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Determination of fenbendazole, praziquantel and pyrantel pamoate in dog plasma by high-performance liquid chromatography

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Abstract

Simple and rapid high-performance liquid chromatographic methods were developed for the determination of fenbendazole, praziquantel and pyrantel pamoate in dog plasma. The combination of these drugs is the most powerful treatment against most types of worms. Blood plasma samples obtained in a pharmacokinetic trial were prepared using solid-phase extraction. Fenbendazole and praziquantel were analyzed simultaneously by reversed-phase high-performance liquid chromatography on an octadecyl-modified silica stationary phase employing acetonitrile–phosphate buffer (pH 3.0) eluent and ultraviolet detection at 220 nm. Pyrantel was analyzed separately on a base-deactivated reversed-phase column using methanol–tetrahydrofuran–ammonium acetate buffer (pH 4.6) eluent and ultraviolet detection at 317 nm. Average recoveries for fenbendazole, praziquantel and pyrantel pamoate were 76.8, 93.4 and 90.5%, respectively. Limits of quantitation were in the range of 15–25 ng/ml plasma. © 1998 Elsevier Science B.V.

Keywords: Fenbendazole; Praziquantel; Pyrantel pamoate

1. Introduction

Helminthiases are common parasitic diseases of great economical and public health importance. Since the anthelmintic spectra of most drugs used for treatment is limited, combinations of more than one active ingredient are required to control mixed helminthic infections effectively. Evaluation of such preparations thus require the determination of their active ingredients in drug formulations as well as in biological matrices.

In the study reported in this paper, high-performance liquid chromatographic methods were developed for the determination of three common veterinary anthelmintics, fenbendazole, praziquantel and pyrantel pamoate (salt of pyrantel with 4,4′-methylenebis-[3-hydroxy-2-naphtoic acid]) in dog plasma. These compounds are widely applied in veterinary practice. However, praziquantel, pyrantel pamoate and some of the benzimidazole carbamates having similar structure to that of fenbendazole (e.g. mebendazole, albendazole, flubendazole) have applications in human therapy as well.

For the determination of the aforementioned compounds in body fluids or tissues, different analytical methods were published. For the assay of fenbendazole and related compounds, reversed-phase (RP) HPLC was successfully used with solid-phase [1] or liquid–liquid [2–4] extraction sample preparation and clean-up. Matrix solid-phase extraction (SPE) procedures using octadecyl-modified silica were developed for the determination of benzimidazole carbamate anthelmintics in beef liver or in milk [6,7]. Extraction of albendazole was performed with

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ethylacetate or diethylether during the analysis of plasma or cytochrome P450-derived enzymatic reaction mixtures [8,9]. Determination of the prodrug febantel and its metabolites fenbendazole, oxfendazole and fenbendazole sulphone in lamb plasma was carried out by RP-HPLC after extraction with diethylether [4]. For the assay of mebendazole and its metabolites in eel tissues, RP-HPLC with SPE on aminopropyl silica cartridges was used [5]. LC coupled with mass spectrometry was applied to the analysis of fenbendazole and oxfendazole in bovine tissue samples [3]. Ion-pair chromatography with octanesulphonate ion-pairing reagent was used for the determination of fenbendazole in bovine milk [2].

Praziquantel was determined in body fluids by gas chromatography with flame ionization detection after liquid–liquid extraction with methylacetate–diisopropylether mixture and alkaline hydrolysis [10]. For the determination of praziquantel, a colorimetric or fluorometric method employing derivatization after alkaline hydrolysis has also been developed [11,12]. A sensitive LC assay for praziquantel in plasma, urine and liver homogenates with solid-phase sample preparation has also been reported [13]. LC was successfully applied to the determination of praziquantel in fish feed and tissues [14].

LC analysis of oxantel and pyrantel pamoate in anthelmintic tablets was carried out using an octyl-modified silica column with acetonitrile–water–butylamine eluents [15]. Determination of pyrantel tartarate in serum and milk of cows was performed by thin-layer chromatographic sample preparation and LC [16].

No reference was found in the literature however for the simultaneous and rapid assay of the three compounds, the combination of which is of the most powerful anthelmintic known today. In the present study, RP-HPLC methods were developed for the assay of three common veterinary anthelmintic drugs fenbendazole, praziquantel and pyrantel pamoate in blood plasma of dogs treated with a tablet containing the three compounds. For sample preparation, SPE was used. Simultaneous determination of fenbendazole and praziquantel was carried out by RP-HPLC with ultraviolet detection at 220 nm. Assay of pyrantel pamoate using a base-deactivated reversed-phase column without using ion-pairing or silanol-masking reagents is also reported.

2. Experimental

2.1. Reagents and disposables

The solvents acetonitrile, methanol, tetrahydrofuran and concentrated phosphoric acid were of HPLC grade (E. Merck, Darmstadt, Germany). N,N-Dimethylformamide was of HiPersolv quality (BDH, Poole, UK). Double-distilled water was further purified using a Milli-Q water purification system (Millipore, Milford, MA, USA). Potassium dihydrogenphosphate, ammonium acetate and anhydrous sodium carbonate were of analytical grade (Carlo Erba, Milan, Italy). Blood samples were collected into 10-ml heparinized vacuum-tubes (Greiner Labortechnik, Germany). For sample preparation, Waters Sep-Pak Vac C_{18} (100 mg) SPE cartridges were used. Praziquantel, fenbendazole, oxfendazole and pyrantel pamoate reference materials used in external standard solutions were obtained from Sigma (St. Louis, MO, USA).

2.2. Instrumental

All HPLC separations were performed using a Waters LC Module I (Waters, Milford, MA, USA). Data were recorded and evaluated using a Waters Maxima 820 Data Station running on an IBM PC/AT computer. SPE was performed on a Supelco VisiPrep vacuum manifold (Supelco, Bellefonte, PA, USA).

2.3. Clinical trial

Three male and three female German Shepherd dogs weighing about 25 kg were treated simultaneously with 20 mg/kg body mass (b.w.) fenbendazole, 10 mg/kg b.w. pyrantel pamoate and 5 mg/kg b.w. praziquantel per os in the form of commercially-available tablets containing the three drugs. Feed was withdrawn from the animals the day before the treatment. Water was provided ad libitum during the experiment. Blood samples (7–8 ml) were collected in heparinized tubes immediately before and 0.5, 1.0, 1.5, 2.0, 4, 8, 16, 24, 48 h after the dosing of the drugs. Blank blood samples (5 ml each) were collected also one week before the main experiment from the six animals, were pooled and plasma was obtained. Blood specimens were cen-

trifuged at 2000*g* for 15 min (4°C) and plasma was collected and stored at −20°C until sample preparation (maximum two weeks). All plasma samples were protected from light during collection, storage and processing.

2.4. Sample preparation procedures

2.4.1. Preparation of standard solutions

Stock solutions of fenbendazole, praziquantel and pyrantel pamoate were prepared weekly in N,N-dimethylformamide at a concentration of 1 mg/ml. All standard solutions were kept protected from light and were refrigerated at 4°C. Intermediate standard solutions of fenbendazole and praziquantel were diluted first with acetonitrile (to 4000 ng/ml). Working standard solutions were prepared by dilution with the chromatographic mobile phase (50–1000 ng/ml). Pyrantel pamoate intermediate standard solutions were prepared from the stock by dilution first with acetonitrile–water (25:75, v/v) solvent mixture (4000 ng/ml), then the second dilution was made using the mobile phase to obtain a working standard solution (50–2000 ng/ml). Intermediate standards solutions were also used for fortification in recovery studies.

2.4.2. Clean-up procedure for the determination of fenbendazole and praziquantel

Fenbendazole and praziquantel were extracted from the blood plasma by SPE. A volume of 1.5 ml of heparinized plasma was mixed with 1.5 ml of 100 m*M* sodium carbonate solution. SPE cartridges (Waters Sep-Pak Vac C_{18}, 100 mg) were conditioned with 3 ml methanol, 1 ml of water, and 5 ml of 100 m*M* sodium carbonate solution. The sample was passed through the column which was subsequently washed with 5 ml of 100 m*M* sodium carbonate solution. The column was dried in air for 30 s and the adsorbed compounds were eluted with 0.75 ml of acetonitrile–0.2% (v/v) phosphoric acid (50:50, v/v) solution. Eluates were thoroughly mixed using a Vortex mixer and were stored refrigerated and protected from the light until analysis (max. 8 h). Twelve samples were processed simultaneously using an SPE vacuum manifold.

2.4.3. Sample preparation for the determination of pyrantel

For the determination of pyrantel, dog plasma (0.5 ml) and 0.5 ml of 100 m*M* sodium carbonate solution were mixed. The SPE cartridge (Waters Sep-Pak Vac C_{18}, 100 mg) was conditioned as described for fenbendazole and praziquantel clean-up. The sample was passed through the column which was then washed with 5 ml of 100 m*M* sodium carbonate solution. Adsorbed compounds were eluted with 0.75 ml of acetonitrile–1% (v/v) phosphoric acid (18:82, v/v) solution after air-drying for 30 s. Eluates was mixed with 0.25 ml 20% (w/v) ammonium acetate solution (pH 4.6 with phosphoric acid) and were stored in the dark until analyzed.

Under the conditions of pyrantel sample preparation, sodium pamoate (sodium embonate) was not retained on the sorbent and was washed out from the SPE cartridge in the third (rinsing with sodium carbonate solution) step of sample preparation.

2.5. Chromatographic conditions

2.5.1. Simultaneous HPLC determination of fenbendazole and praziquantel

Determination of fenbendazole and praziquantel was carried out using a Waters Novapak C_{18} (4 μm, 150×3.9 mm I.D.) column and a BST (Budapest, Hungary) Nucleosil C_{18} precolumn (20×4.6 mm, 5 μm) with an eluent comprising acetonitrile–50 m*M* potassium dihydrogenphosphate (33:67, v/v) solution. The pH of the buffer was adjusted to 3.0 with 20% (v/v) phosphoric acid. The flow-rate was 1.5 ml/min. Fifty-microliter aliquots of the eluate from the SPE were injected. Fenbendazole and praziquantel were detected at 220 nm.

2.5.2. Liquid chromatographic determination of pyrantel

For the determination of pyrantel, a Waters Symmetry C_{18} (5-μm, 150×2.1-mm I.D.) column was used together with a precolumn (20×3.9-mm I.D.) with the same packing. This deactivated column provided good peak shape without the use of ion-pairing or silanol masking reagents. The eluent was comprised of methanol–tetrahydrofuran–50 m*M* ammonium acetate buffer [pH 4.6, adjusted with 20% (v/v) phosphoric acid] (2.5:2:95.5, v/v/v). The flow-rate was 0.4 ml/min. Fifty-microliter aliquots were

injected. The determination was carried out with UV detection at 317 nm.

2.6. Data evaluation

Measurements were evaluated using external standard procedure, based on peak areas. Before analyses, working standard solutions of 250 ng/ml dissolved in the mobile phase (prepared as described Section 2.4.1) were injected to establish system suitability. Standard solutions were injected on a regular basis after each 5–7 samples to ensure the repeatability of retention times and reponse factors. Linear calibration curves were constructed each day for the evaluation of measurements in the range of 50–500 ng/ml at four levels. Plasma concentrations were calculated using the actual recovery data rather than preparing a plasma calibration since recoveries were found to be practically constant in the range of 75–500 mg/ml plasma.

In the trial reported here, the chromatographic assay of pyrantel pamoate was based on the determination of pyrantel (being the active component of the salt). After the sample preparation, pyrantel was present in the sample extract as pyrantel phosphate since sodium pamoate (sodium embonate) was not retained on the SPE cartridge. Results of the analyses were expressed as pyrantel pamoate throughout the study since this salt was actually used in the pharmaceutical specialty, administered to the animals and it was used for calibration.

2.7. Conditioning the chromatographic system

During normal operation, the column backpressure was approximately 1800 p.s.i. (1 p.s.i.=6894.76 Pa). However, after performing 15–20 analyses, a significant (approx. 500 p.s.i.) increase was observed. Thus a procedure was established to prevent an increase in the column backpressure caused possibly by the accumulation of strongly retained compounds. After each day the chromatographic columns were rinsed to remove strongly adsorbed compounds from the stationary phase. The precolumn was removed and was rinsed separately. Analytical columns were reversed and were rinsed at half flow-rate (0.75 and 0.2 ml/min, respectively) with acetonitrile–water (30:70, v/v) and acetonitrile–water (85:15, v/v) mixture for 30 min each. After this washing, columns were flushed with acetonitrile–water (30:70, v/v) mixture.

3. Results and discussion

The aim of a preliminary study was to determine the proper conditions for SPEs of the three compounds with different hydrophobicity. In our first attempt, solid-phase extractions were carried out as described under Section 2.4 with the only difference being that the plasma was mixed with 100 m*M* potassium dihydrogenphosphate buffer instead of sodium carbonate. The pH of the buffer was varied between 2.8–4.5. Although recovery of the neutral analyte praziquantel was satisfactory under these conditions, recoveries for fenbendazole and pyrantel were low, generally less than 45%. To improve the recovery of the strongly basic compound pyrantel, the pH of the sample applied to the SPE cartridge was adjusted with sodium carbonate solution to 9.0–9.5. At this pH pyrantel is deprotonated and was retained on the packing of the cartridge. Elution of this basic compound was done at acidic pH, thus facilitating the salt formation and almost complete recovery of the analyte. We found that by applying acidic elution solvent during the sample preparation for fenbendazole and praziquantel determination, it was possible to decrease the elution volume and the reproducibility of the sample preparation was improved for fenbendazole as well.

Initially attempts were made to develop a method capable of simultaneous assay of the three analytes. However, this approach would require multifactorial optimization of system variables (column, solvent and ionic strength, temperature, ion-pairing reagents etc.). Instead of this optimization, analysis of pyrantel was carried out separately. Determination of this analyte was carried out with acetonitrile–ammonium acetate buffer (pH 4.6) eluents. Since interfering compounds were detected in the dog plasma during the analysis of pyrantel pamoate, an isoeluotropic mixture of tetrahydrofuran and methanol was chosen instead of acetonitrile. Using this mobile phase, no interferences were observed. To achieve acceptable detection limits, injections of 50 μl volume were made. To allow the use of these relatively large

injection volumes, care was taken to ensure that acetonitrile concentration, ionic strength and pH of the sample and mobile phase be almost identical. In the case of pyrantel, this was accomplished by addition of 20% (w/v) ammonium acetate solution to the sample. Although fenbendazole has UV absorption at 295–300 nm as well, use of low wavelength (220 nm) UV detection allowed the sensitive detection of praziquantel, which has no chromophore without derivatization. As it can be concluded from Fig. 1B, an unknown endogenous compound (peak 3) was detected during the assay of fenbendazole and praziquantel. However, this compound eluted at the end of the chromatogram and did not interfere either with the peak of fenbendazole, or with praziquantel.

3.1. Analytical performance of the methods

Representative chromatograms of the determination of fenbendazole and praziquantel and assay of pyrantel pamoate are shown on Figs. 1 and 2, respectively. Fig. 1A and Fig. 2A show the chromatograms of reference solutions. Fig. 1B and Fig. 2B show the chromatograms of blank dog plasma. Fig. 1C and Fig. 2C show chromatograms of plasma sample obtained during the clinical trial. Specificity of the methods was regularly checked by analysing plasma samples obtained before treatment to check for the presence of possible interferences. However, such interference were not detected. The linearity and range of the assay methods were tested by constructing calibration curves for each compound. Calibration was tested and was found to be linear in the range of 15–2500 ng/ml, 15–2500 ng/ml and 18–2750 ng/ml for fenbendazole, praziquantel and pyrantel, respectively ($r>0.992$). Limits of detection (LODs) in plasma giving a peak height three times the baseline noise of the chromatographic system were 5, 4 and 4 ng/ml for fenbendazole, praziquantel and pyrantel pamoate, respectively. Limits of quantitation (LOQs), defined as four times the area of the matrix peak in blank plasma, corresponded to 18, 25 and 15 ng/ml plasma for fenbendazole, praziquantel and pyrantel, respectively. Recoveries of the analytes were determined by fortification at 50, 250 and 500 ng/ml level in triplicates during method development. Further fortifications were done during the analysis of clinical samples, using plasma obtained before the administration of the drugs, whenever it was allowed by the sample amount available. Recoveries were found to be practically constant in the concentration range tested. Average recoveries for fenbendazole, praziquantel and pyrantel were found to be 77.3% (R.S.D. 6.40%, $n=7$) 94.1% (R.S.D.

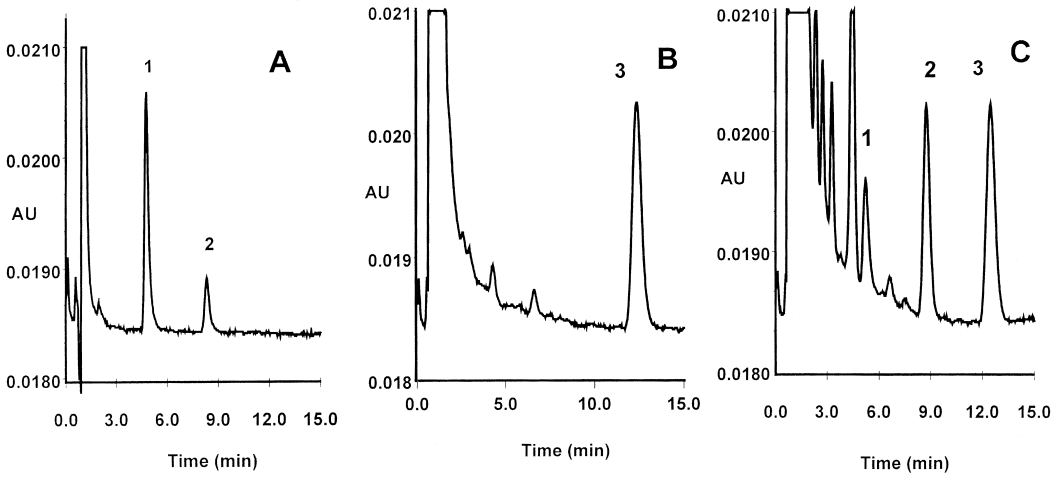

Fig. 1. Chromatograms of fenbendazole and praziquantel determination. (A) Reference solution containing 150 ng/ml fenbendazole and 80 ng/ml praziquantel; (B) Blank dog plasma; (C) Dog plasma 1 h after treatment. Conditions: eluent, acetonitrile–50 m*M* potassium dihydrogenphosphate (33:67, v/v) solution, (pH 3.0); column, Novapak C_{18}, 4 μm, 150×3.9-mm I.D.; flow-rate, 1.5 ml/min; detection, UV 220 nm. Components: 1=fenbendazole, 2=praziquantel, 3=unknown.

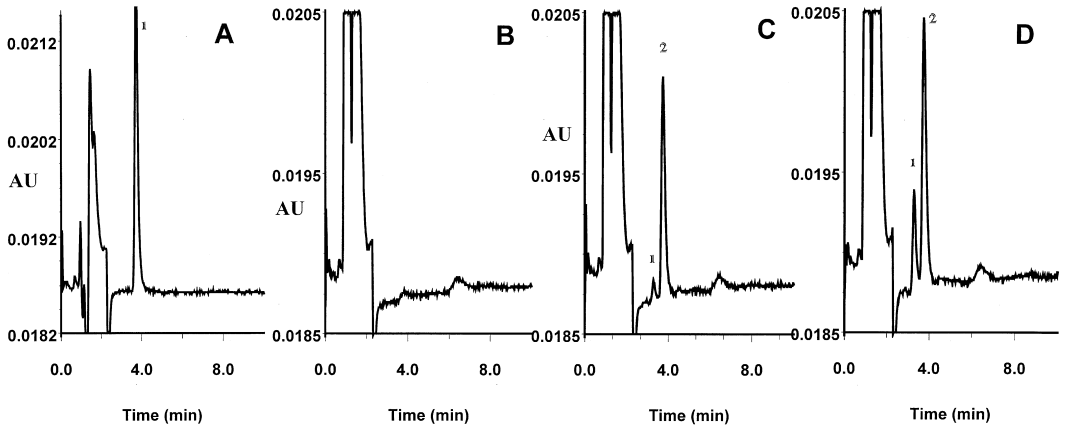

Fig. 2. Chromatograms of pyrantel determination. (A) Reference solution (200 ng/ml pyrantel pamoate); (B) Dog plasma before treatment; (C,D) Dog plasma after 1 h (C) and 4 h (D) after treatment. Conditions: eluent, methanol–tetrahydrofuran–aqueous ammonium acetate (2.5:2.0:95.5, v/v/v) solution (pH 4.6); column, Symmetry C_{18}, 5 μm, 150×2.1-mm I.D.; detection, UV 317 nm. Components: 1=unknown metabolite, 2=pyrantel.

4.1%, $n=7$) and 98.7% (R.S.D. 4.88%, $n=5$). Inter-day accuracy of the methods was determined to be 104.9±8.63, 101.8±4.7 and 98.7±4.9% for fenbendazole, praziquantel and pyrantel, respectively. Sample stability was evaluated by storing prepared samples and standard solutions in room light and dark and at room temperature and at 4°C for up to 24 h. We found that samples and reference solutions were stable at least for 12 h when stored in the dark and at 4°C. Pyrantel pamoate was found to rapidly decompose in room light and also under ultraviolet light of 254 or 360 nm.

3.2. Pharmacokinetic data obtained

Pharmacokinetic data of the three drugs are demonstrated in Table 1. Fenbendazole reached only low concentration (max. fenbendazole concentration 105 ng/ml) in plasma. Praziquantel was absorbed rapidly from the gastrointestinal tract and its maximum

Table 1
Average plasma concentrations of fenbendazole, praziquantel and pyrantel pamoate in dogs after per os treatment with 20 mg/kg b.w. fenbendazole, 10 mg/kg b.w. pyrantel pamoate and 5 mg/kg b.w. praziquantel

Time (h)	Plasma concentration (ng/ml)		
	Fenbendazole	Praziquantel	Pyrantel pamoate
0	0	0	0
0.5	18.0	260.0	90.1
1.0	35.7	339.0	178.0
1.5	46.3	413.8	311.8
2.0	57.7	400.5	302.5
4.0	36.5	237.8	192.5
8.0	21.2	127.4	73.9
24	n.d.	n.q.	n.d.
48	n.d.	n.d.	n.d.

n.d., not detected.
n.q., not quantified.

concentration of 300–700 ng/ml plasma was reached within 2–3 h after treatment. Pyrantel was also well absorbed and its peak concentration in plasma was between 300–500 ng/ml.

3.3. Metabolism study of oxfendazole

It is known from the literature, that all three compounds are rapidly metabolized in the living organism [4,13,16]. Among these compounds an active metabolite of fenbendazole, oxfendazole could be identified as being formed by the partial oxidation of the phenylthio moiety of the fenbendazole molecule. Attempts were made to determine oxfendazole in dog plasma. Sample preparation was identical to that of used for sample preparation of fenbendazole and praziquantel except that acetonitrile–0.2% phosphoric acid (25:75, v/v) solution was used for elution. Chromatographic determination of oxfendazole was carried out using a Novapak C_{18} column (150 mm×4.6 mm I.D., 4 μm) with an eluent comprising acetonitrile–50 m*M* potassium dihydrogenphosphate (18:82, v/v) solution (pH 3.0). Detection wavelength was 220 nm. Due to the limited amount of plasma specimens, oxfendazole assays could only be performed for samples of two animals. Maximum concentration of oxfendazole reached 20 ng/ml only (data not shown).

Unfortunately, at the time of this study, metabolites of fenbendazole (except oxfendazole), praziquantel and pyrantel were not available for us, thus further metabolite studies were not possible.

4. Conclusions

Fast and reproducible sample preparation and HPLC methods were developed for the analysis of three anthelmintics (active ingredients of a very effective drug-combination against worms) in dog plasma.

SPE using octadecyl-modified silica cartridges from alkaline solution (pH 9.5) provided reasonably high and persistent recovery for the three analytes. It is noteworthy that consistent recoveries for fenbendazole and pyrantel were obtained only when acidified eluting solvents were applied.

Low-wavelength ultraviolet detection (at 220 nm) was successfully applied in this case to the sensitive detection of praziquantel which has no chromophore. At this wavelength, fenbendazole was also detectable with increased sensitivity.

A narrow-bore column filled with base-deactivated stationary phase was used for the determination of pyrantel pamoate without using ion-pairing or silanol-masking reagents. Relatively low peak dispersion and acceptable peak symmetry was observed even with large injection volumes and high flow-rates, provided that the pH, ionic strength and solvent concentration of the sample and mobile phase were adjusted to be almost identical.

Data obtained during the testing and validation indicated that with minor modification of the sample extraction, the described SPE and HPLC methods can be applied to the assay of fenbendazole, praziquantel and pyrantel pamoate in animal tissues as well. Since praziquantel and pyrantel pamoate are used also in human medicaments, with minor modifications the described methods may be applied in human pharmacokinetic studies as well.

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References

[1] L.W. LeVan, C.J. Barnes, J. Assoc. Off. Anal. Chem. 74 (1994) 487.
[2] D.J. Fetouris, N.A. Botsoglou, I.E. Psomas, A.I. Mantis, Analyst 119 (1994) 2801.
[3] W.J. Blanchflower, A. Cannavan, D.G. Kennedy, Analyst 119 (1994) 1325.
[4] J. Landyt, M. Delbackere, F. Delbeke, Q. McKellar, Biomed. Chromatogr. 7(2) (1993) 78.
[5] C.A.J. Hajee, N. Haagsma, J. Assoc. Off. Anal. Chem. 79 (1996) 645.
[6] A.R. Long, M.S. Malbrough, L.C. Hsieh, C.R. Short, S.A. Barker, J. Assoc. Off. Anal. Chem. 73 (1990) 860.
[7] A.R. Long, L.C. Hsieh, M.S. Malbrough, C.R. Short, S.A. Barker, J. Assoc. Off. Anal. Chem. 72 (1989) 739.
[8] P. Galtier, M. Alvinerie, J.L. Steimer, P. Francheteau, Y. Plusquellec, G. Houin, J. Pharm. Sci. 80 (1991) 3.

[9] H.S. Amri, O. Mothe, M. Totis, C. Masson, A. Batt, P. Delatour, G. Siest, J. Pharmacol. Exp. Ther. 246 (1988) 758.
[10] H.W. Diekmann, Eur. J. Drug Metab. Pharmacokinet. 4 (1979) 139.
[11] J. Putter, Eur. J. Drug Metab. Pharmacokinet. 4 (1979) 143.
[12] H. Saleh, J. Schnekenburger, Analyst 117 (1992) 87.
[13] D.F. González-Esquivel, C.M. Okuno, M. Sánchez Rodríguez, J. Solelo Morales, H.J. Cook, J. Chromatogr 613 (1993) 174.
[14] V. Hormazábal, M. Yndestad, J. Liq. Chromatogr. 18 (1995) 1231.
[15] W.J. Allender, J. Chromatogr Sci. 26 (1988) 470.
[16] R. Gauch, U. Leuenberger, W. Limacher, U. Müller, M. Schallibaum, Z. Lebensm. Unters. Forsch. 177 (1983) 117.