_4^{2-}/H_2PO_4^-$) were optimized using square-wave voltammetry (SWV). Repetitive flow-injection amperometric measurements at +1.25 V for furosemide showed a continuous decrease in the peak current, probably as a consequence of the microelectrode surface fouling. However, a suitable amperometric detection of furosemide was achieved using a PAD program consisting of a two-step potential waveform with alternating anodic and cathodic polarization. The anodic (detection) potential was +1.25 V (time of application 0.1 s), and the cathodic (cleaning) potential was −0.20 V (t = 0.2 s). A linear calibration graph was obtained for furosemide in the 5.0×10^{-7}–1.0×10^{-4} mol l^{-1} concentration range, with a limit of detection of 1.7×10^{-7} mol l^{-1}. HPLC-PAD at carbon fibre microelectrodes was used for the determination of furosemide in the presence of several thiouracil drugs and oxytetracycline (OTC). The mobile phase selected was a 25:75 acetonitrile:5.0×10^{-3} mol l^{-1} NaH_2PO_4 (pH 5.0) mixture. A linear calibration graph was obtained for furosemide in the 1–100 μM range, with a limit of detection of 0.55 μM. The usefulness of this method for the determination of furosemide in real samples was evaluated by performing the analysis of commercial milk samples spiked with furosemide at a concentration level of 4.5×10^{-7} mol l^{-1} (150 ng ml^{-1}), as well as with other thiouracil drugs and OTC. A mean recovery of 95±5% furosemide was obtained.

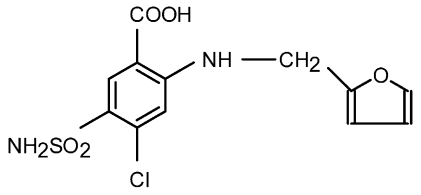
Keywords: Furosemide; Pulsed amperometric detection; Microelectrodes; Milk

1. Introduction

Furosemide (4-chloro-*N*-furfuryl-5-sulfamoyl-anthranillic acid, see Scheme 1) is a powerful, short acting diuretic used for diverse treatments in

* Corresponding author. Tel.: +34-91-394-4315; fax: +34-91-394-4329.
E-mail address: pingarro@quim.ucm.es (J.M. Pingarrón).

0731-7085/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00422-9



Scheme 1.

humans, as well as in veterinary medicine. Because it can be administered to dairy cattle for the treatment of the mammary gland edema, harmful residue concentrations can be found in milk for human consumption [1]. Most of the described methods in the literature for the determination of furosemide employ LC with UV or fluorimetric detection [2–7], and they have been applied only to the determination of this diuretic in urine, serum or plasma and pharmaceuticals. Only one application has been found for the determination of furosemide in bovine milk [8]. Also HPLC with mass spectrometry detection has been used for the determination and characterization of diuretics in human urine [9], as well as for the analysis of furosemide and its use in pharmacokinetic studies [10]. Moreover, a rapid analysis of furosemide in human urine by capillary electrophoresis with laser-induced fluorescence and electrospray ionization-ion trap mass spectrometric detection has been recently reported [11]. On the other hand, electrochemical detection has been very scarcely applied, probably due to the adsorption of this compound on the surface of carbon electrodes [12]. However, an HPLC method with amperometric detection at a glassy carbon electrode using a detection potential of +1.20 V versus Ag/AgCl, and electrochemical or chemical pretreatment of the electrode surface has been reported [13]. This method was applied to the determination of furosemide in pharmaceuticals and urine. Moreover, an improved electrochemical detection of furosemide and other diuretics based on postcolumn online photolysis, and applying a detection potential of +0.20 V versus Pd has been also described [14].

In this work, pulsed amperometric detection (PAD) at cylindrical carbon fibre microelectrodes (CFMEs) has been employed for the determination of furosemide. PAD has greatly enhanced the scope of electrochemical detection under flowing conditions by coupling the process of anodic detection with electrochemical cleaning steps, thus assuring a continuously cleaned and active electrode surface [15]. Moreover, microelectrodes are specially well suited for continuous electrochemical monitoring in flow-injection analysis, liquid chromatography or capillary electrophoresis [15,16], because they generally allow an improvement of the signal-to-noise ratio with respect to that obtained at conventional size electrodes. Furthermore, fast responses, minimally influenced by changes in the flow rate are usually obtained. Among the different types of microelectrodes used for electrochemical detection, cylindrical CFMEs have largely demonstrated their usefulness in several applications, mainly related with the LC determination of important biological compounds [17–19]. Therefore, all these advantages have been joined in this work to develop an improved amperometric detection scheme of furosemide at CFMEs in flowing systems by applying to the microelectrode a simple PAD program. Moreover, an HPLC-PAD method for the determination of this diuretic in the presence of other drugs, which can be present in animal food samples, was also developed. A flow-cell specially adapted for the work with CFMEs has been used for the first-time under HPLC conditions. The cell design was previously reported for the determination of antithyroid drugs by flow injection with amperometric detection [20], and allows the use of microelectrodes of variable lengths which permits the obtention of good signal-to-noise ratios as well as stable and rapidly reachable baselines. Moreover, PAD has been scarcely employed with microelectrodes in liquid chromatography. In fact, only one paper has been found in the literature on this subject, in which the determination of sulfide compounds at a gold microelectrode of 138 μm diameter was reported [21]. However, no previous reports are available on PAD at CFMEs.

2. Experimental

2.1. Apparatus

Voltammetric and flow injection measurements were carried out with an Autolab (Eco Chemie B.V.) PGSTAT 12 potentiostat provided with an ECD low current module. The electrochemical software used was the general purpose electrochemical system (GPES) (Eco Chemie B.V.). The flow-injection arrangement consisted on a Gilson Minipuls-3 peristaltic pump and an Omnifit 1106 valve with variable injection volumes.

Chromatographic experiments were performed using a Jasco PU-980 HPLC pump and a Rheodyne 7725i valve provided with a 20 μl coil which was connected to a Kromasil C_{18} column (150 × 4.6 mm i.d., 5 μm particle size) (Scharlab). Electrochemical detection was carried out by means of a BAS Epsilon Multichannel detector, and the CHROMGRAPH 2.0.01 software (Liquid Chromatography Control Software) from BAS was used to record data.

2.2. Electrodes and electrochemical cells

Cylindrical microelectrodes prepared from single carbon fibres (Union Carbide Corp., Danbury, CT, USA, 8 μm o.d.), pretreated as described previously [22], were used as working electrodes. A BAS MF 2052 Ag/AgCl reference electrode, and a Pt wire auxiliary electrode, were used for voltammetric experiments, as well as BAS VC-2 × 10 ml and home-made 1 ml electrochemical cells.

The flow-cell used, specially adapted for the work with cylindrical microelectrodes, has been described in a previous paper [20], and consisted of a home-made methacrylate (flow-injection) or Teflon (LC) block provided with a 2-mm diameter flow channel where the microelectrode is inserted. The designed cell allowed the flow solution to pass through the whole microelectrode surface. A BAS MW 2030 Ag/AgCl reference electrode was also inserted into the flow cell, and a steel flow outlet tube acted as the auxiliary electrode.

2.3. Reagents and solutions

Stock 1.0×10^{-2} mol l^{-1} furosemide (Sigma, 99%) solutions were prepared in acetonitrile (SDS, HPLC reagent grade). Stock 1.0×10^{-3} mol l^{-1} oxytetracycline (OTC; Sigma, min 95%), 1.0×10^{-2} mol l^{-1} methylthiouracil (MTU; Aldrich, 95%), 1.0×10^{-3} mol l^{-1} propylthiouracil (PTU; Sigma, 95%) and 1.0×10^{-3} mol l^{-1} phenylthiouracil (PhTU; Sigma, min 95%) solutions were also prepared in acetonitrile (OTC), and in methanol (SDS, HPLC reagent grade) (all the others). Working solutions were prepared from these by suitable dilution with acetonitrile or with distilled water. Tetraethylammonium perchlorate (TEAP) (Fluka, 99%), tetrabutylammonium tetrafluoroborate ($TBABF_4$) (Aldrich) and phosphate buffer of pH 6.5 were used as the supporting electrolytes in acetonitrile, ethyl acetate and aqueous media, respectively. A 0.2 mol l^{-1} Britton–Robinson buffer was also used. Other solvents and chemicals used were of analytical reagent grade and water was obtained from a Millipore Milli-Q purification system.

2.4. Procedures

2.4.1. Flow injection with pulsed amperometric detection

Flow injection measurements were performed using a 0.05 mol l^{-1} $HPO_4^{2-}/H_2PO_4^-$ buffer solution of pH 6.5 as the carrier at a flow rate of 1.0 ml min^{-1}. PAD was accomplished using a detection potential, E_1, of +1.25 V ($t_1 = 0.1$ s), and a cleaning potential, E_2, of −0.2 V ($t_2 = 0.2$ s).

2.4.2. HPLC with pulsed amperometric detection

Chromatographic separation of mixtures of furosemide, MTU, PTU, PhTU and OTC was performed using an acetonitrile–water (25/75) mixture containing 5.0×10^{-3} mol l^{-1} NaH_2PO_4, as the mobile phase, which was previously filtered through a 0.1 μm membrane. A flow rate of 1.4 ml min^{-1} and an injection volume of 20 μl were used. PAD was employed with the same experimental conditions mentioned above.

2.4.3. Determination of furosemide in milk samples

24.0 ml of skimmed milk purchased in a local supermarket was spiked with furosemide, MTU, PTU, PhTU and OTC at concentration levels of 150 ng ml^{-1} (furosemide), 65 ng ml^{-1} (MTU), 77 ng ml^{-1} (PTU), 93 ng ml^{-1} (PhTU) and 225 ng ml^{-1} (OTC). 56 ml of ethyl acetate were then added and the mixture was shaken for 10–15 s. Separation of phases was allowed for 5 min, and 50 ml of the ethyl acetate extract were evaporated to dryness under a nitrogen gas stream. Then, the residue was dissolved with 5 ml of the mobile phase (the theoretical furosemide concentration in this solution was of 2.0×10^{-6} mol l^{-1}), and 20 μl of this solution were injected in the LC column. The determination of furosemide was carried out by applying the standard additions method using PAD under the same conditions mentioned above.

3. Results and discussion

3.1. Voltammetry at cylindrical CFMEs

Voltammetry of furosemide at cylindrical CFMEs was tested both in aqueous and nonaqueous media. Net square-wave voltammetry (SWV) was used as the voltammetric technique because of its high sensitivity and good performance when employed with CFMEs [20]. Fig. 1 shows net SW voltammograms for 1.0×10^{-4} mol l^{-1} furosemide in acetonitrile containing 0.05 mol l^{-1} TEAP as supporting electrolyte (a), ethyl acetate using 0.05 mol l^{-1} $TBABF_4$ (b), and 0.05 mol l^{-1} phosphate buffer aqueous solution of pH 7.0 (c). The organic solvents were chosen because they can be used for the extraction of furosemide from samples, and the supporting electrolytes were those selected previously for working with CFMEs in these solvents [22,23]. As can be observed, a well-defined sharp SW oxidation peak at approximately +1.6 V appeared in acetonitrile. This voltammetric response exhibited a higher current density (peak current-to-microelectrode surface area) than that obtained in ethyl acetate, and, consequently, acetonitrile would be the solvent of choice to carry out direct voltammetric analysis of the diuretic drug in the solvent extracts from pharmaceutical or biological samples, according to similar analytical methodologies described previously by our group [20,23].

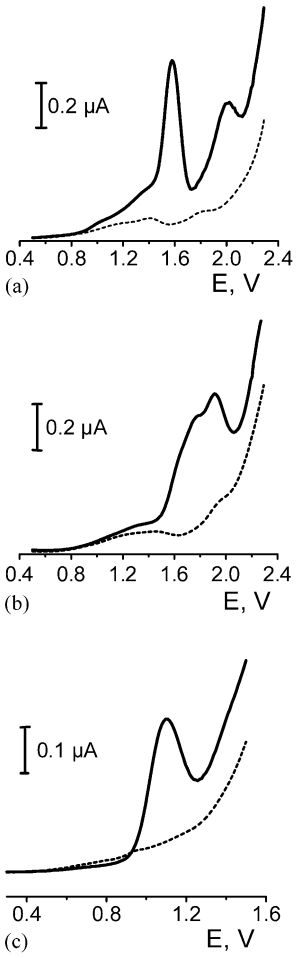


Fig. 1. Square wave voltammograms of 1.0×10^{-4} mol l^{-1} furosemide at CFMEs in acetonitrile–0.05 mol l^{-1} TEAP (a), ethyl acetate–0.05 mol l^{-1} $TBABF_4$ (b), 0.05 mol l^{-1} phosphate buffer solution of pH 7.0 (c). $E_{sw} = 50$ mV; $\Delta E_s = 10$ mV; $f = 25$ Hz.

Regarding aqueous media, a similar voltammogram to that shown in Fig. 1c was also obtained in a 0.05 mol l^{-1} Britton–Robinson buffer medium. A well-defined SW oxidation peak appeared at + 1.05 V, with a lower peak current than that of the voltammogram observed in acetonitrile. Despite this lower sensitivity, this aqueous medium was selected for further work because it is more adequate for a further HPLC application.

Using a 0.05 mol l^{-1} Britton–Robinson buffer as supporting electrolyte, the effect of pH on the furosemide SW oxidation wave was examined over the 1.2–11.0 range (Fig. 2). The net current varied only slightly with pH over the whole range, whereas the peak potential (E_p) decreased as pH was increased. The changes in the slopes of the E_p versus pH plot are related with the dissociation acidity constants of furosemide, involving the imine, carboxylic and sulfonamide groups [13]. Furthermore, a maximum net current-to-peak width at half height ($i/W_{1/2}$) ratio was obtained for pH 6.5, with an E_p value of +1.05 V. At this pH value, ten successive SW voltammograms for 1.0×10^{-5} mol l^{-1} furosemide yielded a relative standard deviation (R.S.D.) for i_p of 4.7%, indicating a good repeatibility of the voltammograms with no pretreatment of the CFME.

The effect of the buffer concentration on the voltammetric response was also tested over the 1.0×10^{-3}–0.1 mol l^{-1} concentration range. Five repetitive measurements from each solution showed than a slight increase of both net current and peak potential occurred as the buffer concentration was increased up to 0.05 mol l^{-1}, then these values leveled off. Consequently, a 0.05 mol l^{-1} $HPO_4^{2-}/H_2PO_4^-$ buffer of pH 6.5 was selected to be used for further work.

Finally, the reproducibility of the SW voltammograms obtained at five different CFMEs was also checked under the above mentioned conditions. A R.S.D. value for i_p of 4.8% indicated that reproducible responses were obtained with different microelectrodes constructed in the same manner.

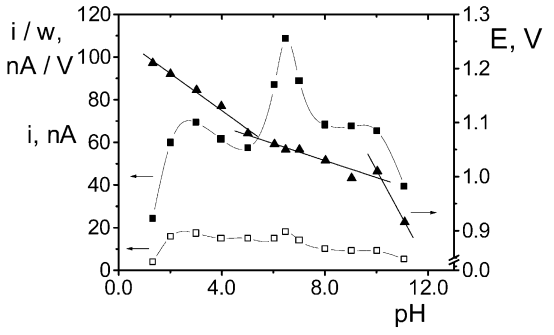


Fig. 2. Influence of pH on net peak current (□), peak potential (▲), and peak current-to-peak width at half height ratio (■) obtained by SW voltammetry at CFMEs for 1.0×10^{-5} mol l^{-1} furosemide. Background electrolyte: 0.05 mol l^{-1} Britton–Robinson buffer. SW parameters as in Fig. 1.

3.2. Flow-injection with amperometric detection

A hydrodynamic voltammogram was constructed by measuring the peak current at different applied potential values, when 84 μl of 1.0×10^{-5} mol l^{-1} furosemide were injected into a carrier consisting of 0.05 mol l^{-1} phosphate buffer solution of pH 6.5. Fig. 3 shows as a narrow current plateau was obtained for potentials between 1.25 and 1.30 V. Moreover, a very stable background current was found over the whole potential range tested. The influence of the flow rate on the amperometric signal obtained at +1.25 V was examined in the 0.24–1.65 ml min^{-1} range. A slight increase in the peak current together with a decrease in both residence time and peak width were observed as the flow rate increased over the whole range studied. As a compromise between sensitivity and resolution, a flow rate of 1.0 ml min^{-1} was chosen for further work. Under these conditions, a stable baseline was reached in approximately 100 s. Concerning the injection volume, its influence on the amperometric re-

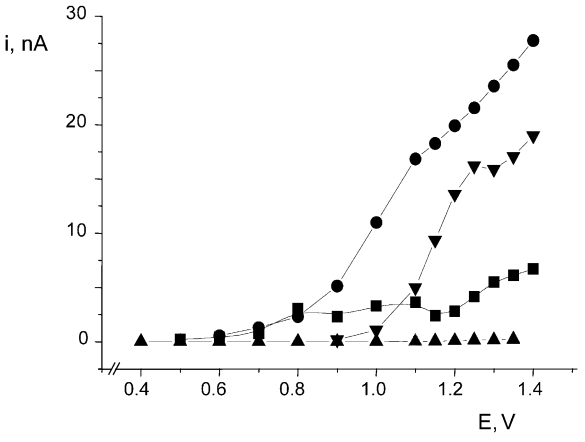


Fig. 3. Peak current vs. applied potential plot obtained by FI with amperometric detection at a CFME. Injections of 84 μl of 1.0×10^{-5} mol l^{-1} (▼) furosemide, (■) OTC, (●) MTU in 0.05 mol l^{-1} phosphate buffer solution of pH 6.5. (▲) Background electrolyte; flow-rate 1.0 ml min^{-1}.

sponse was also tested in the 84–884 μl range. The peak current-to-peak width ratio showed a maximum for 84 μl, this value being the dead volume of the injection valve, that is with no loop connected.

However, repetitive amperometric measurements under these conditions for 1.0×10^{-6} mol l^{-1} furosemide showed a continuous decrease in the peak current, probably as a consequence of the CFME surface fouling by the electrode reaction products under potentiostatic conditions. Thus, the R.S.D. value for i_p and $n = 10$ was of 15%. This type of drawback sometimes can be avoided by application of a PAD program [15].

3.3. Pulsed amperometric detection at CFMEs

A good amperometric detection of furosemide at CFMEs could be achieved by applying to the microelectrode a simple PAD program, consisting of a two-step potential waveform with alternating anodic and cathodic polarization. The anodic potential (E_1) was that chosen before from the hydrodynamic voltammogram, +1.25 V. The cathodic potential applied for the cleaning of the microelectrode surface, E_2, and the time periods for application of both potentials, t_1 and t_2, were optimized by testing their influence on the repeatability of the FI amperometric responses obtained from repetitive injections ($n = 10$), of 1.0×10^{-6} mol l^{-1} furosemide. The lowest R.S.D. value obtained, 5.8%, was achieved for $E_2 = -0.20$ V, $t_1 = 0.1$ s and $t_2 = 0.2$ s. Under these conditions, a linear calibration graph ($r = 0.998$) was obtained for furosemide in the 5.0×10^{-7}–1.0×10^{-4} mol l^{-1} concentration range, with a slope of $(4.49 \pm 0.05) \times 10^3$ μA l mol^{-1} and an intercept of (5 ± 2) nA. A limit of detection of 1.7×10^{-7} mol l^{-1} (56 μg l^{-1}) was calculated according to the $3s_b/m$ criterion, where m is the slope of the calibration graph and s_b was estimated as the standard deviation ($n = 10$) of the amperometric responses from 5.0×10^{-7} mol l^{-1} furosemide. Furthermore, a limit of determination of 5.6×10^{-7} mol l^{-1} (185 μg l^{-1}) was calculated according to the $10 \times$ standard deviation criterion. The good sensitivity achieved allows the determination of residues of this diuretic drug in real samples, provided the absence of other electroactive compounds at the applied potential, as it will be demonstrated below.

3.4. Interferences

The influence of the presence of other drugs used in veterinary on the furosemide FI amperometric response at CFMEs, was evaluated using MTU, a thyreostat drug, and OTC, an antibiotic, as possible interferents. At the experimental conditions used for furosemide detection, both MTU and OTC gave good amperometric signals, as it can be deduced from their corresponding hydrodynamic voltammograms shown in Fig. 3. Indeed, linear calibration plots were obtained for these compounds over the $(2.0–10) \times 10^{-6}$ mol l^{-1} concentration range, with slope values of $(1.03 \pm 0.03) \times 10^4$ μA l mol^{-1} and $(5.6 \pm 0.5) \times 10^3$ μA l mol^{-1}, respectively. As can be observed, these values are higher than that obtained for furosemide, and, consequently, the presence of those compounds in a sample together with furosemide would affect strongly the analyte response. The presence of binary or even ternary mixtures of these veterinary drugs in animal feed or milk is not a very unusual situation, and therefore, the resolution of this analytical problem should imply a chromatographic separation step.

3.5. Liquid chromatography with pulsed amperometric detection at CFMEs

HPLC-PAD at CFMEs was used for the determination of furosemide in the presence of several thiouracil drugs and OTC. C_{18} and ODS reversed-phase columns have been employed in the literature for the separation of diuretics [8,13]. Moreover, acetonitrile/aqueous buffer mixtures have been currently used as mobile phases for the chromatographic separation of diuretics [8,13], antibiotics [24] and antithyroid drugs [25]. Therefore, the separation of furosemide, MTU, PTU, PhTU and OTC was tested using the two types of columns mentioned above and different acetonitrile/phosphate buffer mixtures as mobile phase. Using an ODS column no good separation between the thiouracil drugs could be achieved,

whereas, as it will be shown below, it was possible to separate adequately all the five compounds when the C_{18} column was used. Consequently, an isochratic elution using a C_{18} reversed-phase column was employed for further work. The same PAD program described for FI measurements was applied to the microelectrode. Although this program was optimized for such FI measurements using an aqueous solution, the use of acetonitrile/phosphate buffer mixtures as the mobile phase in HPLC did not imply the need of change of the detection conditions, as the detection potential used was appropriate for a good amperometric detection of all the thiouracil drugs, as it will be shown below.

The influence of the acetonitrile content in the mobile phase consisting of acetonitrile–aqueous phosphate buffer mixtures on the separation of furosemide, MTU, PTU, PhTU and OTC was tested using a phosphate buffer of pH 4.25 as a 3.0–4.25 pH range has been recommended in the literature regarding chromatography of furosemide and related compounds [5,13,8]. Phosphate buffer was used instead of acetate buffer because we observed significant higher peak areas for these compounds in the phosphate medium. As expected, the retention time and the peak area for these compounds decreased when the content of acetonitrile was increased in the whole 10–50% (v/v) acetonitrile range. Furthermore, no resolution between OTC and MTU peaks was achieved for acetonitrile percentages of 30% and higher. Therefore, as a compromise between a good separation, sensitivity and short retention times, a mobile phase containing 25% (v/v) acetonitrile was selected for further work.

On the other hand, the effect of the phosphate buffer pH value on the chromatographic separation of furosemide, MTU, PTU, PhTU and OTC, was evaluated by injecting aliquots of a solution containing each drug at a 5.0×10^{-5} mol l^{-1} concentration in different mobile phases consisting of 25:75 acetonitrile–5.0×10^{-3} mol l^{-1} phosphate buffer whose pH ranged between 3.0 and 6.0. Fig. 4a shows a very different chromatographic behavior for furosemide in comparison with the other drugs tested. So, a slight decrease of the peak area and a rapid shortening of the retention time were observed for furosemide as the pH value increased. However, practically constant retention times (always lower than that of furosemide) and bigger peak areas were obtained for MTU, PTU, PhTU and OTC when the pH raised from 3.0 to 6.0. Chromatograms obtained at pH 4.0, 5.0 and 6.0 are displayed in Fig. 4b. Elution order observed was MTU, OTC, PTU, PhTU and furosemide. As a compromise between sensitivity and time of analysis for furosemide, pH 5.0 was selected for further work and, consequently, NaH_2PO_4 was used to prepare directly the solutions. Also, it was verified that both peak areas and retention times of all the separated drugs did not vary significantly with the phosphate concentration within the 1.0×10^{-3}–5.0×10^{-2} mol l^{-1} range. Concerning the effect of the mobile phase flow rate on the PAD chromatographic responses of these drugs, both retention times and peak width decreased, as expected, for all the compounds when the flow rate increased over the whole 0.6–1.5 ml min^{-1} range studied. Furthermore higher peak area-to-peak width ratios were obtained for higher flow rates, and, therefore, 1.4 ml min^{-1} was the value selected for further work. Under these conditions retention times of 1.35, 2.13, 2.62, 4.69 and 7.00 min were obtained for MTU, OTC, PTU, PhTU and furosemide, respectively.

Calibration graphs were obtained for furosemide, MTU, PhTU, PTU and OTC with the ranges of linearity between peak area and concentration, and the analytical characteristics that are summarized in Table 1. R.S.D. values for peak areas and retention times were calculated from chromatograms of five different mixtures of the drugs at a concentration of 1.0×10^{-5} mol l^{-1} each. The limits of detection were calculated according to the $3s_b/m$ criterium, where m was the slope of the calibration graph for each analyte, and s_b was estimated as the standard deviation ($n = 10$) of the peak areas obtained for the lowest concentration of the corresponding linearity range.

Another interesting point to be remarked is that other tetracyclines besides OTC, such as chlortetracycline and doxytetracycline were also eluted under the same chromatographic conditions used for furosemide and the other drugs. Overlapped

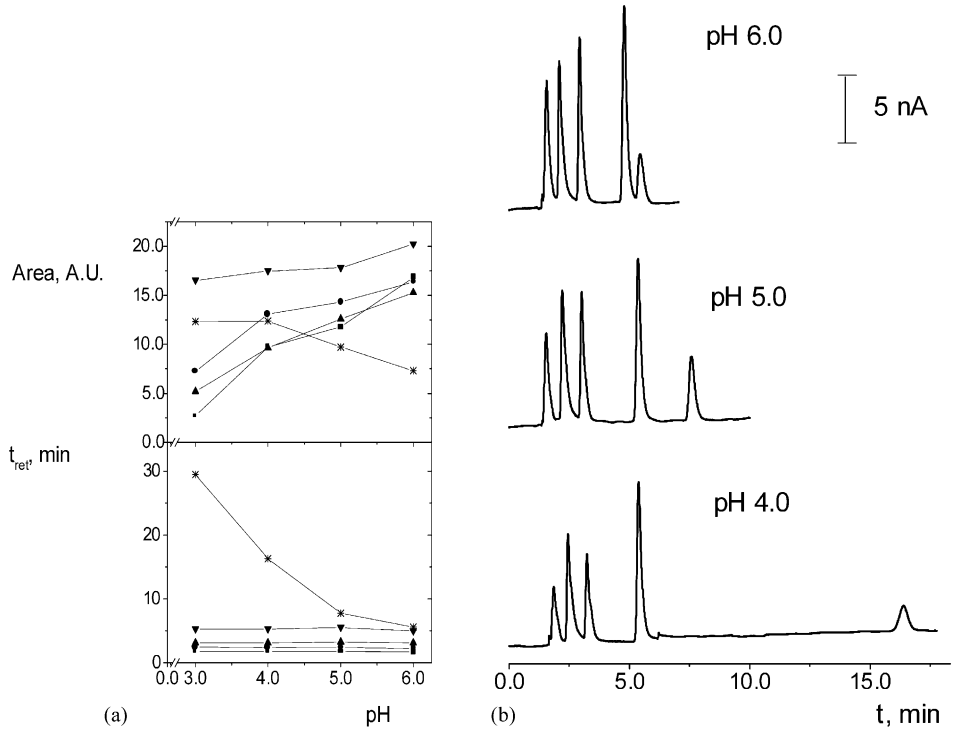


Fig. 4. (a) Influence of pH on peak area (top) and retention time (bottom) of chromatograms obtained by HPLC-PAD at CFMEs 20 μl of 5.0×10^{-5} mol l^{-1} furosemide (*), MTU (■), OTC (●), PTU (▲), and PhTU (▼). (b) Chromatograms obtained at pH 4.0, 5.0 and 6.0. Flow rate, 1.4 ml min^{-1}. Mobile phase, 25:75 acetonitrile–5.0×10^{-3} mol l^{-1} NaH_2PO_4; PAD: $E_1 = +1.25$ V; $t_1 = 100$ ms; $E_2 = -0.200$ V; $t_2 = 200$ ms. 1, MTU; 2, OTC; 3, PTU; 4. PhTU; 5, furosemide.

peaks with that of OTC were found, which can be useful for a qualitative detection of the presence of tetracyclines in the sample in which furosemide is to be analyzed.

Concerning metabolic products of furosemide, it is established that two degradation ways can be considered: conjugation with glucuronic acid and loss of the side chain linked to the anthranillic

Table 1
Analytical characteristics of the calibration graphs for furosemide (FM), thiouracils (MTU, PhTU, PTU) and OTC obtained by HPLC-PAD at a CFME

	Range of linearity (μM)	Slope[a]	Intercept[b]	r	R.S.D. % [1]area; [2]t_{ret}	Detection limit (μM)
FM	1–100	$(1.54 \pm 0.02) \times 10^7$	20 ± 10	0.998	7.5^1 1.1^2	0.55
MTU	1–100	$(1.89 \pm 0.02) \times 10^7$	6 ± 9	0.998	7.5^1 2.1^2	0.50
PhTU	1–60	$(3.45 \pm 0.06) \times 10^7$	50 ± 20	0.998	5.4^1 1.2^2	0.35
PTU	1–80	$(2.70 \pm 0.08) \times 10^7$	10 ± 30	0.993	3.4^1 1.5^2	0.45
OTC	1–100	$(2.52 \pm 0.03) \times 10^7$	-20 ± 10	0.998	4.8^1 1.7^2	0.50

Mobile phase: 25:75 acetonitrile–5×10^{-3} mol l^{-1} NaH_2PO_4 (pH 5.0) $E_1 = +1.25$ V; $t_1 = 100$ ms; $E_2 = -0.200$ V; $t_2 = 200$ ms.
[a] Area units/concentration (mol l^{-1}).
[b] Area units.

group [26]. The product of the first process, furosemide acylglucuronide, cannot be found in milk [8] and, consequently, we did not consider it. The second degradation way involves photochemical degradation giving rise to a series of compounds [2]. Thus, degradation studies of furosemide by exposing this drug to standard laboratory lighting and to sunlight were performed. Two degradation media were checked: pure acetonitrile, in which the furosemide concentration was 1.0×10^{-2} mol l^{-1}, and a 65:35 acetonitrile:5.0×10^{-3} mol l^{-1} NaH_2PO_4 mixture in which furosemide was soluble at a concentration level of 5.0×10^{-3} mol l^{-1} was soluble. After exposure, aliquots of the degradation solutions were diluted with the selected mobile phase and then injected in the chromatographic system.

After 135 min exposing to laboratory lighting in both media, the chromatographic peak area of furosemide decreased approximately to a 17% of the initial value and no additional peaks appeared in the chromatograms. However, after 16 h exposing, a 38% decrease was observed when degradation was carried out in acetonitrile and a small peak with a retention time of 3.93 min appeared.

Regarding sunlight exposing, furosemide solutions in both media turned to a brown–yellow color after 1 h exposing. After 2 h, only 27 and 14% of the initial furosemide peak area remained when degradation occurred in acetonitrile and in the acetonitrile:phosphate mixture, respectively. In the first case, two chromatographic peaks at 1.11 and 1.20 min appeared, whereas four peaks at 1.03, 1.23, 2.61 and 3.94 min were found when degradation occurred in the acetonitrile:phosphate mixture. In the literature, the identity of the photochemical degradation products of furosemide has been discussed extensively, being subjected to controversy, also because most of these products are not commercially available. Actually, only two of them, furfuryl alcohol and 2,4-dichloro-5-sulfamoylbenzoic acid could be purchased. Injections of 4.6×10^{-4} mol l^{-1} furfuryl alcohol in the mobile phase used yielded a peak at 2.16 min, and of 1.0×10^{-3} mol l^{-1} 2,4-dichloro-5-sulfamoylbenzoic acid gave rise to two small peaks at 0.99 and 1.22 min. This seems to suggest that some of the degradation products observed can be assigned to these compounds or to very similar compounds. Unfortunately, the lack of standards for other degradation products avoided a wider study.

3.6. Determination of furosemide in spiked milk samples

The usefulness of the chromatographic method with PAD detection at CFMEs for the determination of furosemide in real samples was evaluated by performing the analysis of commercial skimmed milk samples which were spiked with furosemide at a concentration level of 150 ng ml^{-1}. This concentration is the mean concentration of diurethic that can be detected in the next 8 h after administration of the usual 500 mg dosage of drug [8]. Moreover, the samples were also spiked with 65 ng ml^{-1} MTU, 77 ng ml^{-1} PTU, 93 ng ml^{-1} PhTU and 225 ng ml^{-1} OTC. All these concentrations yielded a 2.0×10^{-6} mol l^{-1} final concentration in the analytical solution.

Samples were treated following the procedure described in Section 2, which is a modification of that proposed by Shaikh [8] for the chromatographic determination of furosemide using fluorescence detection. This method consisted on milk deproteination with acetonitrile, followed by phases separation and volume reduction. However, when this same procedure was employed with PAD detection, a big endogenous peak appeared in the chromatograms (see Fig. 5). This big peak overlapped with those of MTU and OTC, which would avoid their quantification. Moreover, the large peak tail also affected the baseline for the measurement of the other peaks. However, the sample endogenous peak decreased largely when ethyl acetate was employed for deproteination (Fig. 5), and then this solvent was used in the sample treatment.

Results obtained for the analysis of three spiked milk samples yielded a mean furosemide concentration value of 147 ± 8 ng ml^{-1}, with a mean recovery of $95 \pm 5\%$, thus demonstrating the suitability of the developed method for the determination of this diuretic in this kind of samples.

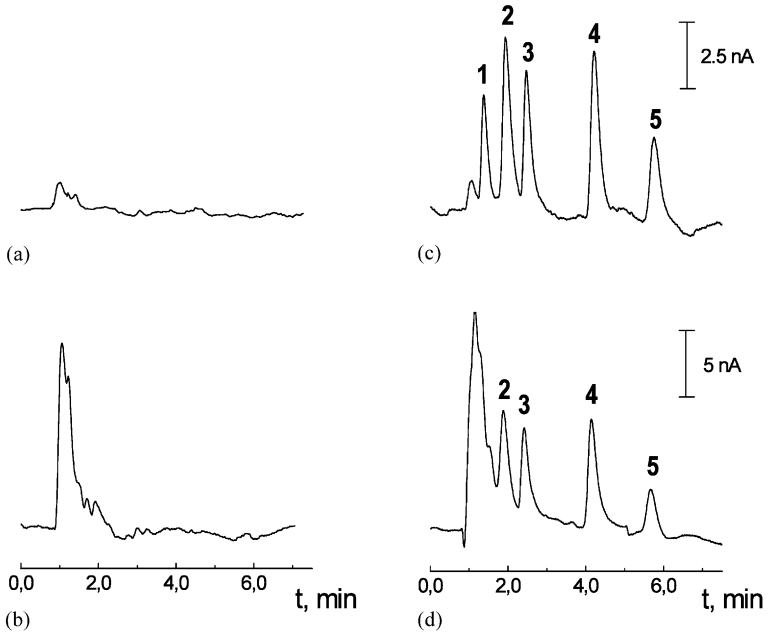


Fig. 5. Chromatograms obtained from a sample of milk deproteinated with ethyl acetate (a, c), or with acetonitrile (b, d). Chromatograms a and b correspond to blank milk samples. Chromatographic conditions as in Fig. 4, including the pH of the mobile phase.

4. Conclusions

PAD at CFMEs constitutes an adequate approach for the flow injection and HPLC determination of the diurethic drug furosemide in the absence or presence of other veterinary drugs such as thiouracils and tetracyclines, respectively. The two step potential waveform involving successively a measurement and a cleaning potential, allows to obtain reproducible amperometric measurements by prevention of the microelectrode surface fouling. Moreover, the HPLC-PAD method has demonstrated to be useful for the determination of furosemide in real samples such as milk, at a concentration level corresponding to the mean concentration of diurethic that can be detected in the next 8 h after administration of an usual 500 mg dosage of drug, and in the presence of other veterinary drugs.

Acknowledgements

Financial support from the Ministerio de Ciencia y Tecnología (Project BQU 2001-2050 No 9765) is gratefully acknowledged.

References

[1] B. Shaikh, in: W.A. Moats, M.B. Medina (Eds.), Veterinary Drug Residues. Food safetyACS Symp. Ser., Ch. 17., 1995, p. 161ACS Symp. Ser., Ch. 17..
[2] S. Cardá-Broch, J. Esteve-Romero, M.C. García Álvarez-Coque, J. Pharm. Biomed. Anal. 23 (2000) 803.
[3] H.-J. Guchelaar, L. Chandi, O. Schouten, W.A. van den Brand, Fresenius' J. Anal. Chem. 363 (1999) 700.
[4] A. Nava-Ocampo, E.Y. Velázquez-Armenta, H. Reyes-Pérez, E. Ramírez-López, H. Ponce-Monter, J. Chromatogr., B 730 (1999) 49.
[5] C.D. Mills, C. Whitworth, L.P. Ribak, C.M. Henley, J. Chromatogr., B 701 (1997) 65.

[6] S. Cardá-Broch, J.S. Esteve-Romero, M.C. García Álvarez-Coque, Anal. Chim. Acta 375 (1998) 143.
[7] P. Campins-Falcó, R. Herráez-Hernández, M.D. Pastor-Navarro, J. Liquid Chromatogr. 20 (1867) 1997.
[8] B. Shaikh, J. Agric. Food Chem. 43 (1995) 2117.
[9] V. Sanz-Nebot, I. Toro, R. Berges, R. Ventura, J. Segura, J. Barbosa, J. Mass Spectrom. 36 (2001) 652.
[10] M.E. Abdel-Hamid, Farmaco 55 (2000) 448.
[11] J. Carlavska, W. Thorman, J. Chromatogr., B 770 (2002) 207.
[12] M.B. Barroso, R.M. Alonso, R.M. Jiménez, Anal. Chim. Acta 305 (1995) 332.
[13] M.B. Barroso, R.M. Alonso, R.M. Jiménez, E. Ortiz, J. Chromatogr., B 675 (1996) 303.
[14] M. Macher, R. Wintersteiger, J. Chromatogr., A 709 (1995) 257.
[15] J. Wang, Analytical Electrochemistry, second ed, Wiley-VCH, New York, 2000, p. 92.
[16] F.M. Matysik, A. Meister, G. Werner, Anal. Chim. Acta 305 (1995) 114.
[17] C. Hua, H.K. Lee, A.K. Hsieh, Electroanalysis 6 (1994) 1147.
[18] K. Pihel, S.C. Hsieh, J.W. Jorgenson, R.M. Wightman, Anal. Chem. 67 (1995) 4514.
[19] K.A. Sagar, M.R. Smyth, Analyst 125 (2000) 439.
[20] A. Guzmán, L. Agüí, M. Pedrero, P. Yáñez-Sedeño, J.M. Pingarrón, Electroanalysis 13 (2001) 1301.
[21] P.J. Vandeberg, D.C. Johnson, Anal. Chem. 65 (1993) 2713.
[22] L. Agüí, J. Reviejo, P. Yáñez-Sedeño, J.M. Pingarrón, Anal. Chem. 67 (1995) 2195.
[23] M.L. Agüí, J.E. López-Guzmán, A. González-Cortés, P. Yáñez-Sedeño, J.M. Pingarrón, Anal. Chim. Acta 385 (1999) 241.
[24] H. Oka, Y. Ito, H. Matsumoto, J. Chromatogr., A 882 (2000) 109.
[25] R.K. Buick, C. Barry, I.M. Traynor, W.J. McCaughey, C.T. Elliot, J. Chromatogr., B 720 (1998) 71.
[26] R. Ventura, J. Segura, J. Chromatogr., B 687 (1996) 127.