Validation of an HPLC method for the simultaneous determination of diminazene diaceturate and phenazone in injectable veterinary granules and bulk powders

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Abstract: A validated HPLC method for the simultaneous determination of diminazene diaceturate and phenazone has been established for the analysis of the compounds in veterinary granules for injection and in bulk powders. The compounds were separated using a Symmetry RP 18 analytical column and detected by UV absorbance at 250 nm. Linearity, accuracy as well as the intra-assay precision, inter-day precision and specificity of the method were established. The limits of detection and quantification were 3.2 and 9.7 µg mL^{-1} for diminazene diaceturate and 9.57 and 28.99 µg mL^{-1} for phenazone. Method had the potential to determine these drugs simultaneously from dosage forms without any interference with each other.

Keywords: Diminazene; phenazone; simultaneous determination; HPLC method.

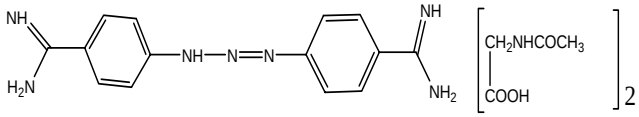
INTRODUCTION

Diminazene diaceturate [4,4'-(diazoamino)dibenza-amidine]; diminazeneaceturate (fig. 1a) is an aromatic diamidine used extensively as a veterinary trypanocide and babesiacide in affected areas of the world (Turnipseed *et al*., 2006; Schad *et al*., 2008). Although not licensed for human use, it has been successfully employed in the treatment of early stage cases of human African sleeping sickness. However, its efficacy has been shortened by widespread drug resistance (Atsriku *et al*., 2002).

Diminazene is soluble in 14 parts of water (20^{o}C), is slightly soluble in alcohol and very slightly soluble in ether or chloroform. Due to the fact that diminazene diaceturate consists of an organic base and an organic acid, once it is dissolved in water it dissociates and each component has its own characteristics (Martindale, 1989).

Phenazone, (1,2-Dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one); commonly called antipyrine, (fig. 1b), is an organic base, tubular crystal or white powder with slightly bitter taste. One gram of phenazone dissolves in less than 1mL water, 1.3 mL alcohol, 1 mL chloroform and 43 mL ether (Merck Index, 1989). Besides to its anesthetic effect (Macharia *et al.*, 2004), phenazone is added to diminazene at a concentration of 55%W/V as a stabilizer in most formulations, since diminazene is unstable in water. Aqueous solutions of these preparations may remain stable for 10-15 days at room temperature (Boothe *et al*., 1994).

Despite its use for over four decades, there are no pharmacopoieal specifications for the quality control of the product. However, through the years multiple analytical methods have been used to quantify diminazene in dosage forms, plasma and animal tissues. These methods include spectrophotometric determination using a sensitive diphenylamine color reaction (Alvi *et al*., 1985), through pre labeling the diminazene with ^{14}C and determining the level of radioactivity (Gilbert, 1983), High performance Liquid chromatographic (UV and/or MS detector) methods (Aliu *et al*., 1983; Atsriku *et al*., 2002; Turnipseed *et al*., 2006), GCMS (Fouda *et al*., 1977) and a competitive Enzyme Linked Immunosorbent Assay (ELISA) method (Karanja *et al*., 2002).



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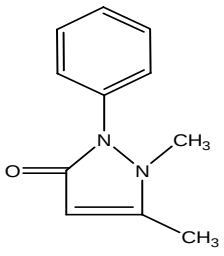

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Fig. 1: Chemical structure of diminazenediaceturate (A) and Phenazone (B).

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The objective of this study was to validate an HPLC method for the simultaneous determination of diminazene diaceturate and pehnazone from their bulk powder and dosage form, veterinary granules for injection. The method has employed the use of isocratic binary compositions of mobile phase. Moreover, constituents of mobile phase as well as type of analytical column used are commonly available in any drug quality control laboratory. Therefore, presented method can be used as an alternative for the already existing HPLC methods.

MATERIALS AND METHODS

Materials and reagents

Diminazene diaceturate (assay value = 98.89 %) and phenazone (assay value = 100%) working reference standards, multi solvent methanol (Scharlau, Spain), monobasic potassium phosphate (Sigma-Aldrich, Germany), ortho-phosphoric acid (Scharlau, Spain), Two brands (Brand A - Mfg: MAR/2009, Exp: FEB/2014 and Brand B - Mfg: AUG/2009, Exp: AUG/2012) containing diminazene diaceturate and phenazone were used during the study.

Chromatographic System

The chromatographic system consisted of a LC-20AD pump, SIL-20A auto sampler, CTO-20AC column oven and SPD-M20A diode array detector (Shimadzu, Japan). The separation was performed by a Symmetry RP 18, 5µm, 15×0.46cm (Waters, Ireland) which was placed at 30^{o}C column oven. Data recording was carried out by LC Solution Software (Shimadzu, Japan). Microsoft excel was also used to treat data statistically.

The mobile phase was isocratic with phosphate buffer 0.085M (pH 3.0) and methanol (78:22, v/v). Mobile phase was filtered through 0.45µm cellulose nitrate membrane filter (Micro science, Germany) and degassed by sonication for 20 minutes prior to use. The flow-rate was 1.0 mL/min. The volume of injection was 20µL and the total run time was 10 min. The eluate was monitored by UV absorbance at 250 nm.

Standard Preparation

Synthetic working standard solution (1 mg mL^{-1}) was prepared by dissolving 250 mg of diminazene diaceturate and 250 mg of phenazone in 250 ml of distilled water. Twenty different concentration levels of working standard solutions (0.002 mg mL^{-1} to 1.0 mL^{-1}) were freshly prepared by diluting suitable volumes of the stock standard solution in appropriate volumetric flasks with distilled water.

Sample preparations

The contents of ten sachets were uniformly mixed and 54.0 mg of the sample was quantitatively transferred and dissolved in 100 ml volumetric flask with distilled water and diluted to volume with the same solvent. Portion of the resulting solution was then transferred into a 1mL glass HPLC vial for analysis.

Method validation

The method was validated in compliance with ICH guidelines (ICH 1994, 1996). The following parameters were evaluated: Linearity, precision (intra and inter-day), accuracy, limit of detection and quantitation, specificity and robustness. Solutions of diminazene diaceturate and phenazone 50, 100, 500 and 1000 µg mL^{-1} were prepared from the dosage form (Brand A) in distilled water for the study of intra and inter-assay precision. These different concentration samples were prepared on three different days in triplicate and analyzed. Some parameters like pH of the mobile phase, flow rate, and column oven temperature were varied ± 10% of the optimized chromatographic conditions to check the robustness of the method. The effect of mobile phase composition variation was checked at ± 5%. However, detection wavelength was tested at 250 ± 3nm. Peak purity of the obtained chromatograms was evaluated by the LC solution software for the study of specificity. For the study of accuracy, 5, 10 and 20 mg of diminazene diaceturate and phenazone were spiked to 20.24 mg of the dosage form (Brand A) and diluted to 100 ml with distilled water.

RESULTS

Resolution and system suitability

The UV-spectrum of diminazene diaceturate and phenazone was presented in fig. 2. Successful resolution of diminazene and phenazone was achieved with a reverse phase analytical column. Fig. 3 shows a chromatogram of the dosage form, while table 1 shows the system suitability data.

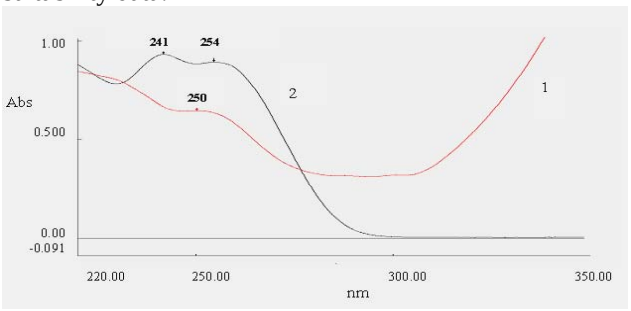

Fig. 2: UV-spectrum of diminazene diaceturate (1) and phenazone (2)

Method validation

Linearity

Summary of linear regression data for calibration curves has been presented in table 2.

Precision study

Precision of the method was studied with respect to intra and inter-day variations. Precision was expressed in terms of coefficient of variation (CV) of peak areas. Results for intra-day and inter-day precision (table 3) have shown

that the method has good precision in the ranges of 50-1000 µg mL^{-1}.

Accuracy study

The accuracy of the presented method has been determined by studying the recoveries of the method at three levels of concentration at 80, 100 and 120% of the assay concentration. The obtained recovery result for diminazene diaceturate was between 97.4 and 103.7% whereas for phenazone it was in the range of 98.1 and 101.5%. Detailed recovery results for both compounds have been presented in table 4.

Table 1: Retention time and peak performance parameters for diminazene diaceturate and phenazone when analyzed by the mentioned chromatographic conditions (n = 5)

Compound	Retention time (min)	USP Tailing factor	USP theoretical plate	USP resolution
Diminazene diaceturate	2.41 ± 0.01	1.41 ± 0.04	2640 ± 134	1.00 ± 0.00
Phenazone	8.35 ± 0.01	1.31 ± 0.01	5888 ± 7	17.27 ± 0.53

All values are mean ± S.D.

Table 2: Summary of linear regression data for calibration curves using peak areas

Parameters	Diminazene diaceturate	Phenazone
Linearity range	0.002 – 1 mg mL^{-1}	0.002 – 1 mg mL^{-1}
Linear regression equation Determination coefficient (r^2)	Y= 27249602X -32798 0.9999 ± 0.0000577	Y = 55645936X ± 300513 0.9994 ± 0.000252
Correlation coefficient (r) Slope ± SD	0.99997 27249602 ± 52232	0.9997 55645936 ± 317505
Intercept ± SD	-32798 ± 26539	300513 ± 161328
Limit of Detection	3.2 µg mL^{-1}	9.57 µg mL^{-1}
Limit of Quantification	9.7 µg mL^{-1}	28.99 µg mL^{-1}

Table 3: Results of intra and inter-day precision for diminazene diaceturate and phenazone

Compound	Conc. (µg mL^{-1})	Intra-day precision (n = 3)			Inter-day precision (n =3)		
		Calculated Conc. (µg mL^{-1})	SD[a]	CV[b]	Calculated Conc. (µg mL^{-1})	SD[a]	CV[b]
Diminazene diaceturate	50	49	1.2	2.50	48	1.4	2.9
	100	104	1.0	0.97	103	1.9	1.8
	500	534	7.5	1.40	534	7.5	1.4
	1000	1059	6.0	0.57	1031	25.8	2.5
Phenazone	50	51	0.8	1.50	50	1.4	2.9
	100	101	0.5	0.51	102	2.2	2.2
	500	504	3.5	0.70	514	11.8	2.3
	1000	1049	2.6	0.25	1035	24.8	2.4

[a]Standard deviation and [b]Coefficient of variation

Table 4 Recovery study of the method for diminazene diaceturate and phenazone (n = 3)

Compound	Levels of addition	Initial amount (mg)	Added Amount (mg)	Amount Recovered (mg)	Recovery (%)
Diminazene diaceturate	80%	20.24	5	5.18	103.7
	100%	20.24	10	10.26	102.6
	120%	20.24	20	19.60	97.4
Phenazone	80%	20.24	5	4.91	98.1
	100%	20.24	10	10.02	100.3
	120%	20.24	20	21.71	101.5

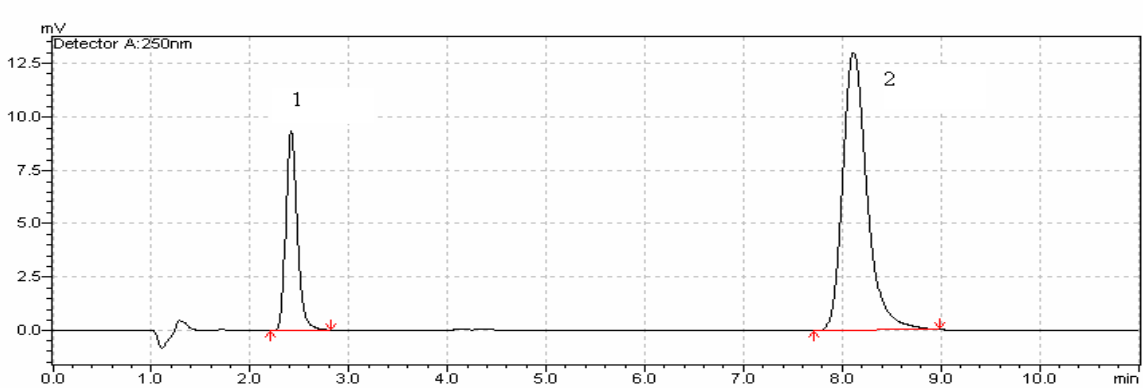

Fig. 3: Typical HPLC chromatogram of diminazene diaceturate (1) and phenazone (2) in sample.

Table 5: Results of robustness study for the presented method (n=3)

Parameters	Diminazene diaceturate		Phenazone	
	SD[a]	CV[a]	SD[a]	CV[a]
Mobile phase composition	59101	0.83	92913	0.59
Mobile phase pH	33709	0.47	153261	0.96
Flow rate	35462	0.52	42345	0.34
Wave length	77649	1.10	294297	1.82
Oven temperature	37483	0.53	37489	0.24

[a] SD and CV were calculated from the peak areas of the chromatograms.

Table 6: Assay results for the commercial dosage forms (n =6)

Compound	Analyzed commercial brands					
	Brand A			Brand B		
	Assay value (%)	SD	CV	Assay value (%)	SD	CV
Diminazene diaceturate	92.97	1.13	1.21	92.66	1.74	1.88
Phenazone	98.6	1.63	1.65	99.30	1.21	1.22

2.36 g of the dosage form contains 1.05 gm of diminazene diaceturate and 1.31 g of phenazone.

Specificity study

The dosage form Brand A was analyzed and the purity index of the obtained peaks (fig. 3) were calculated by the LC solution software. Hence, the purity index for diminazene diaceturate peak is 1.000 and for phenazone is 0.9922. Therefore, the method has the ability to determine diminazene diaceturate in the presence of phenazone and vice versa.

Robustness

The obtained results for robustness study have been presented in table 5. Statistical analysis proves that the method is robust for all of the tested parameters.

Analysis of commercial dosage form

Two commercial brands of diminazene diaceturate that contained phenazone as excipient were analyzed by the presented method and acceptable assay results were obtained. The assay results have been presented in table 6.

DISCUSSION

An efficient method for the simultaneous determination of diminazene diaceturate and phenazone has been described in the study. The UV-spectra of both substances have been collected (fig. 2.) in the range of 220 and 350 nm using the standard solutions in order to determine the working wavelength. The wavelength used in the study was found to be suitable for the assay of the two compounds simultaneously. The choice of 250 nm was shown to be close to the absorption maxima of both diminazene diaceturate and phenazone. This wavelength is the most appropriate in order to get better and relatively steady response for both compounds.

Successful resolution of diminazene and phenazone was achieved with a reverse phase analytical column. Both diminazene and phenazone are basic in nature (Martindale, 1989). The presence of ‘free silanol’ groups in silica based reversed phase columns may give rise to residual interactions with basic analytes which occurs

mainly as a result of incomplete end capping. Residual interactions can weaken quantification process and result in tailed peaks. However, in the presented study, the ionization of free silanol groups of the stationery phase has been suppressed by adjusting the pH of the mobile phase to lower pH value (pH 3.0). Therefore, residual interactions of the free silanol groups with the basic moieties of diminazene and phenazone will be reduced significantly and as a result relatively sharper peaks for both compounds will be produced.

The presented method is linear in the range of 0.002-1 mg $mL^{-1}$for both drug substances. The determination coefficient and correlation coefficient are 0.9999 and 0.99997 for diminazene diaceturate and 0.9994 and 0.9997 for phenazone respectively. The method offers excellent repeatability and intra-assay precision at low (50 µg mL^{-1}) as well as high concentrations (1000 µg mL^{-1}) for both drug substances. The peak purity index values of diminazene and phenazone peaks in chromatograms of the sample solution were above 0.99 indicating that there was no interference from excipient and providing evidence of the selectivity of the proposed HPLC method (Atsriku *et al*., 2002). Mobile phase composition, flow rate, pH change, wave length variation and oven temperature have been varied to study the robustness of the method (Heyden *et al*., 2001). These deliberate variations didn't produce any major changes on the retention times and peak areas of the two drug substances, demonstrating the robustness of the method.

The presented method has been applied in the analysis of two generic products (injectable veterinary granules) of diminazene diaceturate and phenazone. Within a tolerance window of ±/10% of label claim of diminazene diaceturate and phenazone contents (i.e. 90-110%), both of the products have satisfied the criteria.

CONCLUSION

The presented method is simple, accurate and precise and hence it can be applied for the simultaneous determination of diminazene diaceturate and phenazone in their dosage forms and bulk powders.

ACKNOWLEDGMENTS

The authors wish to thank the Egyptian Fund for Technical Cooperation with Africa, Ministry of Foreign Affairs, Egypt, for the financial support that enabled Prof. Adnan Bekhit to be available in School of Pharmacy, Addis Ababa University.

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