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Determination of nitrofurantoin, furazolidone and furaltadone in milk by high-performance liquid chromatography with electrochemical detection

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Abstract

A HPLC method with coulometric detection has been established to carry out the separation of the three nitrofuran derivatives, nitrofurantoin, furazolidone and furaltadone. A Nova-Pak C_{18} column (150×3.9 mm) and a Coulochem II detector from ESA have been used. After obtaining the hydrodynamic curves of the three compounds in the porous graphite electrode a potential of −600 mV was selected as the working potential. The influence of other variables such as mobile phase composition and flow-rate were studied. The mobile phase considered as an optimum was acetonitrile–0.1 *M* aqueous solution of sodium perchlorate (28:72), with 0.5% glacial acetic acid. The oxygen of the mobile phase was removed with a vacuum system on-line and a nitrogen stream was used to remove the oxygen of the samples. The calibration graphs and the detection limits were established. The method proposed was used, with good results, for the determination of the three compounds in milk.

Keywords: Milk; Food analysis; Nitrofuran

1. Introduction

Liquid chromatography with electrochemical detection allows selective and sensitive detection of a considerable number of compounds in complex matrices, the linear dynamic range and safety of electrochemical detectors being at least comparable with those of other detectors, whereas the detection limits obtained reach the order of nanograms. The disadvantage of this method of detection is the limit of the technique's applicability (which can be considered an advantage from the point of view of selectivity). Furthermore, it should be taken into account that the signal (intensity of oxidation or reduction) is sensitive to changes in the electrode surface or interferences like the one due to the presence of oxygen when we work in the reduction mode. For these reasons, the number of applications is limited compared with other systems of detection. This is shown, for example, when a bibliographical revision is carried out on the analysis of nitrofuran derivatives, which are easily reduced compounds whose reduction wave is close to that of oxygen, by means of HPLC with electrochemical detection. The

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three compounds under study in this paper are furaltadone [5-morpholinomethyl-3-(5-nitrofurfurylideneamine)-2-oxazolidinone] (FD), furazolidone [3-(5-nitrofurfurylidenamino)-2-oxazolidinone] (FZ) and nitrofurantoin [N-(5-nitro-2-furfurylidene)-1-aminohydantoin] (NF), nitrofuran derivatives that have been used in medicine separately or in combination or with other drugs for the treatment of gastrointestinal infections in animals and humans. The three compounds are formulated together in our country and are administered in the treatment of infectious diseases common to humans and animals, especially domestic and farm animals. Mostly they are administered orally, mixed with animal feed or drinking water. Their use is regulated and there is an established withdrawal time which must be respected if the meat and its by-products or other animal by-products such as milk or eggs are to be used for human consumption. Therefore, the determination of their residues in body fluids and in foods of animal origin is of great interest. The toxic effects (also mutagenic and carcinogenic) of most of the nitrofurans are well documented [1–3], but their precise mode of action is not fully clarified (and neither is their fate). Because of the transient nature of the metabolites and the structural diversity of the nitrofurans the determination of these drugs as the parent molecules has been recommended [4]. However, some papers have been published on the determination of cyano and amino metabolites of these compounds [5–7].

The electrochemical behaviour of these compounds has been examined [8–11]; it has also been examined by using carbon electrodes [12,13] but only the liquid chromatographic analysis with electrochemical detection of furazolidone is reported in the literature ([14–16]).

For this reason, it is worth developing the possibilities this technique offers for the simple analysis of samples like milk, particularly since the presence of at least nitrofurantoin in breast milk has been proved (in some of the patients examined at levels in the range 0.3–0.5 μg/ml [17]). The lack of confirmation of its presence in all cases could be due to the lack of sufficiently sensitive methods. The legislation of the European Community relating to the presence of some of these compounds in edible animal products refers to the presence of furazolidone in liver, muscle, kidney and fat, the provisional maximal residue limit being 5 ng/g [18]; the current position is that the other nitrofurans are forbidden for use in food-producing animals. The US Food and Drugs Administration has also decided to withdraw approval of furazolidone for use in food-producing animals [19].

Other liquid chromatographic procedures for the determination of one or more of these three compounds in milk can be found in the literature. Different pretreatment procedures such as liquid–liquid partitioning [20–23], matrix solid-phase dispersion extraction [24], lyophilization [25] or dialysis [26], are used. Detection is carried out photometrically in all cases and the detection limits are low (in the order of ng/ml) although these procedures are, in general, quite tedious and time consuming. In one of these procedures for the pretreatment of the sample, an automated technique is used consisting of dialysis coupled on-line with trace enrichment by column-switching HPLC; however, milk samples must be decreamed prior to their introduction into the system and nitrofurantoin cannot be accurately determined owing to an unidentified matrix interference. In this paper, since the selectivity and sensitivity of the detection technique allows it, a simple procedure consisting of precipitation in acid medium and solid–liquid extraction is proposed.

2. Experimental

2.1. Apparatus and chromatographic conditions

The studies carried out were performed in a chromatograph made up of a solvent reservoir, a degassing system, 79700 in-line vacuum degasser from Waters to remove the oxygen, a double piston HPLC pump 420 from Kontron equipped with a damper, a six-way injection valve (Rheodyne) containing a 20 μl loop, a Nova-Pak C_{18} analytical column (150×3.9 mm, 4 μm) with a Symmetry™ C_{18} guard column and a coulometric detection system which consists of a conditioning cell Model 5021 from ESA, a high-performance analytical cell (70% efficiency) Model 5011 from ESA composed of two porous carbon working electrodes controlled

by a Coulochem II (from ESA) potentiostat, the signal being picked up by a personal computer equipped with the Integration Pak software supplied by Kontron Instruments for chromatogram manipulation.

The eluent was prepared by mixing an 0.1 *M* aqueous solution of sodium perchlorate with acetonitrile in a 72:28 proportion and adding glacial acetic acid in a 0.5% concentration. The eluent was filtered and degassed before use. The flow-rate was 1.0 ml/min.

2.2. Chemicals and reagents

Furaltadone hydrochloride, furazolidone and nitrofurantoin from Sigma (St. Louis, MO, USA) were used. HPLC-grade water was obtained from a Water Pro^{TM} PS (Labconco) system. Acetonitrile for chromatography and sodium perchlorate from Merck and glacial acetic acid from Romil were also used. All other chemicals were of analytical reagent grade.

2.3. Procedure for the analysis of milk samples

Aliquots of 50 ml of cow milk are deproteinized with 25 ml of 20% (w/v) trichloroacetic acid. After 15 min the samples are filtered and washed with water. The pH of the serum solution is adjusted with sodium hydroxide between 4.5 and 5 and subsequently the solution is diluted with water in a 100 ml calibrated flask. Aliquots of 25 ml are taken to apply the standard addition method fortifying these with appropriate amounts of the three analytes. These aliquots are passed through a C_{18} cartridge (Sep-Pak Plus C_{18} from Waters) to isolate the nitrofurans and the analytes are finally eluted with 2.5 ml of mobile phase.

3. Results and discussion

Initially, the efficiency in removing oxygen in the mobile phase was seen to be high with the degassing system used, providing stable background currents. Likewise, the presence of oxygen in the samples gave rise to the appearance of signals in the shape of wide peaks and very pronounced tails. The retention time of these peaks depended on the flow-rate and the mobile-phase composition, appearing at approximately 3 min when the flow-rate was 1 ml/min and the mobile phase contained about 30% acetonitrile. For this reason, oxygen removal was carried out in all the samples prior to the injection by passing a nitrogen stream for a minimum time of 2 min.

In previous studies [25], great importance is attached to ionic strength in the chromatographic behaviour of furaltadone. On the other hand, the presence of the electrolyte is essential in the case of electrochemical detection. That is why we chose as mobile phase an acetonitrile–aqueous solution of sodium perchlorate in order to obtain the hydrodynamic curves for the three compounds (Fig. 1). This step is necessary in any method of electrochemical detection it must be performed; on the other hand, each time an exhaustive cleaning of the electrode surface (6 *M* NO_3H) is carried. An appreciable signal is observed from −500 mV, and the maximum value of intensity rose at about −700 mV. We chose a value of −600 mV as a compromise, since at higher potential values the intensity corresponding to the baseline is too high and presents a considerable drift.

Once the working potential was selected, we

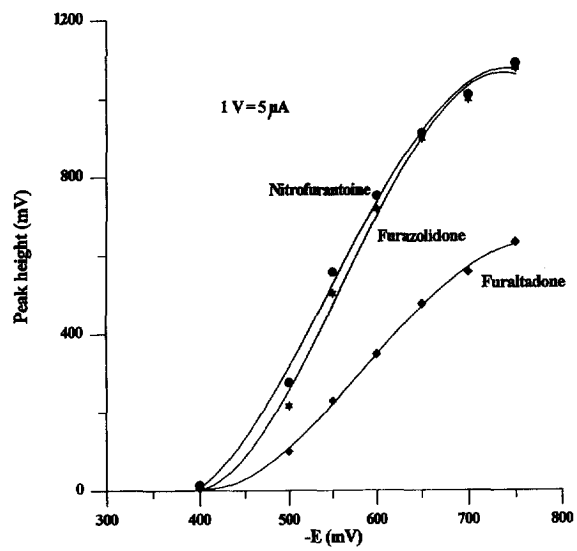

Fig. 1. Hydrodynamic curves of nitrofurantoin, furazolidone and furaltadone (1.0 μg/ml of each) in the porous carbon electrode of the analytical cell. Chromatographic conditions: MeCN–0.1 *M* $NaClO_4$ (28:72), with 0.5% glacial acetic acid, flow-rate, 1.0 ml/min.

optimized the mobile-phase composition, varying the concentration of sodium perchlorate and acetic acid in this, as well as the acetonitrile–aqueous ratio. Only the furaltadone retention is affected by the ionic strength, as shown in Fig. 2. We selected a 0.1 *M* sodium perchlorate concentration in the aqueous portion. Also, the acidity of the mobile phase only affects slightly the retention of furaltadone and a 0.5% concentration of acetic acid was chosen as suitable.

The percentage of acetonitrile was only varied between 27 and 30%, because at a lower percentage an overlapping between the furazolidone and the oxygen signals is observed and at a higher percentage the resolution between the nitrofurantoin and furaltadone peaks is small. An acetonitrile content of 28% was chosen. No significant changes in capacity factors or resolution between peak values (always greater than 1.5) were observed with the variation of the flow-rate (between 0.5 and 1.0 ml/min), and a flow-rate of 1 ml/min was used. The time of analysis was lower than 3 min in the selected conditions, as shown in the final chromatogram at the bottom of Fig. 2.

In Fig. 3 chromatograms obtained with different deoxygenation times (by means of a nitrogen stream) are shown. It is noticeable that although the three peaks are well separated from the oxygen signal, the presence of oxygen in the samples requires a long waiting period until the signal reaches its original value. This fact makes the elimination of oxygen from the samples advisable.

3.1. Analytical figures of merit

The calibration graphs were obtained under the selected conditions. For this purpose samples were prepared in triplicate with increasing concentrations of the three analytes. Each sample was injected three times into the chromatograph and the mean of the area obtained or of height values was plotted against the concentration. This study was performed with two different sensitivity values selected in the detector (1 μA and 100 nA), and, therefore, in two different concentration ranges 10–60 ppb and 0.1–2.2 ppm. The correlation coefficient and the R.S.D. values are similar in both cases. The results corresponding to the first range are summarized in Table

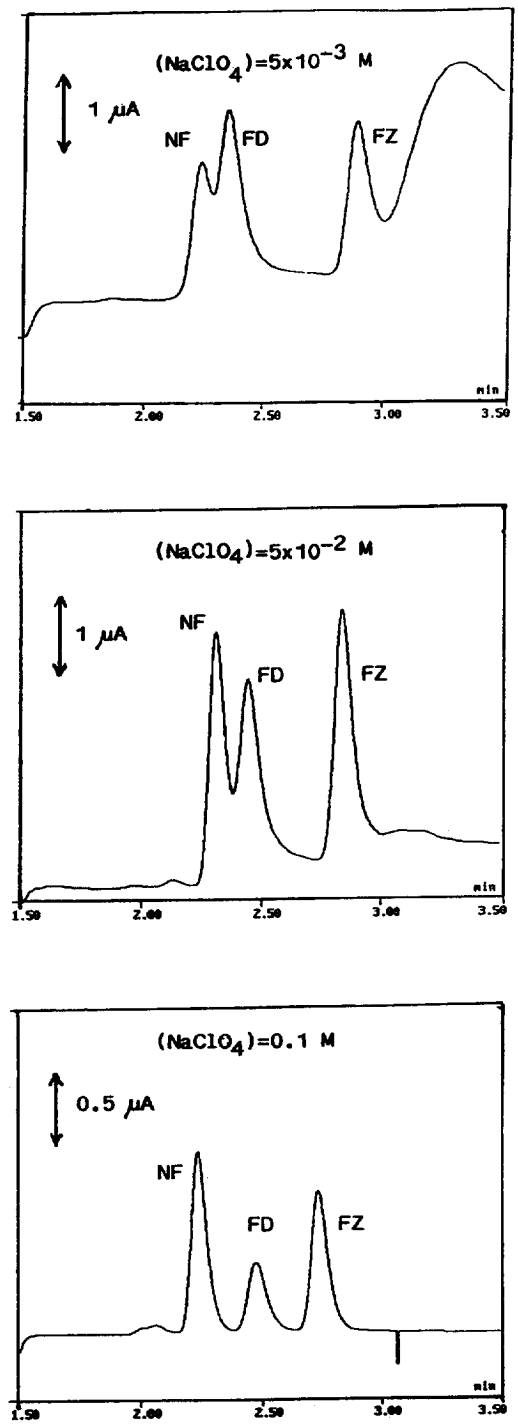

Fig. 2. Influence of the ionic strength on the retention of analytes (1.0 ppm of each). Mobile phase: MeCN–$NaClO_4$ of different concentrations (28:72), with 0.5% glacial acetic acid. Flow-rate, 1.0 ml/min.

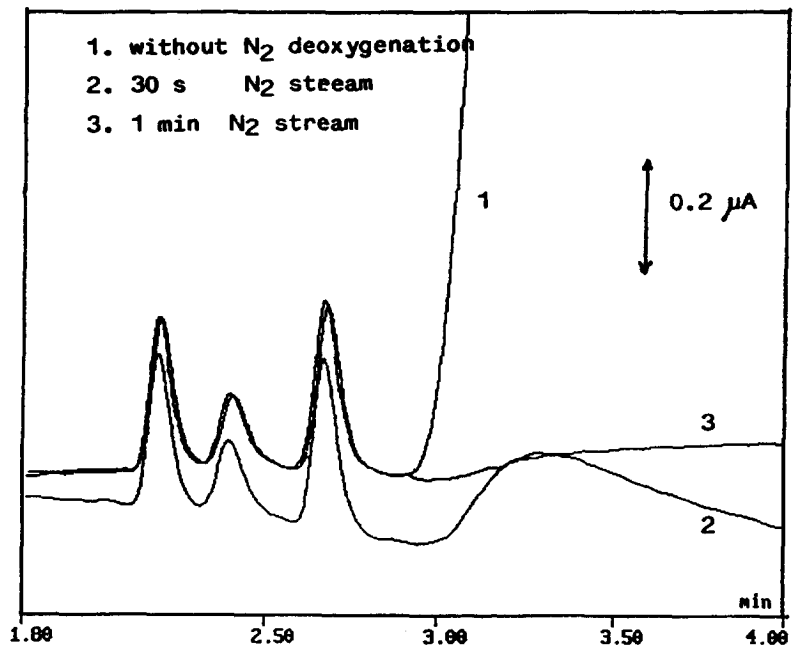

Fig. 3. Variation of the signal due to the oxygen in the sample with the time of the N_2 stream used. Mobile phase: MeCN–0.1 *M* $NaClO_4$ (28:72), with 0.5% glacial acetic acid. Flow-rate, 1.0 ml/min.

1; this also shows the detection limit values, calculated by using the standard deviation values of the slope and the origin intercept and choosing false positive and false negative probability values of 0.05 (Clayton's method) [27].

3.2. Applications

The developed method has been applied to the analysis of nitrofurans in cow milk samples. Two different procedures for the pretreatment of the samples were assayed with spiked milk samples. The first consists of lyophilization of the milk, washing with acetonitrile and extraction with dimethylformamide. After washing again with acetonitrile the extract was evaporated and the sample was diluted with water. No acceptable results were obtained following this procedure due to the signal of the milk matrix. The second method consists of deproteinization of the milk, isolation of the nitrofurans in the

Table 1
Analytical figures of merit

Compound	Signal	Correlation coefficient (r)	R.S.D. (%) $n=11$ (30 ppb)	Analytical sensitivity[a] (ppb^{-1})	DL[b] (ppb)
NF	Height	0.9967	1.6	0.7	4
	Area	0.9975	1.9	0.8	4
FD	Height	0.9268	1.6	0.7	4
	Area	0.9970	3.6	0.7	4
FZ	Height	0.9948	1.3	0.5	5
	Area	0.9930	2.1	0.5	6

Concentration range: 10–60 ppb (1 V=100 nA).
[a]Analytical sensitivity: slope of calibration curve/residual mean [28].
[b]Calculated by Clayton's method ($\alpha=\beta=0.05$).

Table 2
Results obtained in the analysis of nitrofuran derivatives in spiked milk samples

Compound	Added (ppb)	Signal	Recovery±R.S.D. (%)
NF	25	Height	95±2
		Area	97±12
	10	Height	98±9
		Area	83±4
	4	Height	98±8
		Area	90±8
FD	25	Height	97±6
		Area	96±17
	10	Height	92±9
		Area	87±1
	4	Height	–
		Area	–
FZ	25	Height	85±11
		Area	96±11
	10	Height	89±6
		Area	97±9
	4	Height	97±6
		Area	86±7

serum with a C_{18} cartridge (Sep-Pak Plus C_{18} from Waters) and subsequent elution of them with the mobile phase. The procedure followed was detailed above and was applied to the analysis of cow milk samples spiked with the three antibacterials before any subsequent treatment. The standard addition method was always used with the solution obtained after deproteinization of the milk to prevent the effects of incomplete retention or elution in the Sep-Pak, as well as any decrease in the electrode signal resulting from its long use. The results obtained were excellent; they are shown in Table 2. Concentrations of the three nitrofurans in the low ppb level can be determined with great simplicity and speed.

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