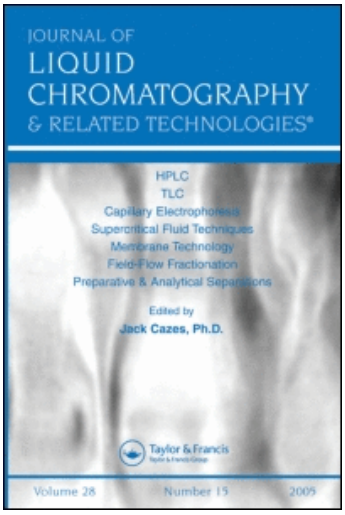

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-ELECTROCHEMICAL DETECTION APPLIED TO MONITORING CLOPAMIDE IN HUMAN URINE AND PHARMACEUTICAL FORMULATIONS

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ABSTRACT

A simple and sensitive HPLC-EC method has been developed for the determination of the benzothiazidic diuretic 4-Chloro-N-(2,6-dimethylpiperidino)-3-sulphamoylbenzamide (Clopamide), using a μBondapack C18 column with a mobile phase of acetonitrile-water (35:65) containing 5mM sodium acetate/acetic acid at a flow rate of 1mL/min. After a simple alkaline liquid-liquid extraction procedure with ethyl acetate, clopamide can be extracted from urine with percentages of recovery greater than 95%. The compound can be detected and measured with good reproducibility and an experimental quantitation limit of 20 ng/mL at a positive potential of +1400 mV. Clopamide has been determined in the pharmaceutical formulation Brinerdine (clopamide 10 mg, reserpine 0.1 mg, dihydroergotoxine mesilate

0.58 mg and tartrazine 0.064 mg). The method developed has been also applied to the determination of clopamide in human urine samples obtained at different time intervals after the ingestion of a single dose of Brinerdine.

INTRODUCTION

4-Chloro-N-(2,6-dimethylpiperidino)-3-sulphamoylbenzamide (Clopamide) is an oral mild diuretic agent of the benzothiazide family that shares the same aromatic sulphonamide base as the thiazide diuretics but without the double-ring structure characteristic of these compounds.[1] Clopamide acts on the proximal convoluted tubule to inhibit the reabsorption of sodium, chloride and hence water, with a mechanism of action similar to that of the thiazide diuretics. Clopamide also causes an increase in potassium excretion in the urine and hypokalemia may occur.[2] The drug is often given with a supplementary potassium to minimize this effect.[3] The diuretic effect is less intense than that of the loop diuretics.

The main therapeutic use is the control of mild to moderate hypertension but it is also used for the management of congestive heart failure and hepatic disorders.[4] For these purposes a daily dose of 5 to 20 mg/day is recommended.[5]

Clopamide causes a diuresis that starts within 2 hours from drug administration. The maximum diuretic effect is usually seen within 3 to 6 hours, corresponding to the peak plasma drug concentrations, and the effect will last for 12-24 hours.[6] Metabolism is complex; the main products are hydroxiderivatives on the piperidinic ring, which then can be conjugated with sulphate or glucuronic acid. Different authors have reported that clopamide is excreted 25% or 30-40% unchanged in urine.[7]

Due to its mild diuretic effect, clopamide is available in oral forms alone or in combination with a b-blocker: pindolol. The combined oral administration of pindolol and clopamide has been found to be more effective than either drug alone on treatment of hypertension.[8] Also the triple combination of clopamide, reserpine and dihidroergocristine was shown to lower blood pressure both at rest and during exercise.

Some methods have been reported for the analysis of clopamide. Most of them involve the determination in plasma using high performance liquid chromatography with photometric detection.[9,10] The determination of clopamide in tablets using spectrophotometric methods has also been reported.[11,12] For the assay of the clopamide-pindolol combination in tablets, a

thin layer chromatography method[13] has been published, which allows the simultaneous determination of the two drugs in pharmaceutical formulations.

The aim of this paper is the development of a HPLC-EC method for the determination of clopamide in pharmaceutical formulations and human urine obtained from healthy volunteers.

MATERIALS AND METHODS

Apparatus and Column

The HPLC system consisted of a Model 2150-LKB (Pharmacia, Barcelona, Spain) HPLC pump, and a Rheodyne (Pharmacia) Model 7125 injector with a loop of 20μL.

An amperometric detector PAR Model 400 equipped with a glassy carbon cell (EG&G Princeton Applied Research, Madrid, Spain) was used to carry out the electrochemical detection. The detector potential was set at +1400 mV vs a Ag/AgCl electrode, in the DC mode with a 5-s low-pass filter, and a current range between 0.2 and 100 nA. The chromatographic response was recorded by a LKB Model 2221 integrator setting with an attenuation of 8 mV full scale and a chart speed of 0.5 cm/min.

The column used was a 125 Å μBondapak C18, 30 cm x 3.9 mm I.D., 10-μm, (Waters Assoc.). A μBondapak C18 precolumn module (Waters Assoc.) was used to protect column from degradation. To study the influence of the temperature, a Waters TMC temperature control system was used.

The extracted urine samples were evaporated to dryness with a Zymark TurboVap LV evaporator (Barcelona, Spain).

Reagents and Stock Solutions

Clopamide was kindly supplied by Sandoz Pharma (AG Basel/Schweiz), and was used without any further purification. HPLC grade acetonitrile and methanol were purchased from Lab-Scan (Dublin, Ireland), and water used was obtained by the Milli-RO and Milli-Q Waters systems. All the reagents used were Merck Suprapur (Bilbao, Spain).

A stock solution containing 1000 μg/mL of clopamide was prepared in pure methanol and stored in the dark under refrigeration. Standard solutions were made from this one and prepared fresh every week.

Urine samples were collected, frozen and kept at -18°C until required for the analysis.

Chromatographic Conditions

A mixture acetonitrile-water (35:65) containing 5 mM sodium acetate/acetic acid was used as mobile phase. pH was adjusted to 5.5 and the buffer served as the supporting electrolyte. This phase was filtered through Millipore membrane filters of 0.45-μm porosity, and the filtrate was degassed by bubbing helium through. The μBondapak C18 column head-pressure was 69 bar at a flow rate of 1.0 mL/min. The injection volume was 20 μL. Chromatographic separations were performed at room temperature.

Electrode Maintenance

The electrode was cleaned electrochemically by keeping it at -800mV for 2 min and after that at +1600 mV for 5 min. This operation was carried out at the end of each working day, using a mobile phase of pure methanol at a flow rate of 1.5 mL/min. When the base line was poorly defined, the glassy carbon electrode was cleaned with a tissue wet with methanol to remove posible adsorbed compounds, and rinsed with deionized water.

Assay of Commercial Tablets

Tablets were reduced to a homogeneous fine powder in a mortar. A suitable amount of this powder was weighed and methanol was added. After shaking for 20 min, the mixture was ultrasonicated for 5 minutes and filtered, and the precipitate washed with the solvent. Solutions obtained after this procedure were made up to 100 mL with methanol. Aliquots of these concentrated solutions were diluted with the mobile phase, and injected into the chromatographic system. Different amounts of initial solid sample were assayed and this procedure was repeated for different tablets in order to obtain a mean value.

Clean-up Procedure for Urine Samples

Urine samples were treated following a liquid-liquid extraction procedure based on the method proposed by Ventura et al.[14] with some modifications. 2 mL urine were alkalinized with 0.5 mL NaOH 5 M, 1.5 mg NaCl(s) were added and 4 mL ethyl acetate. The mixture was shaken for 10 min and centrifuged for 5 min at 734 g. After that, the organic layer was separated and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The dried residue was dissolved in 2 mL of the mobile phase and 20 μL were injected into the HPLC column.

RESULTS AND DISCUSSION

In static conditions, the oxidation of clopamide on a glassy carbon electrode is unable, due to the high potential neccessary for the oxidative process which makes the peak appear behind the cut-off of the electrolyte. However, in the chromatographic system, because of the displacement that takes place in peak potential, it is possible to reach a value which allows the determination of clopamide.

Hydrodynamic voltammetry of the compound was carried out in order to choose the optimum potential value for the amperometric detection of clopamide (Figure 1). An oxidative potential of +1400 mV was chosen as the working potential, since it was the potential which provided the maximum sensitivity for clopamide without increasing the background to high levels.

The chromatographic behaviour of clopamide was not very affected by the pH of the mobile phase in the pH range 3.5-6.5 since the two pKa values of clopamide (2.9 and 9.2)[15] are out of this interval. The study of the influence of pH gave an optimum value of 5.5 which allowed the separation of clopamide from the electrooxidable interferences found in urine, keeping a low retention time (6 min).

The supporting electrolyte used, which is necessary for the amperometric detection, was the buffer sodium acetate/acetic acid. The best signal to noise ratio was provided by an electrolyte concentration of 5mM.

Different ratios of methanol-water and acetonitrile-water containing 5 mM sodium acetate/acetic acid were assayed as the mobile phase. Acetonitrile proved to be better than methanol for the separation of this diuretic since the chromatographic peak was better defined.

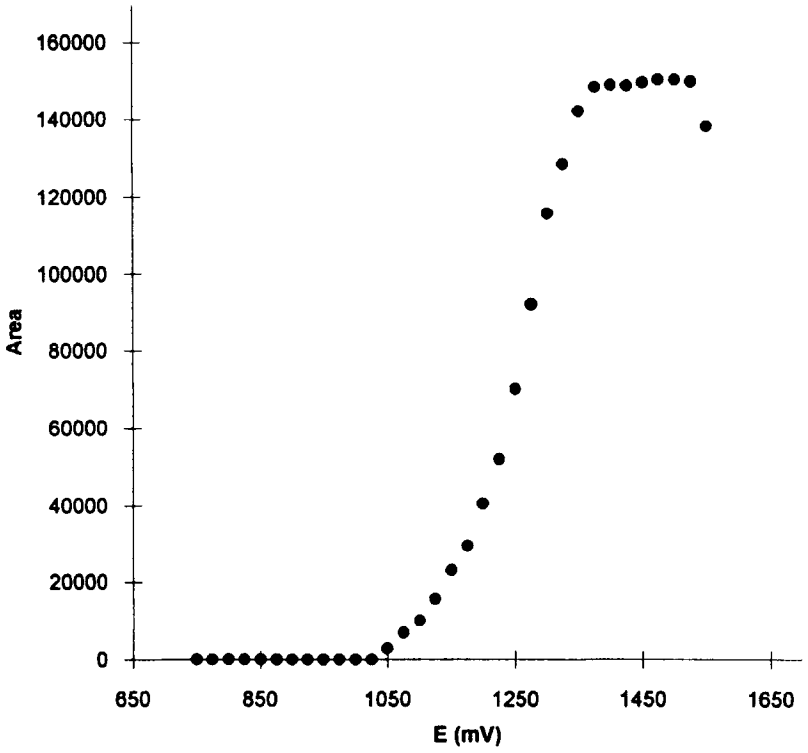

Figure 1. Hydrodynamic voltammogram of clopamide(•). Amount of drug injected: 100 ng in acetonitrile-water (35:65) containing 5mM $NaCH_3COO/CH_3COOH$, pH 5.5.

A study of the influence of the flow rate on the chromatographic behaviour was carried out. As was expected, the peak area decreased with the increase of flow rate, while the effect on the capacity factor k′ (k′=Retention time/injection peak time) was practically negiglible. A value of 1mL/min was chosen as optimum.

A variation in temperature from 26 to 55°C produced small variations on the peak area of chromatograms. A linear relationship between k′-log 1/T was obtained. Since the influence of the temperature was not very relevant, the work was carried out at room temperature.

Table 1

Analytical Parameters for the Determination of Clopamide

Diuretic	Clopamide
Linear concentration range	20 ng/mL- 10μg/mL
Slope of calibration graph (area/[μg/mL])	11501.87
Correlation Coeficient (r^2)	0.9996
Experimental limit of quantitation	20 ng/mL
Reproducibility (%RSD) within-day	1.58 %
Reproducibility (%RSD) day-to-day	8.6 %

The stability of the system was evaluated checking the retention time corresponding to different injections of clopamide. A standard deviation of ± 0.07 min was observed. This deviation is caused by the different integration of the peak area makes by the integrator and not by a real change in the retention time of the compound.

When optimum chromatographic conditions had been established, a quantitative method for the determination of clopamide was developed. In Table 1 are collected the linear regresion of the calibration curve, reproducibility studies (intra-day and inter-day) made on n=10 solutions, in terms of relative standard deviations (% RSD), and the experimental quantitation limit, defined as the minimum concentration of clopamide which gives rise to a signal able to be quantified for the integrator.

The calibration curve for clopamide was linear over the concentration range 20 ng/mL-10 mg/L. The method showed good accuracy since spiked urine samples with different clopamide concentrations gave values very close to the nominal concentrations (Table 2).

Analytical Applications

The method developed was applied to the determination of clopamide in tablets and urine samples obtained from human volunteers.

Table 2

Evaluation of the Precision and Accuracy of the Assay of Clopamide

Nominal Concentration (μg/mL)	Number of Replicates	Observed Concentration (ng/mL)	%Accuracy=(Observ. Conc./Nom. Conc.) x 100
0.250	3	0.253 ± 0.042	101.2 %
0.500	3	0.499± 0.005	99.8 %
1.000	3	0.991 ± 0.027	99.1 %

Table 3

Recoveries for Clopamide Liquid-Liquid Extraction using Different Organic Solvents and NaCl(s)

Organic Solvent	% Recovery
Ethyl acetate	95.96 ± 3.49
Diethyl ether	40.41 ± 2.78
Chloroform	81.12 ± 3.05

Results obtained for pharmaceutical formulations containing clopamide (9.96±0.02 mg/tablet) are in accordance with the values certified by the Pharmaceutical Company (10 mg/tablet). The liquid-liquid extraction procedure of clopamide from urine was evaluated at different concentration levels and with different organic solvents: ethyl acetate, chloroform and diethyl ether. Recovery of clopamide from urine was estimated by comparing the peak area of clopamide in spiked urine samples with those obtained by direct injection of the pure standard solutions of clopamide.

Ethyl acetate provided the best results with high recoveries as can be seen in Table 3 as well as less interfering peaks from endogenous compounds. The extraction procedure was performed with and without NaCl(s), and an increase in recovery was observed with the use of the salt (from 86.19±4.88% to 95.96± 3.49%).

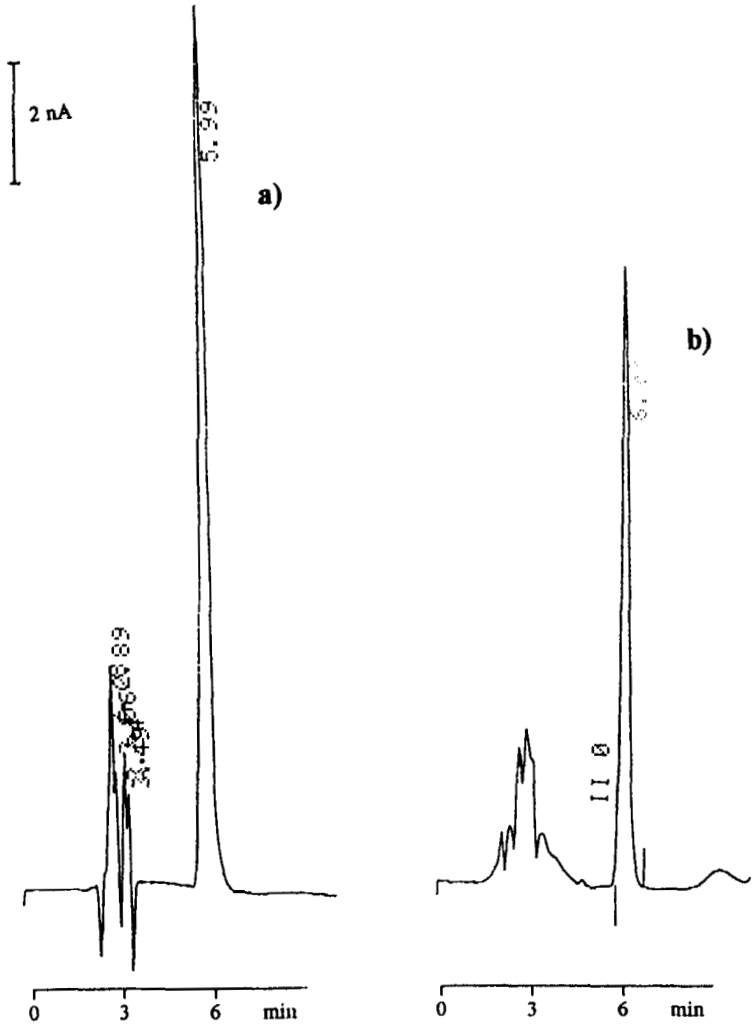

Figure 2. Comparative chromatograms of a) a standard solution of clopamide 5 μg/mL and b) a diluted solution of a pharmaceutical formulation containing clopamide: Brinerdine (-clopamide 10 mg, dyhydroergotoxine mesilate 0.58 mg, reserpine 0.1 mg and tartrazine 0.064 mg-). E= +1400 mV; Flow rate: 1mL/min; Full current scale: 20 nA.

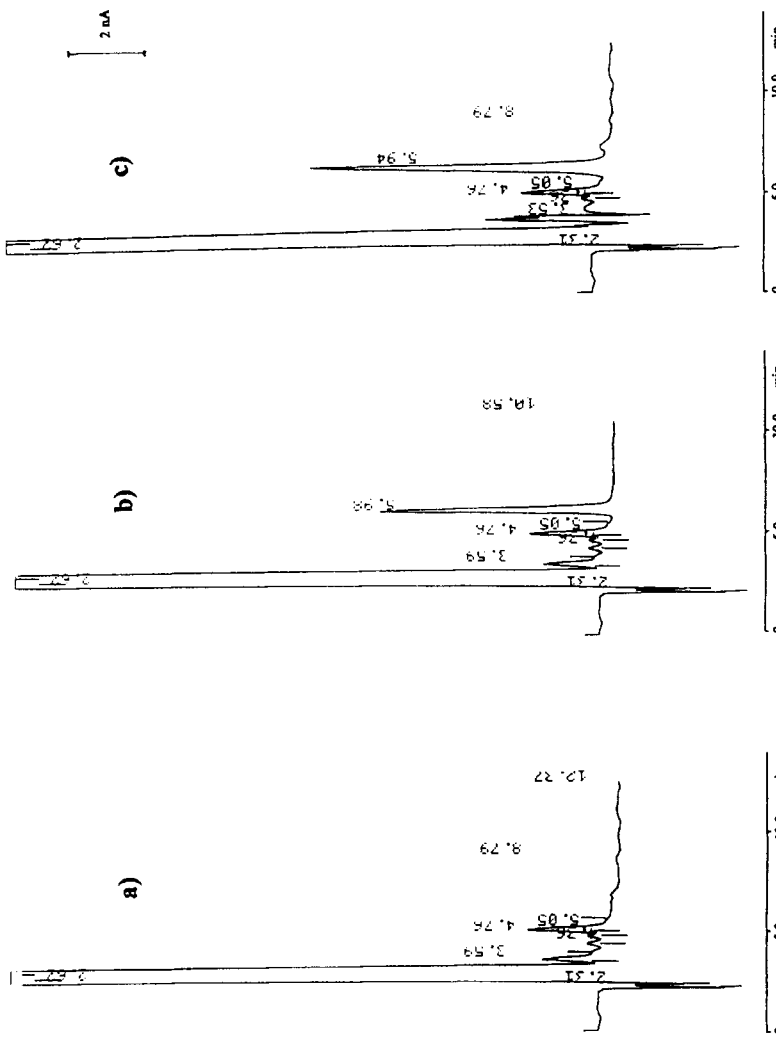

Figure 3. Typical chromatograms of extracts of human urine: a) blank urine sample, b) urine spiked with 1 mg/mL clopamide and c) urine with clopamide from a healthy subject 2-8 hours after an oral dose of Brinerdina (clopamide, dihidroergotoxine mesilate, reserpine and tartrazine). Full current scale: 20 nA. Chromatographic conditions as in Figure 2.

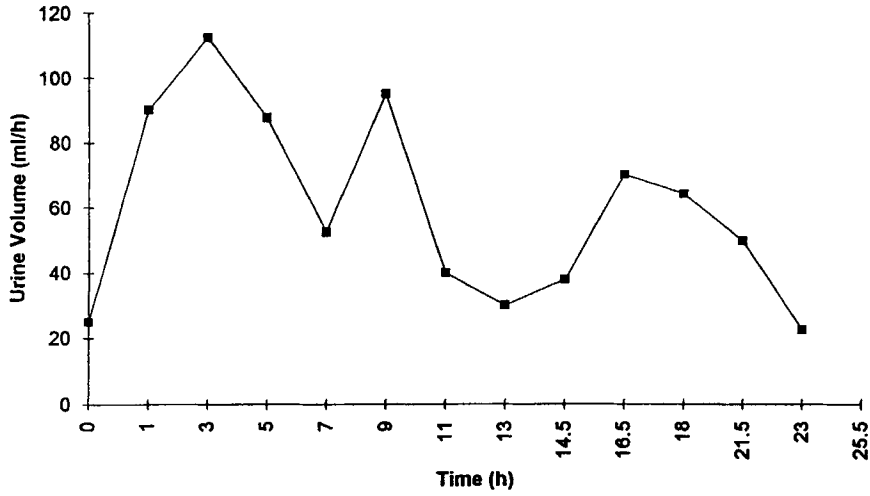

Figure 4. Excretion rate of urine after oral administration of a single dose of Brinerdine (clopamide 10 mg) to a healthy female volunteer.

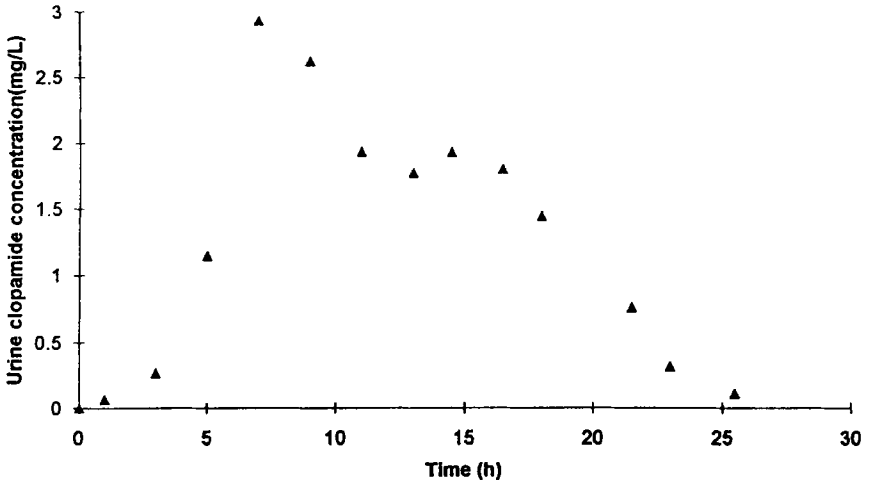

Figure 5. Mean urine concentrations of clopamide versus time in a healthy subject after an oral administration of a single dose of Brinerdine (clopamide 10 mg).

Figure 2 shows the comparative chromatograms of standard clopamide and a dilute solution of the pharmaceutical formulation containing this diuretic.

In Fig. 3, the chromatograms corresponding to blank urine and clopamide in spiked and real urine samples can be observed. In Figs. 4 and 5, the excretion rates of urine and urinary clopamide are plotted.

Both excretion rates represent the average during the collection interval and have been plotted at the midpoint of the interval and are in accordance with the values found in literature.[7] The peak excretion rate of clopamide is about 6 hours which is in agreement with its mild diuretic potency.

DISCUSSION

Clopamide is a usual oral diuretic agent for the treatment of hypertension, alone or in combination with other drugs as beta-blockers. Due to this fact, its determination in urine samples is of great interest.

Different attempts were made to develop a static voltammmetric method for the determination of clopamide. It was impossible, since due to the high potential value neccessary for its oxidation, the peak appears after the cutt-off and only a shoulder was observed in acidic media.

But, with the chromatographic system used, the peak of clopamide can be observed without too many interferences even at high positive potential values.

The method developed allows the determination of clopamide in human urine samples. This paper is not intended to be a study of the pharmacodynamic properties of clopamide, since only one volunteer was used for the sample collection and results may be of no significance. It only shows that the posibility of monitoring this diuretic makes the method useful for pharmacokinetic and pharmacodynamic purposes.

The system was stable despite the high potential neccessary for the oxidation of clopamide. The base line was free from drifts and noises, allowing one to set the scale range at the low nA level necessary for the detection of the small peaks corresponding to very low concentrations of clopamide.

The main interest of this paper is its contribution to a better knowledge of clopamide behaviour since studies about this diuretic are scarce and some of its effects have not been evaluated yet.

ACKNOWLEDGEMENTS

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