Determination of Closantel Residues in Milk and Animal Tissues by HPLC with Fluorescence Detection and SPE with Oasis MAX Cartridges

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Abstract

A liquid chromatographic method for the determination of closantel residues in milk and tissues is developed and validated. An acetonitrile–acetone solution (80:20, v/v) is used for the extraction of closantel residues from milk and animal tissues, and the extract is purified by solid-phase extraction with Oasis MAX cartridges and a mixture of formic acid–acetonitrile (5:95, v/v) as the elution solution. A C_{18} bonded silica column is used for chromatographic separation. The mobile phase consists of acetonitrile–water (85:15, v/v) containing 0.05% triethylamine at pH 2.5, adjusted with phosphoric acid with the flow-rate set at 1.0 mL/min. Using the fluorescence emission of closantel at λ_{ex} = 335 nm and λ_{ex} = 510 nm, the calibration curve is linear, with a correlation coefficient of 0.9999 over the concentration range of 10–5000 µg/kg for the tissue sample and 10–5000 µg/L for the milk sample. The detection limit (s/n = 3) is 3 µg/kg for tissue sample and 3 µg/L for milk sample. The intra- and inter-day repeatabilities are between 3.35–7.66% and 4.04–8.67%, respectively. The proposed method enables the quantitative determination of closantel residues at levels as low as 10 µg/kg in animal tissue samples and 10 µg/L in milk samples.

Introduction

Closantel, *N*-(5-chloro-4-[(4-chlorophenyl)cyano-methyl]-2-methylphenyl)-2-hydroxy-3,5-diiodo-benzamide, is a broad-spectrum antiparasitic agent used against several species of trematodes, nematodes, and arthropods used in the prevention and treatment of parasitic infections in cattle and sheep. It bonds strongly to plasma proteins (1), and thus prolongs drug levels in plasma and protects animals for up to 28 days (2). The European Union has adopted a maximum residue level (MRL) of 100–300 µg/kg for closantel in foodstuffs of animal origin (3).

High-performance liquid chromatography (HPLC) has been used for the determination of closantel in plasma and tissues by UV detection, with the detection limits of 1000 µg/kg and 500 µg/kg in tablets and suspensions (4–6). The fluorescence detection for HPLC was better than the UV detection in terms of detection ability (7). The quantitative determination of closantel residues in plasma and tissues using extraction with acetonitrile and cleanup by SPE with C_{18} cartridges has been described, and the limit of the fluorescence detection was 10–50 µg/kg (7). Another HPLC–fluorescence detection method has been described using a Merck analytical column and an acetonitrile–water (85:15, v/v) mobile phase of pH 2.5 containing 0.05% DEA for the quantitative determination of closantel residues in milk by extraction with acetonitrile and acetone in turn, followed by cleanup with solid phase extraction (SPE) Florisil cartridges and MeOH–CH_2Cl_2 elution reagent. The detection limit was 10 µg/L, and the recovery changed from 66% to 78% (8). The two previously mentioned methods are not suitable for the simultaneous determinations of closantel residues in both tissue and milk samples.

The main purpose of this work was to develop an improved method for the determination of closantel at trace levels in both milk and tissue by HPLC with fluorescence detection using SPE with Oasis MAX cartridges. The mean recovery of closantel in both milk and tissue samples ranged from 81.5% to 87.4%. The detection limit was 3 µg/kg for tissues and 3 µg/L for milk. The linearity of the fluorescence detection in the concentration range of 10–5000 µg/kg for tissue samples or 10–5000 µg/L for milk samples had a correlation coefficient of 0.9999. The proposed method was applied for the determination of closantel residues in milk and tissue samples with satisfactory results.

Experimental

Chemicals and solutions

Acetonitrile, acetone, ethyl acetate, and methanol were HPLC-grade (Dimark, USA). All other chemicals were commercial analytical reagent grade. The water was purified and deionized. The

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solvents for HPLC were filtered using 0.45-µm filters and degassed by an ultrasonic bath. SPE cartridges Oasis MAX (3 cc) were from Waters (Milford, MA).

The standard of closantel (99.0%) was obtained from Sigma (St. Louis, MO). A stock solution of closantel (1.000 g/L) was prepared by dissolving 10.0 mg the standard in 10.0 mL acetonitrile and then stored at –4ºC. The standard calibration solutions in the range of 50–25000 µg/L were prepared freshly by diluting the stock solution with acetonitrile.

Apparatus

An HPLC system was equipped with an LC-10AT$_{vp}$ pump, an DGU-12A degasser, and RF-10A$_{XL}$ fluorescence detector (Shimadzu, Kyoto, Japan), interfaced with a Shimadzu CLASS-VP 6.0 workstation. The injection volume was 20 µL. An HPLC column (C_{18}, 150 mm × 4.6 mm i.d.) was used. The mobile phase was acetonitrile–water (85:15, v/v) containing 0.05% triethylamine, and was adjusted to pH 2.5 with phosphoric acid. The flow rate was 1.0 mL/min at 35°C. The excitation wavelength (λ_{ex}) was 335 nm, and the emission wavelength (λ_{em}) was 510 nm.

The apparatuses used in the sample preparation were as follows: Homogenizer (Ultra Turrax, IKA, Wilmington, DE); nitrogen evaporator (TurboVap LV, Zymark, Hopkington, MA); vacuum manifold processing station (Agilent, Palo Alto, CA); centrifuge (KUBOTA KN-70, Tokyo, Japan); vortex shaker (MS1 Minishaker, IKA); and ultrasonic cleanser (KQ-500E, Kunshan, China).

Sample preparation

The tissue and milk samples were collected from farms in which the animals were treated with and without closantel.

A 5 g sample of tissue or 5 mL sample of milk was weighed or sucked into a 50-mL centrifuge tube, 15 mL mixture of acetonitrile–acetone (80:20, v/v) was added, it was homogenized for 2 min, extracted for 5 min an in ultrasonic bath, and centrifuged for 5 min at 3000 rpm. The extract was filtered into the reservoir. The Oasis MAX cartridge was placed on the vacuum manifold processing station, and the cartridge was preconditioned by 2 mL acetonitrile–acetone (80:20, v/v). Then the extract was passed through the cartridge at the speed of 2 mL/min, and the cartridge was washed with 2.5 mL ethyl acetate and 3 mL methanol in sequence. Finally, the cartridge was eluted with a 4 mL mixture of formic acid–acetonitrile (5:95, v/v), the eluate was collected in a 10-mL test tube, and evaporated to dryness with a nitrogen evaporator in the water bath at 45°C. The residues were dissolved with 1.0 mL acetonitrile using an ultrasonic bath, and the solution was well mixed using a vortex shaker. The final solution was filtered using a 0.45-µm filter before injection for HPLC determination.

HPLC conditions

Twenty microliters of the prepared solution was injected into the HPLC system for analysis of closantel. ODS-U C_{18} HPLC column (150 mm × 4.6 mm i.d. Cloversil Co. USA) was used. The mobile phase was acetonitrile–water (85:15, v/v) containing 0.05% triethylamine, and adjusted to pH 2.5 with phosphoric acid. The flow rate was 1.0 mL/min at 35°C. The (λ_{ex}) and (λ_{em}) were 335 nm and 510 nm, respectively.

Results and Discussion

Optimization of extraction conditions

Acetone showed an excellent deproteinization effect with its carbonyl group, which could attack the hydrogen bonding between amides moieties of proteins, but the recovery of extraction by acetone alone was approximately 60%. Acetonitrile showed satisfactory extraction efficiency but could not deproteinize completely. Based on the previously mentioned considerations, a mixture of acetonitrile and acetone was used as the extraction solvent. The effect of concentration ratios of acetonitrile and acetone on the extraction efficiency was investigated for different matrixes, as shown in Figure 1.

The use of the acetonitrile–acetone mixture (80:20, v/v) enabled optimal recoveries to be obtained. The recoveries of closantel were over 80% for milk, muscle, kidney, and liver samples. This is why the acetonitrile–acetone (80:20, v/v) mixture was used as the extraction in our work.

Choice of cartridge for SPE

Taking into account the pK_a 4.18 of closantel, different polar cartridges were investigated, including AccuBond II Florisil cartridges (1000 mg, 6 mL, Agilent), AccuBond II Amino cartridges (500 mg, 3 mL, Agilent), Strata SAX cartridges (200 mg, 3 mL, Phenomenex, Torrance, CA), and Oasis MAX cartridges for SPE (Waters). After spiking samples at a level of 1000 µg/kg, they were extracted with a mixture of acetonitrile–acetone (80:20, v/v). The extraction solution was purified using the previously mentioned SPE cartridges, with a mixture of formic acid–acetonitrile (5:95, v/v) as the elution solution. The recoveries of closantel are shown in Table I. The recovery of closantel at 0.1 µg/mL was 86, 70.5, 15.1, and 2% for Oasis MAX cartridges, SAX cartridges, Amino cartridges, and Florisil cartridges, respectively.

Oasis MAX cartridges gave the best recovery (86%) and Florisil cartridges gave the lowest recovery (15.1, 2%). Our suggested reasons are listed as follows: Florisil bonded phases interact by polar-dipole/dipole forces between polar functional groups in the closantel and the polar surface of the sorbent. Amino sorbents

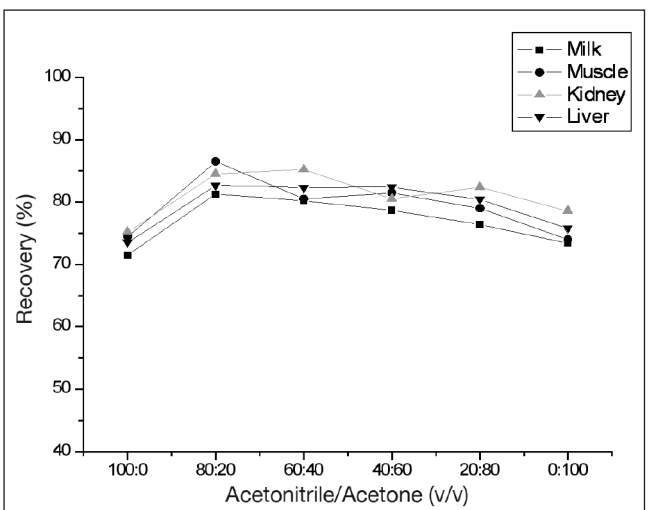

Figure 1. Effect of the extraction solvent in different ratios on the extraction recovery.

interact with analytes by hydrogen bonding, and the sorbents of Oasis MAX cartridges interact primarily through electrostatic attractions between the strong anion-exchange sorbents and closantel. The electrostatic energy of interaction (50–200 kcal/mol) was much higher than the polar-dipole/dipole energy of interaction (1–10 kcal/mol) and the hydrogen bonding energy of interaction (5–10 kcal/mol) (9). Because of this, Oasis MAX cartridges can load closantel from the polar extraction of acetonitrile–acetone (80:20, v/v), and Florisil cartridge sorbents and Amino cartridges only retained a little analyte owing to their weak interaction. At same time, the result of Oasis MAX cartridges was a little better than that of Strata SAX cartridges. Oasis MAX was a strong anion-exchange porous polymer SPE cartridge whose sorbents are divinylbenzene and *n*-vinylpyrrolidone copolymer modified by the trimethylaminopropyl group. The capacity was five times that of trimethylaminopropyl-modified silica sorbent and there was no secondary interaction arising from silanol groups in polymeric sorbents (10). Therefore, the Oasis MAX cartridge was selected for SPE.

The closantel was retained on the Oasis MAX column by ionic interaction with the chloridize quaternary ammonium group. The ionic interactions were much stronger than the non-polar interactions, allowing the use of an extensive wash sequence and giving clear extracts. A mixture of formic acid–acetonitrile (5:95, v/v) was used as the elution reagent, which broke the ionic interaction. An accumulative elution curve of closantel standard (1 µg) was investigated, as shown in Figure 2.

Table I. The Recovery of Closantel Using Different Cartridges (%)

Matrix	MAX cartridges	SAX cartridges	Amino cartridges	Florisil cartridges
Milk	85.9	73.6	17.6	5.7
Muscle	86.7	71.5	15.1	6.8
Liver	84.3	68.7	13.8	4.5
Kidney	83.7	69.8	14.9	4.8

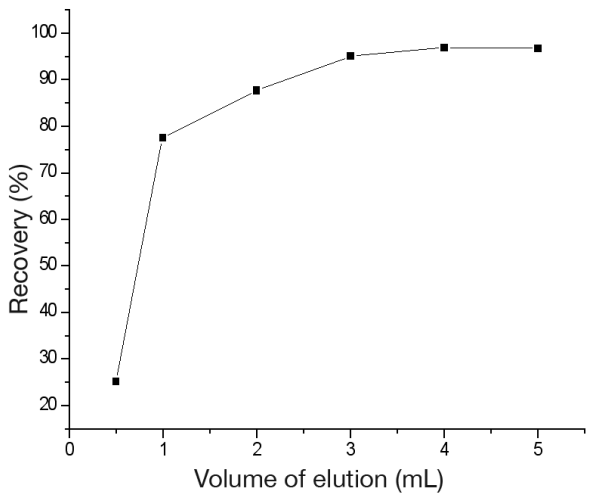

Figure 2. Accumulative elution curve of closantel with a mixture of formic acid–acetonitrile (5:95, v/v) as elution solution.

The results showed that the recovery of closantel obviously increased with the increase of elution solution in the range of 0.5–1.0 mL, and a 3 mL of elution solution was sufficient to elute closantel with 95% of recovery.

Effect of mobile phase on fluorescence

It was indicated that the ortho methoxybenzamides are fixed in a planar conformation by an intramolecular six-membered ring hydrogen bond between the hydrogen of the amide group and the oxygen of the methoxy group, and this association decreased the nonplanarity of the benzamide molecule by decreasing the angle between the aromatic ring and the amide moiety (11,12). The hydroxyl on the phenyl ring of closantel is an acidic group because of the neighboring electron withdrawing iodine (I) atom. At acidic pH values, an intramolecular six-membered ring hydrogen bond could be formed (7,8). It increases the hydrophobicity of closantel, decreasing the number of interaction sites of the closantel available for association with the solvent, which accelerates the analyte to be adsorbed on the C18 column and results in a greater reversed-phase LC retention. The higher pH-medium could obstruct the forming of the six-membered ring H-bond, resulting in a stronger interaction of closantel with the water of the mobile phase and influencing the analyte to be adsorbed. Therefore, the fluorescence emissions of the ortho substitute benzamides were stronger at acidic than at alkaline pH values. The test results showed that maximum fluorescence intensity of closantel was obtained at λex = 335 nm and λem = 510 nm using an acetonitrile–water (85:15, v/v) mobile phase containing 0.05% triethylamine at pH 2.5.

Validation of the method

To support regulatory action, a method must be proven to be accurate and able to identify contaminants with high selectivity. For this purpose, the analytical method evaluation was carried out, including method specificity, linearity, detection limits, quantitation limits, and accuracy in different matrices.

Specificity

The specificity of this method was demonstrated by the representative chromatograms of closantel for a blank sample, a spiked sample, and a standard solution. The chromatograms of closantel for the milk sample and the spiked milk sample are shown in Figure 3.

There were no interferences at the retention times of the compounds of interest, because the probability of another compound with a similar polarity acquiring fluorescence properties at this excitation and emission wavelengths at pH < 4.0 is very small. For the other matrices (bovine liver, kidney, and muscle), there were also no interferences at the retention times of closantel.

Linearity and detection limit

The calibration curve of the peak-area (y) against the closantel content (x) had good linearity. Linear regression analysis was used to calculate the slope, intercept, and correlation coefficient (r) of calibration curves. The equations of linear regression (n = 6) were $y = 1.76 \times 10^6 x - 2.27 \times 10^4$ (r = 0.9999) in the range of 10–5000 µg/kg for tissue samples or 10–5000 µg/L for milk samples. Thus the linearity of the proposed method was quite satis-

factory. Detection limits (LOD) and quantitation limits (LOQ) of the investigated compounds were estimated as the minimum concentration giving a signal–noise ratio (s/n) of 3 and 10, respectively (13,14). The results found were 3 µg/kg and 10 µg/kg for the tissue sample, 3 µg/L and 10 µg/L for the milk sample, respectively.

Precision and accuracy

Three sets (10 each) of blank samples for each matrix were fortified with 10, 50, and 100 µg/kg of closantel. They were analyzed on different days close to each other, with the same instruments and the same operators. The relative standard deviation (RSD) was calculated as repeatability. The analytical results are given in Table II.

The SPE and subsequent HPLC analysis displayed excellent within- and between-day precision for closantel in the milk sample and the tissue sample. For milk samples in the range of 10–100 µg/L, the RSD varied between 4.17% and 7.66% for the intra-day study, and 4.25% and 8.67% for the inter-day study. For tissue samples in the range of 10–100 µg/kg, the RSD varied from 3.35% to 7.24% for the intra-day study and from 4.04% to 8.41% for the inter-day study. The results demonstrate that the accuracy of the present method was acceptable for routine monitoring purposes.

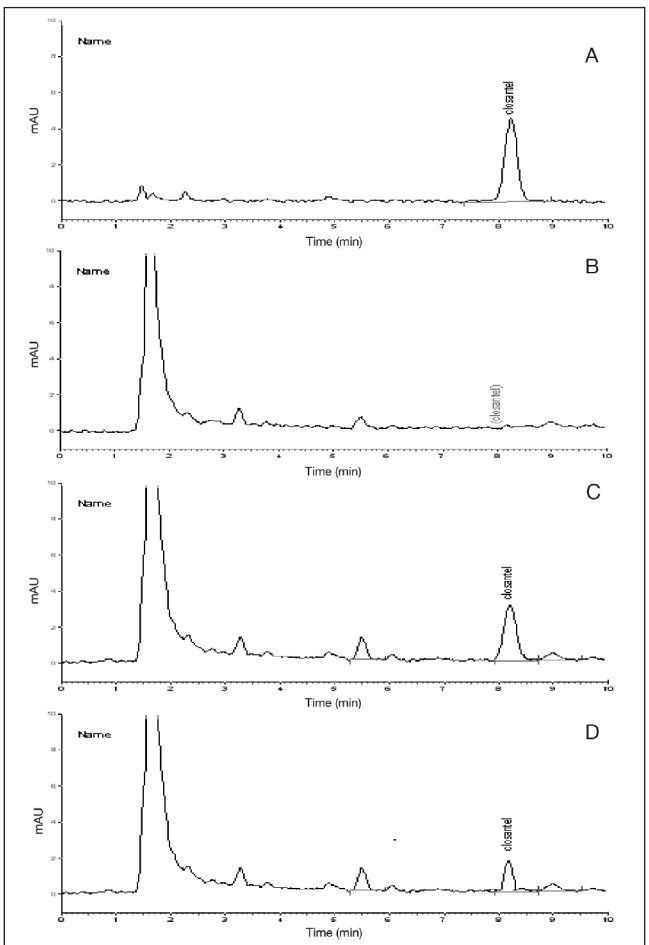

Figure 3. HPLC chromatograms obtained by fluorescence detection at λ_{ex} 335 nm/λ_{em} 510 nm for standard solution of 25 µg/L (A); blank milk extraction (B); spiked milk of 20 µg/L (C); and spiked milk of 10 µg/L (D). HPLC conditions: C18 column 5 µm, 150 × 4.6 mm i.d.; mobile phase 85% AcCN and 15% H_2O; pH 2.5 of the mobile phase was adjusted by H_3PO_4.

Applicability

The method was used to analyze five batches of native milk and tissue samples. The recoveries and contents of closantel are presented in Table III.

For the two batch samples, the contents of closantel were lower than 10 µg/L. A negative sample was discovered, the

Table II. Intra- and Inter-Day Precision and Accuracy for Determination of Closantel in Milk and Tissue Samples

		Intra-day (n = 10)		Inter-day (n = 10)	
Matrix	Fortification level (µg /kg)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)
Milk	10*	80.5	7.66	80.1	8.67
	50*	83.5	6.45	82.1	7.07
	100*	85.9	4.17	83.4	4.25
Liver	10	81.7	7.10	82.1	7.54
	50	82.6	6.45	81.9	6.57
	100	84.8	5.32	83.7	5.33
Kidney	10	83.2	7.24	82.5	7.21
	50	82.1	6.78	83.1	7.11
	100	84.1	5.49	82.8	6.01
Muscle	10	81.2	7.16	81.5	8.41
	50	83.2	5.45	82.1	6.84
	100	84.1	3.35	82.7	4.04

* Concentration unit: µg/L.

Table III. Application of the Proposed Method to Different Samples

Sample	Content µg/L for milk, µg/kg for tissue	Fortification level (µg/L or µg /kg)	Recovery† (%)	RSD† (%)
Milk	–*	10	80.5	7.66
	20	0		7.20
		10	82.1	7.45
	52	0		6.4
		50	83.8	6.49
	123	0		3.80
		100	85.4	4.11
Muscle	–*	10	84.3	6.95
		50	86.7	5.61
		100	87.4	3.50
Kidney	–*	10	82.7	7.71
		50	85.4	6.13
		100	87.2	4.35
Liver	–*	10	81.5	7.45
		50	83.4	7.78
		100	84.4	4.51

* Not detected, < LOQ.
† n = 7.

contents of which were higher than MRL. For the tissue sample, all the contents of closantel were lower than 10 µg/kg.

The mean recovery for the milk sample ranged from 82.1 to 85.4% in the concentration range of 10–100 µg/L, and the mean recovery for muscle, kidney, and liver samples ranged from 81.5 to 87.4% in the concentration range of 10–100 µg/kg. The RSD for seven parallel measurements at content levels of 10–123 µg/kg or µg/L was in the range of 3.50–7.78%.

Conclusion

The proposed method provides a simple and sensitive analysis of closantel residues at trace levels in animal tissues and milk. The use of the extraction with an acetonitrile–acetone mixture combined with Oasis MAX cartridge SPE has the advantages of a simpler procedure and higher extraction efficiency. The LOD has been improved to 3 µg/L as compared to 10 µg/L in previous works. The present method enables closantel residues to be determined at levels of 10 µg/kg in tissues and 10 µg/L in milk. The method can be applied for the analysis of closantel residues at trace levels in different tissue and milk samples.

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