ELSEVIER

Journal of Chromatography A, 846 (1999) 383–386

JOURNAL OF CHROMATOGRAPHY A

Short communication

Quantitative determination of Closantel residues in milk by high-performance liquid chromatography with fluorescence detection

G. Stoev*, T. Dakova, Al. Michailova

Central Laboratory for Veterinary Control and Ecology, Iskarsko Shosse 5, Sofia 1528, Bulgaria

Abstract

A HPLC method with fluorescence detection for quantitative determination of Closantel residues in milk has been developed and validated. The proposed cleaning procedure with acetonitrile and acetone extraction, and solid-phase clean-up with Florisil enables concentrations of Closantel below 50 μg/l to be determined. The method was shown to be sufficient, precise, accurate, selective and rugged. The method was applied in the regular monitoring of Closantel residues in milk and of the pharmacokinetic behavior of Closantel in sheep.

Keywords: Milk; Food analysis; Extraction methods; Closantel

1. Introduction

Closantel, *N*-{5-chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl}-2-hydroxy-3,5-diiodobenzamide (Fig. 1), is a potent anthelmitic drug. It binds strongly to plasma proteins [1], which maintains the level of drug in plasma and protects animals for 28 days [2]. The European Union has adopted 1–3 mg/kg as a Closantel maximum residue level (MRL) in foodstuffs of animal origin [3].

Recently, we have found that Closantel at acidic pH values forms a six-membered ring by hydrogen bonding between the hydroxyl group and the amide moiety, acquiring fluorescence properties without derivatization. Taking into account this fact we proposed a more sensitive and selective high-performance liquid chromatography (HPLC) method with fluorescence detection for determination of Closantel in plasma and tissues with lower detection limits [4] than other HPLC–UV methods [5,6,7]. However, this method was inapplicable to milk samples because the recovery of Closantel was only 35%. Until now we have not found a method for quantitative determination of Closantel residues below 1 mg/l in milk. For this reason we developed a new clean-up procedure for the HPLC method with fluorescence detection.

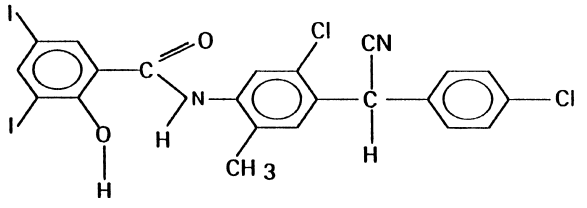

Fig. 1. Structural formula of Closantel.

2. Experimental

2.1. Chemicals and materials

Acetonitrile and methanol were HPLC-grade (Merck). Diethylamine (DEA), methanol (MeOH),

*Corresponding author.

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00135-1

methylene chloride (CH_2Cl_2), orthophosphoric acid (H_3PO_4), *n*-hexane (n-C_6) and acetone, purchased from Fluka were of analytical-grade quality. The water was purified and deionized by a Milli-Q system (Millipore). The solvents for HPLC were filtered using 0.45-μm filters (Millipore) and degassed by an ultrasonic bath.

Closantel (Janssen Pharmaceutica, 99.3%) was used as a standard substance.

Bond Elut cartridges (250 mg, 40 μm): silica gel, Florisil and C_{18} (16% carbon loaded), were purchased from Varian.

2.2. Preparation of standard solutions

A stock solution of Closantel (1 g/l) was prepared by dissolution of 10.0 mg in 10.0 ml acetonitrile and then stored at −4°C. The desired concentrations of 50, 100, 500, 1000 and 3000 μg/l were adjusted by diluting the stock solution with either acetonitrile or cow or sheep milk. Milk samples were purchased from farms, in which the animals were not treated with Closantel.

A sample of 20 μl of each concentration was analyzed five times to define the calibration curve (peak area/concentration).

2.3. Apparatus

The LC system consisted of a Varian Star 9012 solvent delivery pump, Rheodyne injector with 100-μl loop; fluorescence detector (λ_{ex}=335 nm and λ_{em}=510 nm) Waters, Model 470 with Shimadzu C-1R integrator.

A Merck analytical column (150×4 mm LiChrospher 5 μm 100 RP_{18} ODS1) was used in this study. Acetonitrile–water (85:15) mobile phase of pH 2.5 containing 0.05% DEA was pumped at a rate of 1.5 ml/min. The pH value of the mobile phase was adjusted with H_3PO_4 and measured with a pH meter Chemcadet (Cole Parmer).

2.4. Sample preparation

A 5-ml sample of raw milk was extracted with 15 ml acetonitrile (Vortex, 3 min), and filtered (Whatman 541). A second extraction was done by acetone using an ultrasonic bath and the sample was filtered again. The extracts were combined and evaporated in a vacuum evaporator at 50°C to dryness. The residue was dissolved in a mixture of 2–3 ml MeOH–n-C_6(1:9). This volume was purified with a Florisil cartridge. The cartridge was previously conditioned with 10 ml n-C_6. After passing the sample, the cartridge was washed with 5 ml n-C_6. Closantel was eluted with a 10 ml mixture of MeOH–CH_2Cl_2 (60:40), which was evaporated in water bath under nitrogen at 50°C. Dry residues were dissolved in 0.5 ml acetonitrile and 20-μl samples were analyzed by HPLC.

3. Results and discussion

3.1. Sample preparation

Taking into account the high concentration of proteins in milk and their structure, we supposed that the binding of Closantel to them should be stronger than to the proteins in the tissues. An increasing recovery of Closantel by extraction with acetonitrile was achieved by acidification of the sample [4]. However, we ascertained that Closantel is unstable in acid media. At the beginning of the experiments, we extracted the acidified samples with acetonitrile and dissolved the dried residue after the elution of the cartridge in mobile phase of a 0.5 ml (containing H_3PO_4). When we analyzed this sample after 24 h we found that the concentration of Closantel was 25% lower than in immediately analyzed sample. That is why the samples were not acidified and were extracted with acetonitrile. At the second extraction of the samples acetonitrile was replaced with acetone as a more effective extraction solvent. We supposed that acetone with its carbonyl group attacks the hydrogen bonding between amides moieties of proteins and Closantel in a stronger way than acetonitrile. That is why the acidification was not necessary. The recovery at the extraction only by acetone was about 60%. Replacing acetonitrile with acetone at the second extraction of milk samples and using Florisil for solid-phase extraction render a 75% recovery of Closantel at 1000 μg/l. Moreover, acetone showed an excellent deproteinization effect, which resulted in a better purification of the sample and in the absence of peaks in the area of Closantel.

The higher content of fat in milk than in tissues necessitates a more rigorous clean-up procedure. Fats are usually discarded by extraction with light petroleum or n-C_6 before solid-phase clean-up with a C_{18} cartridge. This procedure is necessary because fats block up the C_{18} packing and decrease the recovery of Closantel and limits the possibility of reusing the cartridge. However, we found out that in an acetonitrile–n-C_6 extraction system the ratio between the concentration of Closantel in acetonitrile and that in n-C_6 was 6:1. It means that n-C_6 washes away 16% of Closantel and reduces the recovery of the method. Therefore silica gel and Florisil cartridges were used instead of C_{18} packing. The lipids and the other non-polar compounds, extracted with acetone from milk, were replaced from these packing materials by washing with n-C_6. We found a 100% recovery in Florisil clean-up from solutions of 0.5% cow butter and 50, 100 and 1000 μg/l Closantel in MeOH–n-C_6 (1:9). The recovery in a clean-up with silica gel of the same solutions was 83%. That is why Florisil cartridges were used in further experiments.

Replacing acetonitrile with acetone in the second extraction of milk samples and using Florisil solid-phase clean-up rendered a 75% recovery of Closantel in the sample preparation. The chromatogram of a blank milk sample (Fig. 2A), demonstrates that the cleaning procedure is satisfactory because there are no interfering peaks. The proposed clean-up procedure enables us to determine concentrations of Closantel below 50 μg/l. This level is many times lower than the MRL adopted by the European Union (1.5 mg/l).

The used Florisil cartridges were regenerated by 10 ml of MeOH–CH_2Cl_2 (60:40) and were reused up to five times without decreasing the recovery of Closantel. This fact confirms the success of the cleaning procedure.

3.2. Validation of the method

3.2.1. Recovery, precision, accuracy and sensitivity

The extraction recovery was determined by comparing the peak area of Closantel extracted from milk samples with that of the unextracted standard containing the same amount of Closantel. The mean recovery ranged from 66 to 78% in the concentration range of 50–3000 μg/l, and that of Closantel at 500 μg/l was 73% (Table 1). The peak area was linear between 50 and 2000 μg/l of Closantel. The correlation coefficient, *r*, of the standard curve was 0.995. It was estimated that the limit of quantification of Closantel in milk was 25 μg/l. The detection limit, determined as a concentration resulting in a signal-to-noise ratio of 3, was 10 μg/l, which is many times lower than the MRL adopted by the European Union level: 1.5 mg/l.

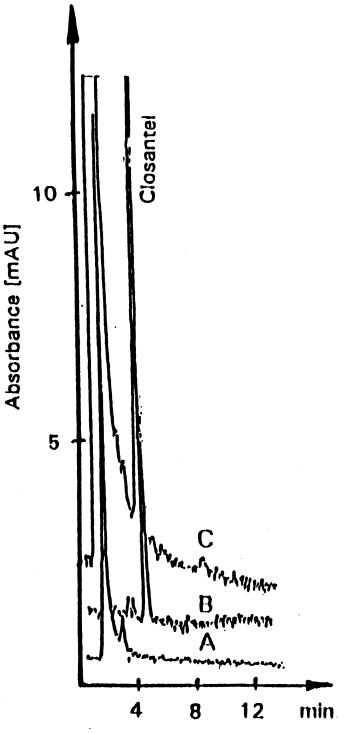

Fig. 2. Chromatograms of milk samples. (A) Blank milk sample, (B) milk sample spiked with 100 μg/l Closantel, (C) milk sample from the monitoring of sheep, containing 560 μg/l Closantel.

3.2.2. Selectivity

Fluorescence detection of Closantel at λ_{ex}=335 nm and λ_{em}=510 nm guaranteed the high selectivity of the method because the probability of another compound acquiring fluorescence properties at this excitation and emission and pH<4.0 is very small.

3.2.3. Ruggedness

Ruggedness was evaluated by six replicate injection of one sample containing 100 and 500 μg/l

Table 1
Extraction efficiency, within- and between-day reference standard deviations (RSDs) as determined with 50–3000 μg/l concentration in a milk sample

Concentration (μg/l)	Extraction recovery (%)	Within-day RSD (%)	Between-day RSD (%)
50	66	3.6	7.6
100	71	3.1	6.4
500	73	1.8	4.2
1000	75	1.1	3.6
78	0.8	4.8	

of Closantel. Analyses were performed during five days with different standard solutions, mobile phases and by two different analysts with two equipments. This procedure was repeated after two months without considerable deviations (Table 1). Over 400 samples were analyzed with the same column. The pre-column was only replaced after the analysis of 120–140 samples, when the pressure increased from 80 bar (fresh pre-column) to 200 bar (dirty pre-column).

Chromatograms of three milk samples are presented in Fig. 2. They have been analyzed in different days. The results show that system and method precision, as well as ruggedness, were within the acceptable range (RSD$<$2.5%) and suggest that the method would be transferable between different laboratories.

Method accuracy results, expressed as percent recoveries, are summarized in Table 1. Acceptable mean recoveries and RSD values are shown, although the recovery parameter displayed values are slightly lower than desired at the lowest tested concentration (50 μg/l). However this fact has little, if any, practical relevance because even these low concentrations can be quantified with acceptable accuracy.

4. Conclusions

A HPLC method with fluorescence detection characterized by good reliability for quantitative determination of Closantel residues in milk has been developed. The proposed cleaning procedure with acetonitrile and acetone extraction and solid-phase clean-up with Florisil enables the quantitative determination of concentrations of Closantel below 50 μg/l. This level is considerably lower than the MRL level of 1.5 mg/l adopted by the European Union.

References

[1] M. Michiels, W. Meuldermans, J. Heykans, Drug Metab. Rev. 18 (1987) 235.
[2] C.A. Hall, J.D. Kelly, H.V. Whitlock, L. Ritchie, Res. Vet. Sci. 31 (1981) 104.
[3] Establishment of Maximum Residue Levels of Veterinary Medical Products in Foodstuffs of Animal Origin, Council Regulation No. 2377/90, European Union, Brussels.
[4] G. Stoev, J. Chromatogr. B 710 (1998) 234.
[5] H.A. Benchaoui, Q.A. McKeller, Biomed. Chromatogr. 7 (1993) 181.
[6] B. Roudant, J. Liq. Chromatogr. Rel. Technol. 19 (1996) 1097.
[7] C.E.-P. Malan, M.M. de Villas, J.S. Handford, E. Swanpool, Pharmazie 51 (1996) 250.