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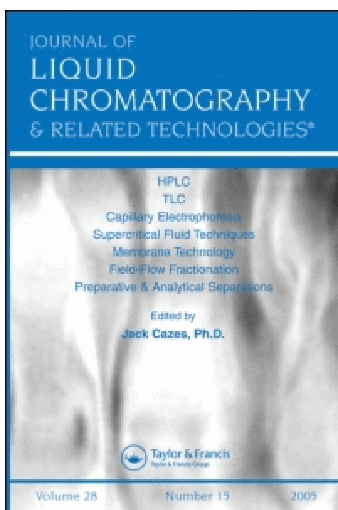

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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Densitometric Determination of Fenbendazole in Veterinarian Suspension: Validation of the Method

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To cite this Article Cholifah, Siti , Noviansari, Anna , Kartinasari, Wiwin Farina and Indrayanto, Gunawan(2007) 'Densitometric Determination of Fenbendazole in Veterinarian Suspension: Validation of the Method', Journal of Liquid Chromatography & Related Technologies, 30: 4, 489 — 498

To link to this Article: DOI: 10.1080/10826070601093788
URL: http://dx.doi.org/10.1080/10826070601093788

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Journal of Liquid Chromatography & Related Technologies®, 30: 489–498, 2007
Copyright © Taylor & Francis Group, LLC
ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070601093788

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Abstract: A simple and rapid densitometric method has been developed for determination of fenbendazole in veterinarian suspension. After extracting the samples with 0.1 N HCL in methanol, the solutions were spotted on precoated silica gel TLC plates, which were eluted with a mixture of dichloromethane-ethyl acetate–formic acid-methanol (12:1.0 : 0.6 : 0.6, v/v). Quantitative evaluation was performed by measuring the absorbance reflectance of the fenbendazole spots at $\lambda = 293$ nm. The TLC densitometric method is cheap, selective, precise, and accurate, and can be used for routine analysis of suspension in veterinary, pharmaceutical industry quality control laboratories.

Keywords: Fenbendazole, Densitometry, Syrup Suspension, TLC, Validation

INTRODUCTION

Fenbendazole, chemically known as methyl [5-(phenylthio)-1H-benzimidazole-2-yl]-carbamic acid methyl ester (CAS 43210–67–9), is active against gastrointestinal nematodes and lungworm. Fenbendazole is used usually as

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suspension, for animals.[1] Fenbendazole, as a veterinarian drug preparation, has been marketed in Indonesia, usually as a drug suspension.

The official method for determination of fenbendazole is by a titration method, which its end-point was observed potentiometrically.[2,3] Mottier et al.[4] reported the quantitative analysis of several benzimidazoles, including fenbendazole in parasite materials using HPLC with UV detection. The analysis of fenbendazole in pig plasma, and other animal tissue homogenates using HPLC was also reported.[5–9] Assay of fenbendazole residue in milk using HPLC was also reported.[10] Analysis of fenbendazole and its metabolites by direct EI-MS and MS/MS in biological matrices was described by Barker et al.[11] Spectrophotometric determination of fenbendazole was described by Rajput et al.[12] and Sastry et al.[13] Analysis of fenbendazole in food animals could be performed also by a monoclonal antibody based ELISA.[14] De Olivera and Stradiotto reported the assay of fenbendazole in veterinarian formulation using the voltammetric method.[15] An LC method for determination of fenbendazole in poultry feed was reported by Botsoglou et al.[16] Determination of impurities of fenbendazole, mebendazole, and albendazole by HPLC was published.[17]

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There are no reports on the determination of fenbendazole by a densitometric method in the CBS data base 51-96 (2005) from Camag.[18] To the best of our knowledge, no publication is available in the present time, which described the quantitative determination of fenbendazole in syrup suspension by using TLC or HPTLC, and its validation.

The objective of the present work is to develop a cheap, rapid, and simple validated TLC densitometry method for determining fenbendazole in veterinarian suspension for pharmaceutical quality control laboratories.

EXPERIMENTAL

Materials and Reagents

Fenbendazole (Changzhou Yabang Veterinary Co. Ltd., Changzhou, Jiangsa, PR of China; Batch 20050202: Assay 99.04%; Expiration date: February 2009) was of pharmaceutical grade substance. The substance was used as received for preparing a laboratory suspension, and standard solutions.

Methanol, dichloromethane, ethyl acetate, HCL (JT. Baker, Philipsburg, NJ, USA), and formic acid (E. Merck, Darmstadt, Germany) were analytical grade reagents; the solvents and reagents were used without further purification. Excipients for laboratory made (LM) suspension (citric acid, sodium hydroxide, propylene glycol, Na_2EDTA, xanthan gum, nipagin) were pharmaceutical grade substances.

For performing accuracy and precision studies, laboratory made (LM) suspension containing five different concentration levels of fenbendazole

(80, 90, 100, 110, and 120% of label claim) were prepared. The label claim is 25 mg mL^{-1}.

Stock standard solutions were prepared daily by dissolving accurately weighed fenbendazole (20.0, 25.0, 35.0 mg) in 10.0 mL in solvent, which was comprised of 0.1 N HCl in methanol. Various standard solutions were prepared from the stock solution by dilution with the solvent. For a basic linearity study, the solutions were prepared containing 250, 300, 350, 400, 450, 500, 550, 600, 650, and 700 μg mL^{-1} fenbendazole, and 2.0 μL of these solutions was spotted onto the TLC plate. The standard solutions were stable at least for 36 hours at room temperature (100.6 $\pm$ 0.79%, n = 4, at 24 $\pm$ 2°C, room humidity 50 $\pm$ 10%).

Sample Preparation

An equivalent weight of syrup suspension, which contained 5.0 mg fenbendazole (ca. 200 mg accurately weighed suspension) was transferred into a 10.0 mL volumetric flask containing about 5 mL of solvent, which was comprised of 0.1 N HCl in methanol, then the mixture was ultrasonicated for 10 min, mixed with a vortex mixer for 5 min, and diluted to 10.0 mL with the solvent, and 2.0 μL of this solution was spotted onto the TLC plate together with the standards.

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Chromatography

Chromatography was performed on precoated silica gel F254 aluminum back sheets (E. Merck. # 1.05554, all the precoated plates were cut into 10 × 20 cm before being used). The plates were used as obtained from the manufacturer without any pretreatment; a Nanomat III (Camag, Muttenz, Switzerland) equipped with a dispenser magazine containing 2.0 glass capillaries (Camag) was used for sample application (as spot with diameter *ca.* 1–2 mm). The mobile phase used in this experiment is dichloromethane-ethyl acetate–formic acid-methanol (12 : 1.0 : 0.6 : 0.6, v/v). The distance from the lower edge was 10 mm; distance from the side was 15 mm, and track distance was 10 mm. Ascending development was performed in a Camag twin-through chamber (for 20 × 10 cm plates) after at least 3 h of saturation; the mobile phase migration distance in all experiments was 8.0 cm. (development time *ca.* 15 min at 24 $\pm$ 2°C). After being air dried for 30 min, the plates were scanned in the TLC scanner.

Densitometric scanning was performed with a Camag TLC-Scanner II. The purity and identity of the analyte spots were determined by scanning the absorbance-reflectance mode from 200 to 400 nm. Quantitative evaluation was performed by measuring the absorbance reflectance of the analyte spots at its λ maximum (ca. 293 nm) (Figure 1). The densitometric scanning

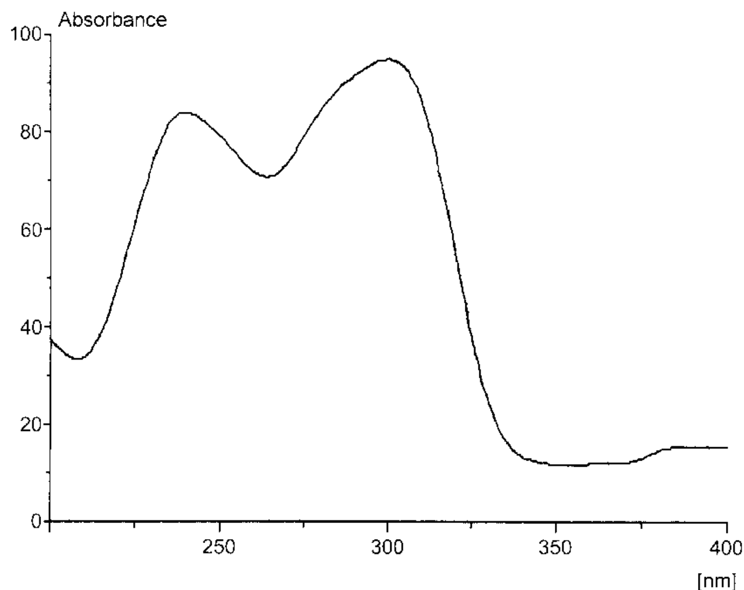

Figure 1. *In situ* absorbance-reflectance UV spectrum of fenbendazole spot (from 200 to 400 nm; maximum absorption wavelengths at ca. 293 nm). TLC conditions, stationary phase: precoated TLC plate silica gel 60 F_{254} (E. Merck); mobile phase: dichloromethane-ethyl acetate–formic acid-methanol (12 : 1.0 : 0.6 : 0.6, v/v).

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parameters were: bandwidth 10 nm, slit width 4, slit length 6, and scanning speed 4 mm s^{-1}. Calculations for identity, purity checks ($r_{S,M}$ and $r_{M,E}$ where S = start, M = center, E = end spectrum), RSD (relative standard deviation) of the linear/calibration curve, and quantification of the analyte spots were performed by CATS version 3.17 (1995) software (Camag). Routine quantitative evaluations were performed via peak areas with linear regression, using 4–5 points' external calibration on each plate (80 to 120% of the targeted value). Each of the extract aliquot samples was spotted at least in duplicate.

Validation

The method was validated for linearity, detection limit (DL), Quantitation limit (QL), accuracy, and range by the modified published methods.[19] In order to assure the selectivity of the method, forced degradation studies using 0.1 N HCl, 1 N NaOH, and H_2O_2 (1%) were performed on *ca.* 1000 mg LM suspension (equivalent to 25 mg of fenbendazole) in an oven (40 to 60°C for 16 hours). The selectivity of the method was proven by identification and purity checks of the analyte spots. In the present work, five point

accuracy studies (80, 90, 100, 110, and 120% of the expected value) were performed for LM suspension. The precision (repeatability and intermediate precision) was evaluated by analyzing six different extract aliquots from the LM suspension (80, 100, and 120% of label claim). Robustness evaluation was performed using Plackett Burman design. In this case, the influence of small variations of the mobile phase composition on the values of Rf of analyte spot, TF (tailing factor), and % recovery (%R) of LM suspension was evaluated. Design and analysis of effect of the robustness data were performed and calculated by using Unscramble 9.6^{TM} (2006) software from CAMO (Bangalore, India).

RESULTS AND DISCUSSION

After the TLC plate was eluted, the densitogram at 293 nm (Figure 2) showed a single spot of fenbendazole (R_f ca. 0.42). This TLC system demonstrated that all analyte spots of the laboratory made suspensions, furnished *in situ* UV spectra, identical with those of standards ($r \geq 0.9999$). Purity check of the analyte spots using CATS software also showed that all analyte spots of the extracts were pure. The values of $r_{S,M}$ and $r_{M,E}$ were $\geq$0.9999, demonstrating that the proposed TLC method is highly selective.

The peak area was observed to be linearity dependent of the amount of fenbendazole within the range of *ca*. 40 to 140% of the expected value (500

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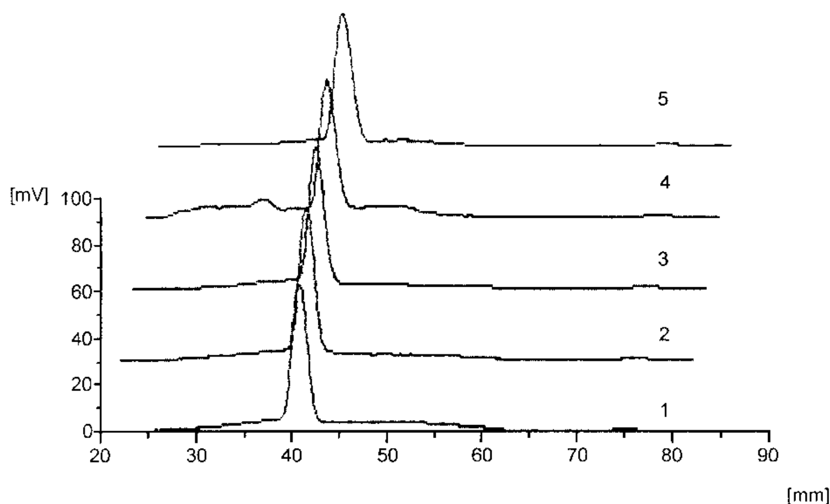

Figure 2. Densitograms measured at 293 nm obtained from: (1) solution of standard fenbendazole, (2) extract of laboratory made tablets, (3) extract of stressed LM suspension using 1 N NAOH, (4) extract of stressed LM suspension using 1% H_2O_2, (5) extract of stressed LM suspension using 0.1 N HCL. TLC conditions: see Figure 1.

to 1400 ng spot^{-1}), with linear regression line $Y = 372.77 + 0.636\ X$ (the relative process standard deviation value V_{XO}[19] was 1.986%; $n = 10$; $sdv = 2.8$; $r = 0.9983$). The calculated value of test parameter X_p (for $p = 0.05$) and r were satisfactory (101 ng spot^{-1} and ≥ 0.99, respectively).[19,20] The ANOVA regression test for linearity testing of the regression line showed significant calculated F-value (2317.4; $p < 0.0001$). The linearity of the basic calibration curve was also proven by the Mandel's fitting test.[14] The plots of the residuals against the quantities of the analyte confirmed the linearity of the basic calibration graph (data not shown). The residuals were distributed at random around the regression lines; neither trend nor unidirectional tendency was found. The basic linear calibration curve showed variance homogeneity over the whole range. The calculated test values PW[19] was 1.395, the PW values less than the F_{table} value (5.35 for $f_1 = 9$, $f_2 = 9$; $p = 0.01$).

All the linear regression calibration curve parameters used in this present work showed satisfactory results (data not shown). All values of the correlation coefficient r in this present work are >0.99; and the values of other parameters such as, Xp (less than lower limit in the calibration range), sdv (<5), Vxo ($<5\%$), and p (<0.05) for ANOVA linear test also showed satisfactory results.[20,21]

Although the validation parameters DL and QL were not required for the assay of active ingredient(s) in pharmaceuticals, those parameters were also determined in this present work. These parameters maybe used for other purposes (e.g., for bioequivalence studies, limit test for adulterants detection, and stability testing etc.). DL was determined by making a linear regression of relatively low concentration of fenbendazole (100 to 450 ng spot^{-1}) according to the method of Funk et al.[19] The calculated equation of the regression line was $Y = 171.09 + 2.05\ X$ ($n = 8$; $V_{XO} = 1.33\%$; $r = 0.9996$; $sdv = 1.0$). The calculated value of test parameter X_p (for $p = 0.05$)[19] was 19.1 ng spot^{-1}. In this case, the value of $DL = X_p$ [19] According to Carr and Wahlich,[22] the value of the QL could be estimated at 3 times of the DL value (57.3 ng spot^{-1}).

Table 1 demonstrated good accuracy as revealed by the percentage of mean recovery data of the assay of the LM suspension. To prove whether systematic errors did not occur, linear regression of recovery curve of X_f (concentration of the analyte measured by the proposed method) against X_c (nominal concentration of the analyte) was constructed. The confidence interval data ($p = 0.05$) of the intercept {VB(a_f)} and slope {VB(b_f)} from the recovery curves did not reveal the occurrence of constant and proportional systematic errors.[19]

All the relative standard deviations (RSD) of the repeatability and intermediate precession evaluations have values less than 2% (see Table 2), and the calculation by using David, Dixon, and Neumann Test[23] showed satisfactory results (data not shown). All the standard deviations (SD) (data not shown) of the precision studies yielded values below the permitted maximum standard

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Table 1. Results from determination of accuracy using laboratory-made suspensions

Nominal concentration of fenbendazole (X_c) (ng spot $^{-1}$)	Measured values (X_f) (ng spot^{-1})
806	796
806	795
904	912
906	902
1005	1004
1006	999
1106	1096
1104	1086
1202	1196
1202	1204
Mean recovery ± SD (%):	99.4 ± 0.76
Line equation of the recovery curve:	$X_f = -6.53 + 1.00\ X_c$
$V^{a}_{b(af)}$:	−6.53 ± 41.27
$V^{a}_{b(bf)}$:	1.00 ± 0.04

[a]For p = 0.05.

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deviation as reported by Ermer (2.43 for specification range 95–105%, basic lower limit 99%, n = 6).[24] The measurements were performed in one laboratory by different analysts, on different plates and days, on the three different concentrations of the analyte in the laboratory made suspension. These results demonstrated that the accuracy and precision of the proposed method were satisfactory in the range of 80 to 120% of the expected concentration in LM suspension.

Table 3 showed that, although the recovery of fenbendazole was reduced 15 to 60% in stressed LM samples, the purity and identity check of the analyte spots using CATS software yielded good values (>0.999); this showed that all

Table 2. Results from evaluation of precision of LM suspensions

	RSD values (%, n = 6)		
Measurements	LM suspension (80%)	LM suspension (100%)	LM suspensions (120%)
1[a]	1.71	1.44	0.55
2[a]	1.34	0.93	1.13
3[a]	0.40	1.02	0.63

[a]Each measurement was performed by a different analyst on the different days, and plates within one laboratory.

Table 3. Results of forced degradation studies of laboratory-made suspensions

Storage condition	Time	% Recovery of[a] (mean ± SD, n = 3)
3 drops of 1 N NaOH	16 hours at 60°C	84.8 ± 1.19
3 drops of 0.1 N HCl	16 hours at 40°C	62.3 ± 0.15
3 drops of 1% H_2O_2	16 hours at 60°C	41.1 ± 0.93

[a]Purity and identity checks of fenbendazole spots using CATS software yielded good values ($r > 0.999$).

the analyte spots were still pure and identical with the standard. This proved that the analyte peaks were not interfered by the degradation products (see Figure 2). It seemed that the degradation product(s) were not detected clearly in the stressed samples measured at 293 nm.

In order to evaluate the robustness of the proposed method, the influence of small variations of the mobile phase composition on percent recovery of the LM suspension were evaluated (Table 4). Analysis of the effect of the data was performed by using Unscrambler 9.6™ software. A higher order interaction effect (HOIE) method showed that the Rf of analyte spot, TF and % R values were significantly not affected by these small variations (Table 5; $p > 0.05$). This data proved that the proposed method was robust enough.

The present work shows that the proposed TLC densitometric method is suitable for the routine analysis of products of similar composition in the pharmaceutical industry quality control laboratories, especially for developing countries like Indonesia. Our experiences showed that the TLC methods are

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Table 4. Effect of the mobile phase compositions[a] on the Rf, TF and % recovery of LM suspension values[b]

Dichloromethane	Ethyl acetate	Formic acid	Methanol	Rf	TF	Recovery (%)
12.50	1.50	0.65	0.55	0.430	1.200	100.7
11.50	1.50	0.65	0.65	0.510	1.180	99.4
11.50	0.50	0.65	0.65	0.430	1.000	100.7
12.50	0.50	0.55	0.65	0.410	0.920	100.8
11.50	1.50	0.55	0.55	0.440	1.000	100.7
12.50	0.50	0.65	0.55	0.455	1.100	101.0
12.50	1.50	0.55	0.65	0.510	1.080	100.7
11.50	0.50	0.55	0.55	0.390	1.250	99.8
12.00	1.00	0.60	0.60	0.415	1.083	101.5

[a]Mobile phase composition presented in v/v.
[b]Mean value of triplicate determinations.

Table 5. Analysis of effect of the robustness data (HOIE method)[a,b]

Variable (mobile phase)	Rf (p)[c]	TF (p)[c]	Recovery (%), (p)[c]
Dichloromethane	NS (0.1020)	NS (0.7659)	NS (0.2451)
Ethyl acetate	NS (0.1583)	NS (0.6991)	NS (0.6874)
Formic acid	NS (0.5431)	NS (0.6077)	NS (0.9187)
Methanol	NS (0.2778)	NS (0.4275)	NS (0.7613)

[a]Calculated from data presented on Table 4.
[b]Calculation was performed by using Unscrambler 9.6 software (CAMO).
[c]Probability value; NS means not significant.

very cheap compared to the LC-MS method, GC-MS, and even with HPLC equipped with DAD/UV detector. The disadvantage of using LC with a fixed UV detector and GC-FID is the inability for proving the identity and purity of the analyte peak(s). To the best of our knowledge, no Indonesian pharmaceutical companies have LC-MS in their QC/R & D laboratory in the present time. For developing countries in which the price of HPLC grade solvents and columns are relatively very expensive, the availability of an alternative cheap method is important.

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Received October 10, 2006
Accepted October 28, 2006
Manuscript 6964

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