ELSEVIER

Journal of Chromatography B, 710 (1998) 234–238

JOURNAL OF CHROMATOGRAPHY B

Short communication

Determination of closantel residues in plasma and tissues by high-performance liquid chromatography with fluorescence detection

G. Stoev

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

Received 6 August 1997; received in revised form 24 February 1998; accepted 24 February 1998

Abstract

The influence of the pH of the mobile phase with some modifiers on the chromatographic behavior and fluorescence properties of closantel have been investigated. At acidic pH values (2–6), the benzamide moiety of the closantel forms a six-membered ring by hydrogen bonding and possesses a native fluorescence. Using the fluorescence emission of closantel at λ_{ex}=335 nm, λ_{em}=510 nm, and pH 2.5 of the mobile phase, a linear calibration curve was estimated over a concentration range of about two orders of magnitude with a correlation coefficient larger than 0.992. The limit of the fluorescence detection was 10 μg/kg. This value was at least 10 times lower than that using UV detection. The method was applied to the determination of closantel in plasma and tissue samples, purified by a solid-phase extraction with C_{18} cartridges.

Keywords: Closantel

1. Introduction

Closantel, *N*-(5-chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl)-2-hydroxy-3, 5-diiodobenzamide (Fig. 1), is a potent antihelminthic drug. It binds strongly to plasma proteins [1], which prolongs drug levels in plasma and protects animals for up to 28 days [2]. The European Community adopted for closantel a maximum residue level of 1–3 mg/kg in foodstuffs of animal origin [3].

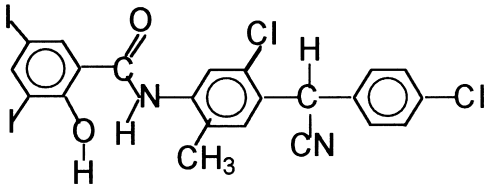

Fig. 1. Structural formula of closantel.

High-performance liquid chromatographic procedures with ultraviolet detection (HPLC–UV) procedures for determination of closantel in plasma and tissues [4–6] as well as in tablets and suspensions [7] were developed. The detection limits of these methods were between 1.0 mg/kg [4,5] and 0.5 mg/kg [6], e.g. below the maximum residue levels. Although solid-phase extraction was used in the analyses of plasma and tissues, the calibration graphs were linear above 3 mg/kg because some impurities passed through the cartridge.

0378-4347/98/$19.00
PII: S0378-4347(98)00114-5

Recently we have found that closantel at acidic pH values exhibits fluorescence properties without derivatization. Taking into account this fact, we proposed a more sensitive and selective HPLC method with fluorescence detection for determination of closantel in biological media with a lower detection limit than other HPLC–UV methods.

2. Experimental

2.1. Chemicals

Acetonitrile and methanol were HPLC-grade (Merck, Darmstadt, Germany). Dibasic potassium phosphate (Merck) and diethylamine, orthophosphoric acid and heptanesulphonic acid natrium salt (Fluka, Buchs, Switzerland) were of analytical-grade quality. The water was purified and deionized by Milli-Q system (Millipore, Milford, MA, USA). The solvents for HPLC were filtered with 0.45-μm filters (Millipore) and degassed by ultrasonic bath.

Closantel obtained from Janssen Pharmaceutica was used as a standard substance. A fresh solution of closantel was prepared in acetonitrile and the desired concentrations were adjusted by diluting.

Bond Elut C_{18} cartridges (250 mg, 18% carbon loaded end-capped silica gel, 40 μm) were purchased from Varian (Walnut Creek, CA, USA).

2.2. Apparatus

The Varian LC system consisted of a Star 9012 solvent delivery pump, Polychrome 9065 diode array detector and Varian Star data system; Rheodyne injector with 100-μl loop; fluorescence detector Waters, mod. 470 with Shimadzu C-1R integrator.

The Merck analytical column (125×4 mm Lichrospher 5-μm 100 RP_{18} ODS1) was used in this study. The mobile phase was pumped at a rate of 1 ml/min. The pH-value of the mobile phase was measured with a pH-meter Chemcadet (Cole Parmer). The void volume of the chromatographic system was determined by injecting an aqueous solution containing sodium nitrate.

The UV spectra of closantel at different pH-values and solvents were obtained by Varian Cary 1E UV–Vis spectrophotometer.

2.3. Sample preparation

A 3-ml sample of plasma or 3-g sample of tissue (ground) was mixed for 3 min with 3 ml of 0.05% acetic acid in water saturated with sodium chloride and 10 ml of acetonitrile using a high-speed blender. The mixture was centrifuged for 5 min at 2400 *g*. The supernatant was transferred into a flask, diluted with 25 ml of acetonitrile for an additional precipitation of proteins, centrifuged again and concentrated to 2–3 ml at 50°C under a vacuum. This volume was purified by C_{18} cartridge. Best results were obtained if the cartridge was previously conditioned with 5 ml of acetonitrile and a 5-ml mixture of 10% acetonitrile and 90% water with 0.1% diethylamine. Closantel was eluted with 5 ml of acetonitrile and the eluate was concentrated to 0.3 ml. A sample of 30 μl was analyzed with HPLC.

3. Results

A considerable decrease of the capacity factor and the electronic density of the moieties of closantel were observed when the pH was changed from acidic to basic values (Fig. 2). There were no significant differences between the spectra of closantel in

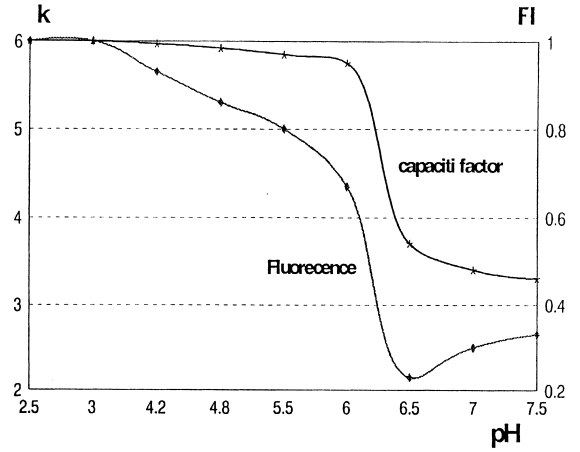

Fig. 2. The effect of the pH of the mobile phase on the capacity factor *k* and the fluorescence of closantel. Chromatographic conditions: Nucleosil 100, C_{18}, 5 μm, 150×4.6 mm; mobile phase: acetonitrile–water (80:20), 10 m*M* K_2HPO_4; pH was adjusted by H_3PO_4; the maximal fluorescence at pH 2.5 was accepted as 1.0.

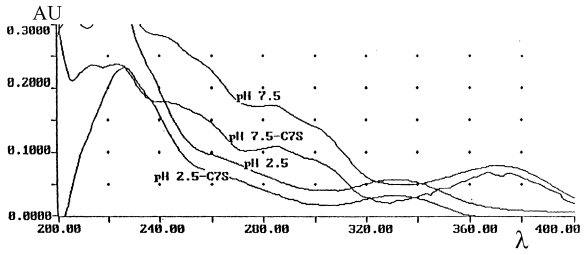

Fig. 3. Effect of pH on the UV absorption of closantel: 1 μmol/l, 1-cm cell; C_7S-heptanesulphonic acid natrium salt (0.05%).

water–acetonitrile or water–methanol mixtures with or without modifiers as phosphate buffer, diethylamine and heptanesulfonic acid sodium salt (Fig. 3). The only difference was the decreasing of the intensity of the band spectra. The asymmetry factor, measured at 5% of the high of the closantel peak at pH 4,0 was less than 3. That is why closantel was analyzed with an acidic mobile phase (pH 2.5) without modifiers.

It was found at UV-absorption maxima of λ_{ex}= 335 and 375 nm that closantel displayed native fluorescence at λ_{em}=510 and 425 nm, respectively. Varying the pH of the acetonitrile–water (80:20) mobile phase, it was established that the fluorescence intensity of closantel (1 μmol/I) at λ_{ex}=335 nm was about thirty times higher than that at λ_{ex}=375 nm, although the electronic absorption at λ=375 nm was stronger than the adsorbtion at λ=335 nm. Maybe for this reason Hennessy et al. [6] analyzed closantel with HPLC at λ=375 nm.

The detection limits of UV and fluorescence were compared by diode array and fluorescence detectors connected in a cascade. Depending on the origin (impurities) of the sample, the limit of UV detection of closantel at λ=254 nm and the signal/noise ratio (S/N)=5, was about 0.2–0.5 mg/kg. The limit of fluorescence detection at λ_{ex}=335 nm and λ_{em}=510 nm was 10–50 μg/kg.

Recovery assays were performed with both detection modes in the ranges of 0.5–10.0 mg/kg fortification for UV detection and 0.05–5.0 mg/kg for fluorescence detection (Table 1). The recovery results were in the interval x=80±11% (plasma, muscle) and x=86±11% (fat, kidney). The lowest recovery value: 73±12% was observed at liver

Table 1
Recovery results of spiked samples (n=9) at HPLC–UV and fluorescence detection

Sample	Fortification level (mg/kg)	Recovery method UV	Recovery method FI
Plasma	0.05	[a]	78.4±12.1%
	0.5	78.9±12.5%	79.3±11.8%
	5.0	79.2±11.6%	81.1±11.0%
	10.0	81.4±10.9%	
Muscle	0.05	[a]	78.9±9.8%
	0.5	78.8±9.9%	80.1±10.8%
	5.0	80.1±11.2%	82.3±10.4%
	10.0	80.6±9.6%	
Fat	0.05	[a]	84.4±12.0%
	0.5	84.2±11.6%	85.1±11.2%
	5.0	86.4±10.3%	85.9±11.0%
	10.0	87.2±12.0%	
Kidney	0.05	[a]	85.1±12.2%
	0.5	86.8±12.3%	85.2±11.1%
	5.0	87.0±10.0%	87.7±10.2%
	10.0	87.9±12.4%	
Liver	0.05	[a]	71.2+14.2%
	0.5	70.6±14.6%	72.9±12.2%
	5.0	72.2±9.6%	74.0±11.3%
	10.0	75.2±11.6%	

[a] Determination was not possible.

samples because extracts give some interfering peaks in spite of the severe purification procedure.

The linearity of the fluorescence detection in the concentration range 0.05–10.0 mg/kg had a correlation coefficient larger than 0.993.

It is necessary to point out that the UV spectrum of closantel at 0.2 mg/kg was destroyed and was not as clean as that at a higher concentration. For this reason, the identification of closantel is not reliable despite using a diode array detector. On the other hand, the fluorescence response of closantel allows selective detection without matrix interference (Fig. 4).

4. Discussion

Waterbeemd and Testa [8] have shown that orthomethoxybenzamides are fixed in a planar conforma-

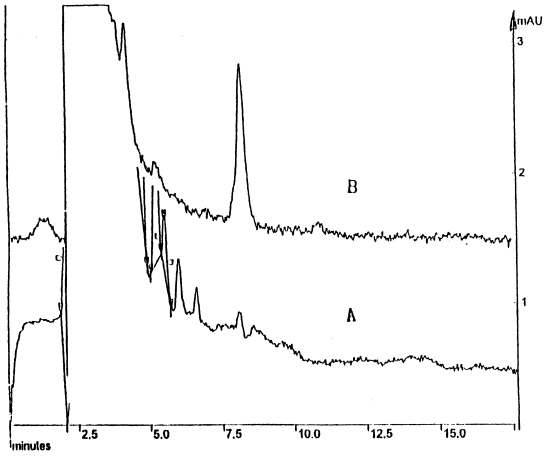

Fig. 4. Chromatograms of liver samples, obtained by UV detection at λ=254nm (A) and fluorescence detection λ_{ex}335 nm/λ_{em}510 nm (B). HPLC conditions: C_{18} Nucleosil 5 μm 100A, 150×4.6 mm column; mobile phase: 85% AcCN and 15% H_2O; pH 2.5 of the mobile phase was adjusted by H_3PO_4.

tion by an intramolecular six-membered ring hydrogen bond between the hydrogen of the amide group and the oxygen of the methoxy group. This association decreased the nonplanarity of the benzamide molecule by decreasing the angle between the aromatic ring and the amide moiety [9]. The formation of the six-membered ring hydrogen bond decreased the number of interaction sites of the analyte available for association with the solvent resulting in a greater reversed-phase LC retention [10].

At acidic pH values the oxygen atom of the orthohydroxyl group of closantel is protonated and is capable of acting as a proton acceptor. The proton at the nitrogen is more movable and the amino group acts as a donor. The six-membered ring hydrogen bond formed (Fig. 5) increases the hydrophobicity of

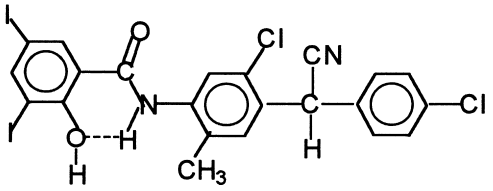

Fig. 5. Six-membered ring hydrogen bond.

closantel which is reflected in the capacity factor. At basic pH values, the hydroxyl group and H-atom at the amide group are free. They interact stronger with the water of the mobile phase and the capacity factor is low (Fig. 2).

The pH strongly influences the UV adsorption (Fig. 3). Modifiers such as diethylamine, heptanesulfonic acid natrium salt and phosphate buffer for (water–acetonitrile or water–methanol) mobile phase for HPLC do not influence the UV adsorption of closantel. They only insufficiently decrease the intensity of the spectra. We assume that diethylamine and heptanesulfonic acid form complexes with the oxygen atom of the hydroxyl group or nitrogen atom at the amide moiety, respectively, and decrease the possibility of an intramolecular hydrogen bond. These facts mean that the formation of the six-membered ring H-bond is possible when the oxygen atom is protonated and is influenced mainly by the pH of the solution.

The fluorescence of closantel depends on the pH value of the mobile phase approximately in the same way as the capacity factor (Fig. 2), and the maximum fluorescence intensity is at λ_{ex}=335 and λ_{em}= 510. We suppose the insufficient fluorescence emission at λ_{ex}=375 (pH 7.5) is a result of the higher pH-medium, which acted as an unfavorable environment and obstructed the forming of a six-membered ring H-bond. The fluorescence emissions of the ortho substitute benzamides were stronger at acidic (1–6) than at alkaline (8–12) pH values, too [11].

The UV absorption of closantel at the universal wavelength λ=254 nm is considerably stronger than at λ=335 nm. Because the noise of the fluorescence detector at λ=254 nm is very strong, this excitation has no practical value. The lower UV adsorption means that the excitation at λ=335 nm is not as strong as at λ_{ex}=254 nm and the intensity of the fluorescence emission would not be large. In spite of everything, the detection limit of the fluorescence detection is considerably lower than the detection limit of the UV detection at λ=254. This prerequisite was used for the quantitative determination of closantel in biological fluids and tissues. The advantages of this method, low detection limit and good reproducibility, were successfully tested in a routine monitoring program for blood, kidney, liver, muscle, fat and skin samples.

5. Conclusion

At acidic pH values, closantel forms an intramolecular hydrogen bond at the benzamide moiety and acquires a native fluorescence. This structure decreases the polarity of closantel and HPLC analysis can be carried out with a mobile phase without modifiers such as heptanesulphonic acid or diethylamine. By virtue of this fact, a HPLC method of quantitative determination of closantel in plasma and tissues with a high sensitivity and reproducibility has been proposed.

Acknowledgements

The author thanks Mrs. Albena Michaylova for her assistance.

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 of EEC.
[4] R. Woestenborghs, J. Hendrickx, L. Michielsen, J. Heykants, Residues of some veterinary drugs in animals and foods, FAO Food and Nutrition, Paper 41/5 (1992) 15.
[5] B. Roudant, J. Liq. Chromatogr. Relat. Technol. 19(7) (1996) 1097.
[6] D.R. Hennessy, E. Lacey, J.W. Steel, R.K. Prichard, J. Vet. Pharmacol. Ther. 10 (1987) 64.
[7] C.E.-P. Malan, M.M. de Villas, J.S. Handford, E. Swanepool, Pharmazie 51(4) (1996) 250.
[8] P. Van de Waterbeemd, B. Testa, J. of Med. Chem, 26 (1983) 203.
[9] C.C.F. Blake, R.W.H. Small, Acta Cryst. B28 (1972) 2201.
[10] C.R. Clark, M.J.M. Wells, R.T. Sansom, J.L. Humerick, W.B. Brown, B.J. Commander, J. Chromatogr. Sci. 22 (1984) 75.
[11] M. Buna, J.-J. Aaron, P. Prognon, G. Mahuzier, Analyst 121 (1996) 1551.