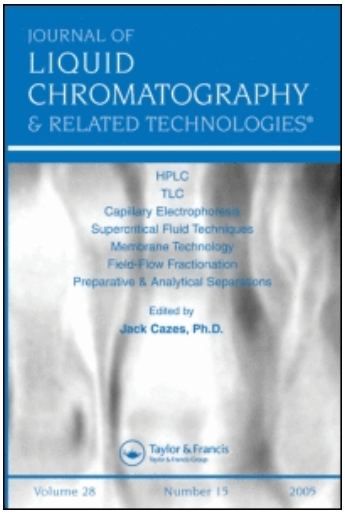

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Simultaneous HPLC Determination of Levamisole Hydrochloride and Anhydrous Niclosamide in Veterinary Powders, and its Validation

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Abstract: A simple, rapid, and validated HPLC method has been developed for simultaneous determination of levamisole hydrochloride and anhydrous niclosamide. A Luna® C-18 column was used with a mobile phase consisting of acetonitrile–buffer solutions (2:8, v/v). Quantitative evaluation was performed at 240 nm. The HPLC method is selective, precise, and accurate and can be used for routine analysis of the preparations in pharmaceutical industry quality control laboratories.

Keywords: Levamisole hydrochloride, Niclosamide anhydrate, HPLC, Veterinarian powders, Validation

INTRODUCTION

Levamisole hydrochloride, chemically known as (6S)-6-phenyl-2,3,5,6-tetrahydroimidazole[2,1-b]thiazole hydrochloride, and anhydrous niclosamide, chemically known as 5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide are both used as anthelmintic drugs. A combination of these drugs,

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which are used for veterinary application, is already marketed in Indonesia as veterinarian powders preparations.

The official determination of levamisole hydrochloride and niclosamide anhydrate is by titration.[1–5] Sari et al.[6] reported the HPLC determination of levamisole and abamectine in sheep plasma, whilst Yamada et al.[7] published the simultaneous LC-MS determination of residual levamisole and abamectine in bovine, porcine, and chicken muscle. The determination of levamisole in sheep muscle tissue by HPLC-DAD was also reported.[8]

Other HPLC methods for determination of levamisole in biological samples were reported.[9–12] A fluorescence sensor based on the supermolecular recognition by glycosylated metalloporphyrin for levamisole assay was reported recently.[13] The HPLC determination of levamisole and mebendazole in tablets were described by Xia Qing et al.[14] Determination of levamisole in urine by TLC was published by Wang et al.[15] Schreier et al.[16] reported the HPLC determination of the niclosamide residues in rainbow trout and channel catfish fillet tissue. Determination of niclosamide by GLC as heptafluorobutyryl derivate was reported by Johnson & Pickering.[17] Alemu et al.[18] reported the determination of niclosamide by the voltametric method. A spectrophotometer method was described for the simultaneous assay of niclosamide and thiabendazole in tablets.[19] Qualitative analysis of niclosamide by TLC was also reported.[20] To the best of our knowledge no report is available for the simultaneous determination of levamisole and niclosamide in veterinarian drug preparations.

The aim of this present work was to develop a simple, validated, and rapid HPLC method for routine analysis of levamisole HCl and anhydrous niclosamide in veterinarian drug preparations by using the HPLC method.

EXPERIMENTAL

Materials and Reagents

Levamisole hydrochloride (Nanjing Baijingyu Pharmaceutical Co., Nanjing, China; Batch no. 060962; assay: 100.3%; manufacturing date: September 2006; expiry date: October 2010), Anhydrous Niclosamide (Wujiang Luosen Chemical Co Ltd., Jangsu Province, China; Batch no. 0603205; assay 99.45%; manufacturing date: March 2006; expire date: March 2010) were of pharmaceutical grade substance. These substances have fulfilled the requirement of the Indonesian Veterinarian Pharmacopoeia[1] and were used as received for preparing laboratory made veterinarian powders and standard solutions.

Acetonitrile (Mallinckrodt Baker Inc. Phillipsburg, NJ, USA), acetic acid glacial, phosphoric acid, *n*-propanol, dibutylamine, (E. Merck, Darmstadt, Germany), sodium hexanesulphonic acid (Tokyo Kasei Kogyo Co., Tokyo, Japan) were analytical grade reagents. The solvents and reagents were used

without further purification. The excipient for laboratory made veterinarian powders (lactose) was of pharmaceutical grade substance.

Laboratory made (LM) veterinarian powder preparations were prepared containing five different concentrations in the range of 80% (LM1), 90% (LM2), 100% (LM3), 110% (LM4), and 120% (LM 5) of the label claim. The concentrations of levamisole HCl in LM1-5 were 37.8, 42.5, 47.2, 51.9, 56.6 mg g^{-1}, and for anhydrous niclosamide were 160, 180, 200, 220, 240 mg g^{-1}, respectively. These LM powders were used for accuracy determination.

Stock standard solutions were prepared daily by dissolving accurately weighed levamisole HCL and anhydrous niclosamide (20.0, 25.0, 30.0, 35.0 mg) in a mixture of equal volume of *n*-propanol and mobile phase (25.0 mL). For linearity study, various standard solutions were prepared from the stock solutions by dilution with the solution mixture (for levamisole HCl: 10.0, 12.0, 14.0, 16.0, 18.0, 20.0, 22.0, 24.0, 26.0, and 28.0 μg mL^{-1}; for anhydrous niclosamide: 10.0, 20.0, 40.0, 60.0, 80.0, 100, 120, 140, 160, and 180 μg mL^{-1}), and each of these solutions (20 μL) was injected into the HPLC. The standard solution was stable at least for 48 hours (at 24 ± 2°C, room humidity 50 ± 10%); the result of analysis of the standard solution that kept 48 hours yielded 100.12 ± 1.50% (levamisole HCl, and 99.71 ± 1.63% (anhydrous niclosamide), respectively (Mean ± SD, n = 4, compared to the fresh solution).

Sample Extraction

About 50.0 mg veterinarian powder (accurate weight) was transferred in a 10.0 mL volumetric flash containing 5 mL *n*-propanol and ultrasonicated for 5 min, than diluted to volume by the mobile phase; 1.0 mL of this solution was transferred to a 10.0 mL volumetric flask and diluted to volume with the mobile phase. The solution was filtered through 0.45 μm Durapore™, membrane filters (Milipore, Ireland) before injection into the HPLC apparatus (20 μL).

Chromatography

The HPLC systems used in this work were comprised of a Hitachi L-6200 (Tokyo, Japan) intelligent pump equipped with a Hitachi LC organizer and dynamic mixer mode 655A, a 20 μL Rheodyne 7125 injector, a Hitachi L-4500 photo diode array detector (DAD detector) and completed with Hitachi model D-6500 chromatography data station software, DAD system manager, and a Shimadzu LC-10 AD VP pump (Kyoto, Japan) equipped with a mixer FCV-10 AL VP, a Degasser DGU-14A, a controller SCL-10

AVP, a Shimadzu SPD-M10A DAD detector, a Shimadzu auto sampler SIL 10-ADVP, and completed with Shimadzu LC-solution software.

The analysis was carried out on a Luna^{TM} C-18 (5 μm, 25 cm) (Phenomenex, Torrance, USA) with flow rate of 0.8 mL/min. As mobile phase, a mixture of buffer solution:acetonitrile (2:8, v/v) was used. The buffer solution consisted of an equal mixture of solution A and B. Solution A consisted of 0.005 M Na hexanesulphonic acid with the addition of acetic acid glacial (8 mL) and adjusted to pH 3 by phosphoric acid, whilst solution B consisted of 0.01 M dibutylamine and adjusted to pH 3 with phosphoric acid. The mobile phase was prepared daily, and filtered through 0.45 μm Ultipor N^{TM} (Pall, Washington, USA) filters and ultrasonicated for 30 min before use.

To confirm the purity and identity of the analyte peak, the eluent was also monitored using a DAD detector in the range of 210–400 nm; all quantitative and qualitative data evaluation (identification, purity check) were performed on Hitachi model D-6500 chromatography data station software, DAD system manager, and Shimadzu LC-solution software. Routine quantification was performed at 240 nm via peak areas with linear regression, using at least three points of external calibration.

Validation

The method was validated for linearity, limit of detection (LOD), limit of quantification limit (LOQ), accuracy, robustness, and range by the modified methods.[21–23] The selectivity of the method was proven by identification and purity checks of the analyte peaks. In order to assure the selectivity of the method, forced degradation studies using 0.1 N HCl, 0.1 N NaOH, and 15% H_2O_2 were performed on *ca.* 1000 mg powdered LM3 veterinarian powders. Five point accuracy studies (80–120% of the expected value) were performed on the LM veterinarian powder preparations. The precision (repeatability, and intermediate precision) was evaluated by analyzing six different extract aliquots from LM1, LM3, and LM5 veterinarian powders preparations, which contained 80, 100, and 120% of expected value, on different days by different analysts, and HPLC equipments. A fractional factorial design and analysis of effect of the robustness evaluation were performed and calculated by using Unscramble 9.6^{TM} (2006) software from CAMO (Bangalore, India).

RESULTS AND DISCUSSION

Extracts of the excipient of the laboratory made veterinarian powders showed no peaks. All HPLC chromatograms of the extracts of laboratory made veterinarian powders showed 2 peaks of levamisole HCl and anhydrous niclosamide. To confirm the identity and purity of the analyte peaks a DAD

detector was used. Figure 1 showed the typical HPLC chromatogram of standard mixtures, extract of LM3, and blank sample. The wave length (λ) at 240 nm was then selected for further work and quantitative evaluation. All the UV-spectra of the analyte peaks showed good correlation to the standard peak ($r > 0.99$). Purity checks of the analyte peaks showed that all the peaks were pure ($r > 0.99$). This affirmed the proposed HPLC method is sufficiently selective.

The relative standard deviation (RSD) of its retention time (R_t) data from this work for levamisole HCl were 0. 82% ($n = 119$, Hitachi's HPLC) and 0.19% ($n = 36$, Shimadzu's HPLC), and for anhydrous niclosamide were 1.59% ($n = 119$, Hitachi's HPLC) and 0.31% and 0.315 ($n = 36$, Shimadzu's HPLC). The tailing factor (TF) at 10% peak height yielded relatively good values for both analytes (1.21–1.40).

Using this HPLC system, the linearity of levamisole HCl and anhydrous niclosamide were achieved in the range of 10.0 to 28.0 $\mu g/mL$, and 10.0 to 180 $\mu g\ mL^{-1}$, respectively. The linear regression line equation for levamisole HCl was: $Y = 19063 + 1814\ X$, $n = 10$, relative process standard deviation

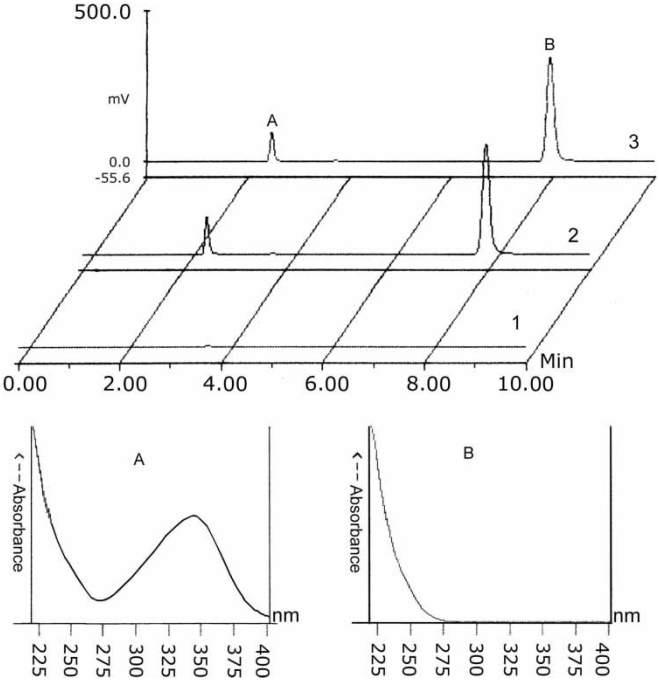

Figure 1. Typical HPLC chromatogram (at λ 2 40 nm) of (1) extract of the blank sample, (2) extract of LM 3 powders, (3) standards mixture solution, and UV spectra of the peaks. Peak identities: (A) levamisole HCl, (B) anhydrous niclosamide. Mobile Phase: acetonitrile – buffer solutions (2:8, v/v).

V_{XO}[21] = 2.34%, r = 0.9976, injection volume 20 μL). The calculated testing value X_p (for p = 0.05)[21] was 2.37 μg mL^{-1}, ANOVA regression test for linearity testing of the regression line showed calculated F-value (20959.5, for p 0.0001), standard error of the intercept (S_a) and slope (S_b) were 22924 (p = 0.406) and 215 (p < 0.0001). For anhydrous niclosamide the data were: Y = 20071 + 31070 X, n = 10, V_{XO}[21] = 1.34%, r = 0.9998, injection volume 20 μL), testing value X_p (for p = 0.05)[21] was 5.27 μg/mL, F-value of ANOVA = 1666.9 for p < 0.0001), S_a = 8955 (p = 0.066), S_b = 451 (p < 0.0001). The plots of the residuals against the quantities of the analyte confirmed the linearity of both basic calibration graphs (data not shown). The residuals were distributed at random around the regression line; neither trend nor unidirectional tendency was found. This basic linear calibration curve showed variance homogeneity over the whole range. The calculated parameter PWs[21] were 0.739 (levamisole HCl) and 1.717 (anhydrous niclosamide), respectively; this PW-value was less than the F_{table}-value (5.35; n = 10, 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, as well as the values of other parameters such as

Table 1. Results from determination of accuracy using LM veterinarian powders

LM	Levamisole Hydrochloride		Anhydrous Niclosamide	
	Xc[a]	X_f[b]	Xc[a]	X_f[b]
1	18.9	18.7	80.0	78.6
1	18.9	18.8	80.0	81.6
2	21.2	20.9	90.0	89.7
2	21.2	20.8	90.0	91.2
3	23.6	23.7	100	101.9
3	23.6	23.7	100	98.3
4	26.0	26.0	110	108.2
4	26.0	26.3	110	109.1
5	28.3	28.3	120	118.0
5	28.3	28.1	120	121.4
	Mean Recovery ± SD (%) = 99.64 ± 0.93 Line equation of the recovery curve: $X_f = -0.678 + 1.026 X_c$ $V^c_{b(af)} = -0.678 \pm 1.052$ $V_{b(bf)}{}^c = 1.025 \pm 0.044$		Mean Recovery ± SD (%) = 99.84 ± 1.59 Line equation of the recovery curve: $X_f = 2.399 + 0.974 X_c$ $V^c_{b(af)} = 2.399 \pm 8.399$ $V^c_{b(bf)} = 0.974 \pm 0.083$	

[a]Nominal concentration in μg mL^{-1}.
[b]Measured Values in μg mL^{-1}.
[c]For p = 0.05.

Table 2. Results from evaluation of precision of LM veterinarian powders preparations

	RSD values (%, n = 6)					
	LM1 Powders (80%)		LM3 Powders (100%)		LM5 Powders (120%)	
Measurements	Levamisole HCl	Niclosamide	Levamisole HCl	Niclosamide	Levamisole HCl	Niclosamide
1[a]	1.04	1.30	0.37	1.37	0.97	0.82
2[a]	0.79	1.62	1.58	0.69	0.92	1.52
3[a]	0.94	1.33	0.91	1.50	1.47	0.88

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

Table 3. Effect of the mobile phase conditions on the Rt. T and % recovery of LM3 values

Buffer (%)	Acetonitrile[a] (%)	pH	Flow (mL min^{-1})	Rt (min)	TF	R (%)
A. Levamisole HCl						
15	85	2.90	0.75	2.776	1.342	101.042
25	75	2.95	0.75	2.811	1.329	101.086
15	85	3.05	0.75	3.141	1.364	99.838
25	75	3.05	0.75	2.940	1.397	99.424
15	85	2.95	0.85	2.860	1.338	101.333
25	75	2.95	0.85	2.869	1.379	100.104
15	85	3.05	0.85	2.945	1.440	100.544
25	75	3.05	0.85	2.579	1.399	100.174
20	80	3.00	0.80	2.643	1.349	100.139
Mean Value				2.618	1.371	100.409
RSD (%)				0.568	0.036	0.637
B. Anhydrous niclosamide						
15	85	2.90	0.75	6.905	1.223	99.314
25	75	2.95	0.75	10.639	1.176	99.340
15	85	3.05	0.75	7.368	1.213	98.847
25	75	3.05	0.75	11.044	1.186	98.169
15	85	2.95	0.85	6.733	1.214	100.799
25	75	2.95	0.85	10.902	1.200	98.976
15	85	3.05	0.85	6.645	1.201	98.412
25	75	3.05	0.85	9.759	1.187	98.964
20	80	3.00	0.80	7.944	1.206	98.129
Mean Value				8.660	1.201	98.994
RSD (%)				1.900	0.015	0.812

[a]%Acetonitrile = 100 − % Buffer solution.

Xp (less than lower limit in the calibration range),Vxo (<5%), and p (<0.05) for ANOVA linear test.[20–23]

Although the validation parameters LOD and LOQ were not required for the assay of active ingredient(s) in tablets,[2] these parameters were also determined in this present work. These parameters perhaps can be used for other purposes (e.g., for *in vitro* bio-equivalence-, stability- studies, etc.). LOD was determined by making a linear regression of relatively low concentration of levamisole HCl (1.00 to 20 μg mL^{-1}, injection volume 20 μL) according to the method of Funk et al.[21] The calculated equation of the regression line was $Y = 3136 + 29184\ X$ ($n = 9$; $V_{XO} = 1.47\%$; $r = 0.9998$). The calculated testing value X_p21 (for $p = 0.05$) was 0.519 μg mL^{-1}. In this case, the value of LOD = X_p.[21] According to Carr and Wahlich,[24] the value of the LOQ could be estimated at 3 times of the LOD value (1.55 μg mL^{-1} for injection volume 20 μL). For anhydrous niclosamide the range of the linear

Table 4. Analysis of Effect of the Robustness data (HOIE method)[a,b]

Variable	Rt (p)[c]	TF (p)[c]	R (%) (p)[c]
A. Levamisole HCL			
Buffer (%)	NS (0.4586)	NS (0.7867)	NS (0.2366)
pH	NS (0.3963)	NS (0.0374)	NS (0.0645)
Flow (mL min^{-1})	NS (0.4289)	NS (0.1472)	NS (0.6176)
B. Anhydrous niclosamide			
Buffer (%)	S (0.0003)	NS (0.0272)	NS (0.3500)
pH	NS (0.7923)	NS (0.4345)	NS (0.0907)
Flow (mL min^{-1})	NS (0.2114)	NS (0.9003)	NS (0.4612)

[a]Calculated from data presented on Table 3.
[b]Calculation was performed by using unscrambler 9.6 software (CAMO).
[c]Probability.

regression line was also 1.00 to 20 μg mL^{-1}, injection volume 20 μL). The calculated equation of the regression line was $Y = -2391 + 53860 \times$ ($n = 9$; $V_{XO} = 2.57\%$; $r = 0.9995$, $X_p = LOD = 0.903$ μg mL^{-1}, $LOQ = 2.71$ μg mL^{-1}).

Table 1 demonstrates the high accuracy as revealed by the percentage of mean recovery data of LM powders (99.64 and 99.84%). To prove whether systemic errors did not occur, linear regression of the recovery curve of X_f (concentration of the analyte measured by the proposed method) against X_c (nominal concentration of the analyte) of the laboratory made tablets was constructed.[21] The confidence interval data ($p = 0.05$) of the intercept {$VB(a_f)$} and slope {$VB(b_f)$} from the recovery curve did not reveal the occurrence of constant- and proportional systematic errors.

Table 5. Results of forced degradation studies of LM3 veterinarian powders

Stress condition	Time	Recovery (%) (Mean ± SD, n = 3)
A. Levamisole HCl[a]		
1 drops of 0.1 N NaOH	16 hours at 60°C	76.30 ± 1.22
1 drops of 0.1 N HCl	16 hours at 60°C	78.67 ± 2.10
1 drops of 15% H_2O_2	16 hours at 60°C	76.36 ± 4.60
B. Anhydrous niclosamide[b]		
1 drops of 0.1 N NaOH	16 hours at 60°C,	80.22 ± 1.78
1 drops of 0. 1 N HCl	16 hours at 60°C	86.29 ± 1.59
1 drops of 15% H_2O_2	16 hours at 60°C	85.55 ± 3.53

[a]Purity and Identity checks of levamisole HCl peaks were evaluated Shimadzu LC solution software yielded good values (peak purity index >0.999).
[b]Purity and Identity checks of anhydrous niclosamide peaks were evaluated Shimadzu LC solution software yielded good values (peak purity index >0.999).

All the RSD values of the repeatability and intermediate precision studies evaluations were less than 2% (see Table 2), and the calculation by using the David, Dixon, and Neumann Test[22] showed satisfactory results (data not shown). The measurements were performed within our laboratory on the different days by different analysts and by using different HPLC equipment. These results demonstrated that the accuracy and precision of the proposed method were satisfactory in the range of 80 to 120% of the expected value.

In order to evaluate the robustness of the proposed method, the influence of small variation on buffer concentration (% volume), pH, and flow rate of the mobile phase on the values of R_t, TF, and % recovery (% R) of the LM3 were evaluated using the same HPLC equipment on the same day. The data were presented in Table 3. Analysis of effect of the data was performed by using Unscrambler 9.6™ software. A higher order interaction effect (HOIE) method showed that the % R values were significantly not affected by these small variations (Table 4; $p > 0.01$). Rt and TF were also not affected, except Rt for anhydrous niclosamide. A good linear correlation between Rt of anhydrous niclosamide (Y axis) and buffer concentration (% volume) was observed ($Y = 1.313 + 0.367\ X$; $n = 9$; $r = 0.967$; calculated F value of ANOVA linearity test $= 100.33$ for $p < 0.0001$).

Table 5 showed that although the recovery of the analytes was reduced about 20–25% in stressed samples (Table 5), purity and identity check of the analyte peaks using DAD detector yielded good values (>0.99), this showed that all the peaks were still pure and identical with the standard. This proved that the analyte peak had no interference from the degradation products.

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