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DETERMINATION OF ACTIVE SUBSTANCES IN MULTICOMPONENT VETERINARY PREPARATIONS OF ANTIPARASITIC ACTION BY HPLC METHOD

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Abstract: The study aimed at the development of an HPLC method enabling the identification and determination of the content of selected compounds occurring in multicomponent veterinary preparations used in parasitic diseases. The studied compounds included: pyrantel embonate, fenbendazole, praziquantel, epsiprantel and febantel. Using the developed HPLC method, a good separation of the above compounds was achieved. The regression analysis has shown linearity of the method in the required concentration range. The determination of the compounds mentioned and statistical evaluation of the results have demonstrated that the method is characterized by a good selectivity and high precision.

Keywords: pyrantel embonate, fenbendazole, praziquantel, epsiprantel, febantel, HPLC method

Parasitic diseases are fairly common in the animal world. This problem concerns both wild and domestic animals. The simplest way of invasion is the transfer of a parasite through contact. This way is encountered with external parasites living on the body surface of a host, for example, louses, itch mites, or living in body cavities, e.g. distomae. The most frequent way of invasion of internal parasites is the alimentary canal. The invasion forms, eggs or larvas, enter the host organism together with contaminated food. Here belongs, for instance, parasite-protozoa, some kinds of distomae (e.g., fluke), several nematodes (e.g., pinworms, worms, trichurises) and some datyhelminthes (e.g., tapeworms).

Treatment of infected animals aims at the liberation them from parasites. A parasite medicament should exhibit efficacy of action not only against mature forms of parasites, but also against their larval forms, and should possibly have a wide range of action. The latter feature is particularly important in the case of mixed invasions. To broaden the antiparasitic action, multicomponent medicines are applied. The substances most commonly used and demonstrating different mechanism and range of action comprise: pyrantel embonate, fenbendazole, praziquantel, epsiprantel and febantel. Structural and summed formulae as well as molecular weights of these compounds are shown in Figure 1.

Analysis of multicomponent medicines contaning substances from different chemical groups can afford serious difficulties. The published literature describes mostly determination of pyrantel embonate, praziquantel, fenbendazole or febantel in body fluids, tissues or food (1–6) but reports only several cases of determination of pyrantel embonate and fenbendazole in drug product (7–10).

The aim of the present studies was the elaboration of a universal HPLC method enabling identification and determination of the content of the following selected compounds: pyrantel embonate, praziquantel, fenbendazole, febantel, epsiprantel in various chemical combinations in pharmaceutical formulations.

EXPERIMENTAL

Materials

The following materials were used: standard substances: epsiprantel, febantel, fenbendazole, praziquantel, pyrantel embonate; drug products: Drontal Plus tablets (praziquantel 50 mg + pyrantel embonate 144 mg + febantel 150 mg) manufactured by Bayer HealthCare AG; Cestal Plus tablets (praziquantel 50 mg + pyrantel embonate 144 mg + fenbendazole 200 mg) manufactured by Lawet Pharmaceuticals; Dosalid 1200 coated tablets

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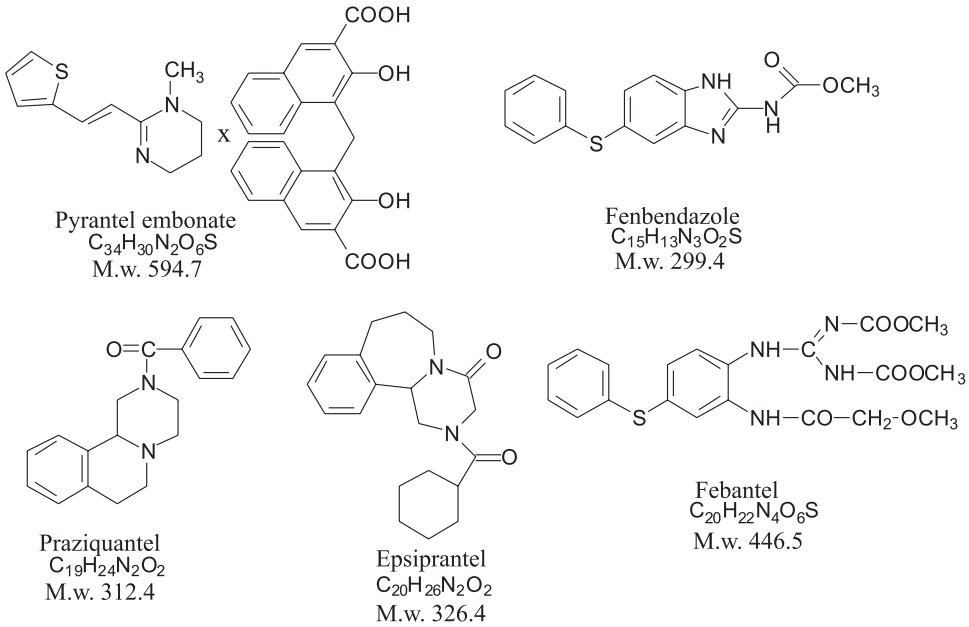

Figure 1. Structural and molecular formulae and molecular weight of the compounds studied

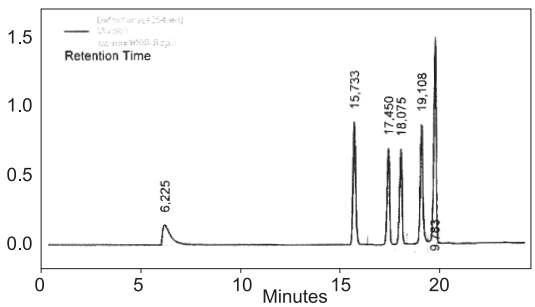

Figure 2. Chromatogram of a mixture of the five studied substances. Retention times (min): pyrantel 6.2; fenbendazole 15.7; praziquantel 17.4; epsiprantel 18.1; embonic acid 19.1; febantel 19.7. Resolution values**:** pyrantel – fenbendazole 20.0; praziquantel – epsiprantel 2.6; epsiprantel – embonic acid 4.4; embonic acid – febantel 2.9**.** Detection wavelengths: pyrantel 312 nm; fenbendazole 288 nm; praziquantel, epsiprantel, embonic acid and febantel 215 nm

(epsiprantel 100 mg + pyrantel embonate 261.6 mg) manufactured by Pfizer Ltd.

Reagents and apparatus

The reagents used were of high purity suitable for HPLC. A liquid chromatograph (Shimadzu) controlled by a computer with an SPD-M10ATVP diode detector, a UV-VIS SPD-10AVP spectrometer with LC-10 ATVP pumps and a DGU-14A degasser, a controller SCL-10AVP and an autosampler SIL-10ADVP were used throughout.

Standard solutions

Standards were dissolved in dimethylformamide (DMF) to obtain the following concentrations:

- 0.72 mg/mL (pyrantel embonate), 0.25 mg/mL (praziquantel), 1.0 mg/mL (fenbendazole) for Cestal Plus tablets;
- 0.72 mg/mL (pyrantel embonate), 0.25 mg/mL (praziquantel), 0.75 mg/mL (febantel) for Drontal Plus tablets;
- 0.65 mg/mL (pyrantel embonate), 0.25 mg/mL (epsiprantel) for Dosalid 1200 coated tablets;

A volume of 10 mL of each solution was diluted to 25 mL with acetonitrile.

Sample solutions

Cestal Plus tablets: an amount of about of 0.35 g mass of tablets was weighed and transfered to a 100 mL volumetric flask.

Drontal Plus tablets: an amount of about of 0.33 g mass of tablets was weighed and transfered to a 100 mL volumetric flask.

Dosalid 1200 coated tablets: an amount of about of 0.60 g mass of tablets was weighed and transfered to a 200 mL volumetric flask.

Each sample was poured with DMF, shaked for about 15 min, diluted to volume with the solvent and filtered. A volume of 10 mL of each solution was transfered into 25 mL volumetric flask and making up to volume with acetonitrile.

RESULTS AND DISCUSSION

In the first phase of the study, an investigation was undertaken to obtain an optimum system which would allow the identification and good separation of the studied compounds occurring simultaneously in pharmaceutical formulations. Experiments were carried out with the use of the diode detector in the wavelength range from 200 to 350 nm. To determine the wavelength enabling the determination of particular compounds, the course of chromatograms was analyzed in the above UV range and spectra were drawn for each substance. In order to choice the best HPLC system, 10 µL of standards solution containing 0.2 mg of epsiprantel, 0.2 mg of praziquantel, 0.3 mg of pyrantel embonate, 0.3 mg of febantel and 0.4 mg of fenbendazole in 1 mL of DMF and acetonitrile (1:1) (Mix) was injected into chromatograph. Many columns and mobile phases were checked. The obtained chromatograms were analyzed and the following system was selected: a Nucleosil 100-5 C18 5 µm, 250 × 4.6 mm column, mobile phase A containing water adjusted to pH 2.5 with 85% phosphoric acid, mobile phase B containing acetonitrile in gradient as follows:

time (min)	phase B (%)
0	20
2	20
18	70
20	70
22	20
25	20

The flow rate of mobile phase was 1.2 mL/min and column temperature was 40^{o}C. The detection was performed at various wavelengths (the UV-VIS detector used) as follows:

time (min)	λ (nm)
0 – 10.00	312
10.01 – 16.50	288
16.51 – 22.00	215
22.01 – 25.00	312

A chromatogram obtained under these conditions for the mixture of standards (Mix) is shown in Figure 2. The wavelengths at which the particular compounds were determined: pyrantel – 312 nm; fenbendazole – 288 nm; praziquantel, epsiprantel, embonic acid, febantel – 215 nm

In the next phase of the study, validation of the method was done.

Specificity

In order to determine specificity of the method, solvent, standards solution (Mix) and three placebo solutions were injected into the HPLC system. No peak was observed on the chromatogram obtained with solvent and placebo solutions at retention time of pyrantel, fenbendazole, praziquantel, epsiprantel and febantel.

Linearity

The linearity of peak area, on the chromatograms in the concentration range of the analyzed substances was examined as follows: 0.039 – 0.576 mg/mL (pyrantel embonate), 0.049 – 0.815 mg/mL (fenbendazole), 0.020 – 0.277 mg/mL (praziquantel), 0.020 – 0.275 mg/mL (epsiprantel), 0.099 – 0.599 mg/mL (febantel).

The results of the statistical analysis of calibration curves and determined correlation coefficients are shown in Table 1.

Limit of detection and limit of quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated, using the values of statistical parameters for adequate calibration curves according to equations: $LOD = 3.3 \times S_y/a$, and $LOQ = 10 \times S_y/a$, where S_y – standard error of estimate, a – slope of a straight line coefficient (Table 1).

Precision

Six replicate injections of standards solution (Mix) were injected into the HPLC system. The results are shown in Table 2.

Accuracy

Known amounts of pyrantel embonate, fenbendazole, praziquantel, epsiprantel and febantel working standards were added to the placebos at three levels (80%, 100% and 120% of label claim) in duplicate. The samples were analyzed as per the proposed method. The results are shown in Tables 3–5.

Quantitative analysis

For quantitative analysis, a 10 µL portions of the prepared standard solutions and test sample solutions were separately injected into the chromatograph. The emerging signals were recorded and analyzed. The obtained results and their statistical evaluation are shown in Table 6. The obtained chromatograms for the particular drug product are shown in Figure 3.

CONCLUSION

On the basis of the presented results it was found that the elaborated HPLC system enables identification and quantitative determination of each of the five examined substances. The advantage of

Table 1. Calibration curve parameters, correlation coefficients, detection limits (LOD) and quantitation limits (LOQ) of the analyzed substances.

Substance determined	Calibration curve parameters y = ax + b	LOD [mg/mL]	LOQ [mg/mL]
Pyrantel embonate	$a \pm \Delta a = 15879 \pm 375$; $S_a = 135$ $b \pm \Delta b = -35722 \pm 111627$; $S_b = 40211$ $S_y = 58746$ r = 0.9999	0.012	0.037
Fenbendazole	$a \pm \Delta a = 19329 \pm 305$; $S_a = 129$ $b \pm \Delta b = 54422 \pm 109376$; $S_b = 46248$ $S_y = 84398$ r = 0.9998	0.014	0.044
Praziquantel	$a \pm \Delta a = 295832 \pm 563$; $S_a = 238$ $b \pm \Delta b = 90753 \pm 64370$; $S_b = 27218$ $S_y = 54093$ r = 0.9999	0.006	0.018
Epsiprantel	$a \pm \Delta a = 27630 \pm 408$; $S_a = 172$ $b \pm \Delta b = 28297 \pm 46100$; $S_b = 19492$ $S_y = 38905$ r = 0.9999	0.005	0.014
Febantel	$a \pm \Delta a = 24346 \pm 1454$; $S_a = 523$ $b \pm \Delta b = 43372 \pm 459170$; $S_b = 165407$ $S_y = 220989$ r = 0.9993	0.030	0.091

a, b – regression coefficients; S_a, S_b – standard deviation of regression coefficients; S_y – standard error of the estimate; r – correlation coefficients

Table 2. Statistical evaluation of the repeatability of injections of the standard solutions.

Substance determined	Average peak area [a.u.]	Number of results S	Standard deviation [%]	Coefficient of variation
Pyrantel embonate	4306422	6	19131	0.44
Fenbendazole	8351407	6	32568	0.39
Praziquantel	6012285	6	47717	0.76
Epsiprantel	6020574	6	45602	0.41
Febantel	12351352	6	86131	0.70

Table 3. Results and statistical evaluation of the recovery in Cestal Plus tablets.

Substance	Recovery level	Added substance [mg]	Determined concentration [mg]	Recovery [%]	x – mean value S – mean standard deviation C_v – coefficient of variation $x \pm \Delta x$ – confidence interval of mean value
Praziquantel	80%	5.70	5.63	98.77	x – 99.04 S – 0.85 C_v – 0.86 $x \pm \Delta x$ – 99.04 ± 0.89
	80%	5.31	5.32	100.18	
	100%	6.83	6.71	98.24	
	100%	6.86	6.84	99.71	
	120%	7.54	7.38	98.01	
	120%	7.72	7.67	99.35	
Pyrantel embonate	80%	13.41	13.27	98.96	x – 99.01 S – 0.74 C_v – 0.74 $x \pm \Delta x$ – 99.01 ± 0.77
	80%	13.21	12.96	98.11	
	100%	18.07	17.99	99.61	
	100%	18.07	17.73	98.12	
	120%	21.65	21.57	99.58	
	120%	21.05	20.98	99.66	
Fenbendazole	80%	19.68	19.96	101.37	x – 100.44 S – 0.72 C_v – 0.72 $x \pm \Delta x$ – 100.44 ± 0.76
	80%	19.68	19.74	100.30	
	100%	23.48	23.73	101.06	
	100%	23.48	23.62	100.60	
	120%	30.22	30.16	99.80	
	120%	29.02	28.87	99.48	

Table 4. Results and statistical evaluation of the recovery in Drontal Plus tablets.

Substance	Recovery level	Added substance [mg]	Determined concentration [mg]	Recovery [%]	x – mean value S – mean standard deviation C_v – coefficient of variation x ± Δx – confidence interval of mean value
Praziquantel	80% 80% 100% 100% 120% 120%	5.34 5.07 6.30 6.23 7.55 7.23	5.28 5.03 6.25 6.17 7.45 7.20	98.87 99.21 99.21 99.06 98.67 99.58	x – 99.10 S – 0.31 C_v – 0.32 x ± Δx – 99.10 ± 0.33
Pyrantel embonate	80% 80% 100% 100% 120% 120%	14.07 14.24 17.65 17.85 21.47 21.27	13.79 13.97 17.38 17.57 21.30 21.16	98.01 98.10 98.47 98.43 99.21 99.48	x – 98.62 S – 0.60 C_v – 0.61 x ± Δx – 98.62 ± 0.63
Febantel	80% 80% 100% 100% 120% 120%	14.78 15.06 18.53 18.66 22.62 22.93	14.57 14.78 18.25 18.34 22.26 22.69	98.58 98.14 98.49 98.28 98.41 98.95	x – 98.48 S – 0.28 C_v – 0.28 x ± Δx – 98.48 ± 0.29

Table 5. Results and statistical evaluation of the recovery in Dosalid 1200 coated tablets.

Substance	Recovery level	Added substance [mg]	Determined concentration [mg]	Recovery [%]	x – mean value S – mean standard deviation C_v – coefficient of variation x ± Δx – confidence interval of mean value
Epsiprantel	80% 80% 100% 100% 120% 120%	9.56 10.46 12.35 12.45 14.94 15.24	9.58 10.53 12.41 12.41 14.80 15.03	100.10 100.67 100.48 99.68 99.06 98.62	x – 99.77 S – 0.81 C_v – 0.81 x ± Δx – 99.77 ± 0.85
Pyrantel embonate	80% 80% 100% 100% 120% 120%	26.22 26.31 32.47 31.78 39.52 39.42	25.91 25.84 31.84 31.38 38.77 38.67	98.82 98.21 98.03 98.71 98.10 98.10	x – 98.33 S – 0.34 C_v – 0.35 x ± Δx – 98.33 ± 0.36

Table 6. Results and statistical evaluation of the determination of Cestal Plus tabl., Drontal Plus tabl. and Dosalid 1200 coated tabl.

Drug product	Substance determined	Declared content [mg]	Average concentration [mg]	Number of results	Standard deviation S	Confidence interval for 95% probability x ± Δx	Coefficient of variation [%]
Cestal Plus	Praziquantel Pyrantel embonate Fenbendazole	50.00 144.00 200.00	49.36 137.93 198.35	6 6 6	0.23 0.84 1.01	49.36 ± 0.25 137.93 ± 0.89 198.35 ± 1.07	0.48 0.61 0.51
Drontal Plus	Praziquantel Pyrantel embonate Fenbantel	50.00 144.00 150.00	50.41 140.55 149.70	6 6 6	0.28 0.74 1.21	50.41 ± 0.30 140.55 ± 0.77 149.70 ± 1.27	0.57 0.52 0.81
Dosalid 1200	Epsiprantel Pyrantel embonate	100.00 261.60	96.96 261.06	6 6	0.48 0.83	96.96 ± 0.50 261.06 ± 0.87	0.49 0.32

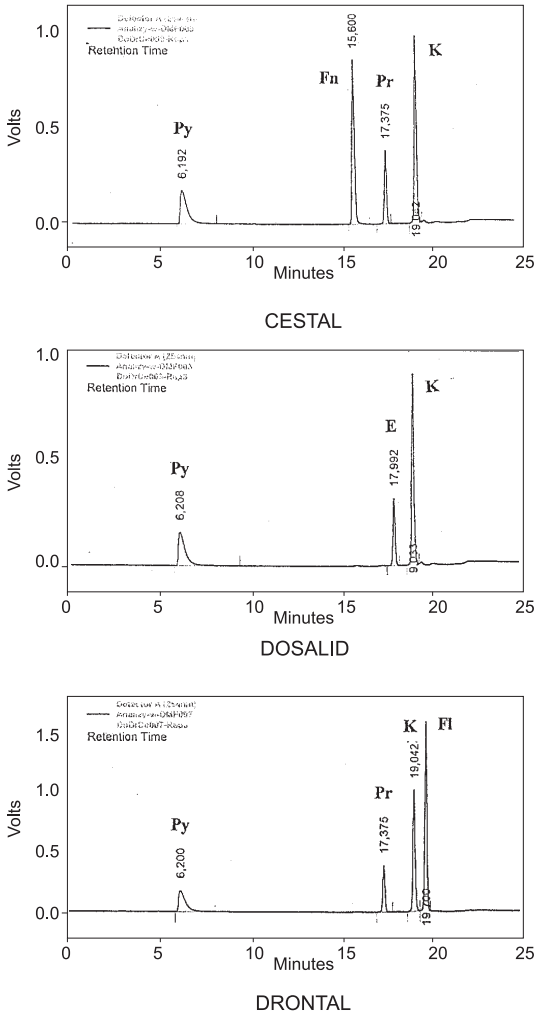

Figure 3. Chromatograms obtained for the drug product: Py – pyrantel, Fn – fenbendazole, Pr – praziquantel, E – epsiprantel, K – embonic acid, Fl – febantel

the method is that all the five compounds can be determined simultaneously. In veterinary pharmaceutical drug products applied in parasitic diseases, the above substances can occur in various combinations.

This method is specific for the determination of pyrantel embonate, fenbendazole, praziquantel, epsiprantel and febantel. No interfering peaks were observed at the retention time of the active substances. The linearity is maintained in the required concentrations range and the correlation coefficients R, are reaching satisfactory values. By determination of the detection limit for each compound, the sensitivity of the method has been estimated.

Recovery percentage of studied substances is within acceptable range (98% – 102%), indicating that this HPLC method is selective. The results of injections of standard solutions confirm the precision of the method.

The determinations of active ingredients are close to the declared content. Standard deviation and coefficient of variation have small values and the method is characterized by a short time of analysis (25 min), which is its additional advantage.

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