

## Paired-ion extraction and high-performance liquid chromatographic determination of diminazene in cattle plasma: a modified method

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### ABSTRACT

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The high-performance liquid chromatographic method published by Aliu & Ødegaard (1983) was found to give poor peak separation when used to determine plasma diminazene concentrations in cattle. Before bioequivalence studies could be carried out, the method had to be modified. Solid-phase extraction with acetonitrile/0,025 M Na-octane sulphonate and 2% acetic acid as eluent, followed by sample concentration, gave recoveries of > 90% for diminazene and the internal standard. A mobile phase of acetonitrile/0,005 M Na-octane sulphonate, 0,1% triethylamine, pH 3,2 with acetic acid on a Nova Pak C-18 column was used for the analysis. Wavelength switching was used to determine the internal standard (imidocarb) and diminazene at their respective wavelengths of maximum absorbance, resulting in a fivefold increase in the limit of detection for diminazene. The modified method attained a detection limit of 2 ng.m<sup>-1</sup> (peak 4x baseline noise), limit of quantitation of 10 ng.m<sup>-1</sup> (coefficient of variation < 15%) and an accuracy of > 96% over the range from 10–5000 ng.m<sup>-1</sup>.

**Keywords:** Paired-ion extraction, liquid chromatographic determination, diminazene, cattle plasma

### INTRODUCTION

Diminazene diacetate, "Berenil" (Hoechst, RSA) (Fig. 1), is a well known trypanocide and babesiacide that has been used extensively throughout the world. In recent years, most of the clinical trials published originated in Africa, where such infections are still common (Aliu, Mamman & Peregrine 1993; Gray, Kimarua, Peregrine & Stevenson 1993; Mamman, Katende, Moloo & Peregrine 1993). Several methods of analysis, including colorimetry, biological assay, radiometry, GC-MS, TLC

and HPLC, have been described for diminazene in biological fluids. According to Pererine & Mamman (1993) and Aliu & Ødegaard (1983), colorimetry, biological assay and radiometry lack specificity, while the TLC method is not sensitive enough. The GC-MS and the HPLC methods described by Fouda (1977; 1978) are tedious and do not determine intact diminazene, but its reduction product, 4-aminobenzamidine. Aliu & Ødegaard (1983) were able to extract and determine intact diminazene with paired-ion solid-phase extraction and reversed-phase HPLC. The method described by the latter authors seemed to be the most sensitive, accurate and precise.

It was therefore decided to apply Aliu & Ødegaard's (1983) method to a bioequivalence study in which cattle plasma would be used (Gummow 1993). However, the method resulted in poor peak shape and

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separation, and the desired levels of precision, accuracy and limit of quantitation could not be achieved. The method was therefore modified to meet the needs of the bioequivalence study.

## METHOD

### Reagents and materials

The following reagents and materials were used:

- 100 mg octadecylsilane solid-phase extraction (SPE) tubes (*Supelclean LC-18, Supelco Inc. Bellefonte, PA*)
- Nova Pak C18 radial compression cartridge, 4  $\mu\text{m}$ , 8 x 100 mm (*Waters Assoc., Milford, MA*)
- Methanol [*Analar, E Merck SA (Pty) Ltd, Johannesburg*]
- Acetonitrile (*HiPerSolv, BDH, Poole, England*)
- Water, HPLC grade, prepared in-house with a Milli-Q system (*Millipore, Milford, MA*)
- 10% methanol/water
- 0,05 M Ethylenediaminetetra-acetic acid disodium salt (Na-EDTA), 1-Octane sulphonic acid sodium salt, triethylamine and glacial acetic acid (*UnivAR, Saarchem, Muldersdrift, RSA*)
- Dynagard fibre syringe filters, 0,45  $\mu\text{m}$  (*Microgon, Inc. Laguna Hills, CA*)
- Membrane filters, 0,45  $\mu\text{m}$  (HVLP, *Millipore, Milford, MA*)
- Diminazene diacetate (*Hoechst, RSA*)
- Imidocarb (*Burroughs Wellcome, RSA*)

### Mobile phase

The mobile phase comprised 30/70 acetonitrile/0,005 M octanesulphonic acid sodium salt in water containing 0,1% triethylamine, and was set to a pH of 3,2. Glacial acetic acid was used. The mobile phase was filtered through a 0,45  $\mu\text{m}$  membrane filter and degassed with helium immediately prior to use.

### HPLC system

A Hewlett Packard 1050 HPLC system equipped with a programmable variable wavelength UV detector, autosampler, isocratic pump and HP MS-DOS chemstation data-analysis system (Hewlett Packard, Palo Alto, CA) was used for analysis. The detector was pro-

grammed to detect diminazene at 370 nm and imidocarb at 254 nm. The separation was achieved on the column mentioned previously, at a flow rate of 1,5  $\text{mL}\cdot\text{min}^{-1}$ .

### Diminazene stock solutions

Five milligrams of pure diminazene diacetate were accurately weighed and dissolved in 50 mL of distilled water. Five mL of this solution was then further diluted to 50 mL with water, and 10 mL of the latter solution was diluted to 50 mL with water to obtain three stock solutions of 50, 5 and 1  $\mu\text{g}\cdot\text{mL}^{-1}$  of diminazene, respectively.

The above stock solutions were used to spike blank plasma to obtain standards containing 10, 20, 50, 100, 250, 500, 1000, 2500 and 5000  $\text{ng}\cdot\text{mL}^{-1}$  of diminazene.

### Internal standard solution

The internal standard used was imidocarb (Fig. 1) which is structurally similar to diminazene. Five milligrams of imidocarb were accurately weighed and dissolved in 200 mL of distilled water to give a stock solution of 25  $\mu\text{g}\cdot\text{mL}^{-1}$  imidocarb for use as internal standard. Fifty microlitres of this solution were added to all standards and samples prior to extraction.

### Preparation of standards and samples

The standards were prepared by spiking 1 mL of blank plasma with 50  $\mu\text{L}$  of the imidocarb internal standard solution and the appropriate amount of diminazene standard solution as mentioned above, and vortexing for 30 s. Three millilitres of 0,05 M Na-EDTA solution was then added to each standard, after which it was again vortexed for 30 s. The samples were prepared in the same manner, but without the addition of diminazene.

### Solid-phase extraction procedure

The SPE tubes were conditioned with 3 mL of methanol, followed by 2 mL of water. Samples were passed through the SPE tubes at a rate of approximately 0,5  $\text{mL}\cdot\text{min}^{-1}$ . Two mL of 10% methanol in water was passed through the tubes as a clean-up step, the effluent discarded, and the tubes dried under full vacuum for 5 min. Analytes were eluted with 3 mL of 90/10 acetonitrile/0,025 M octanesulphonic acid sodium salt with

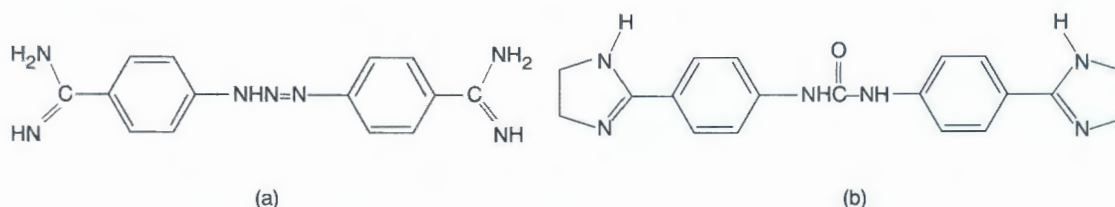


FIG. 1 The chemical structures of diminazene (a) and imidocarb (b)



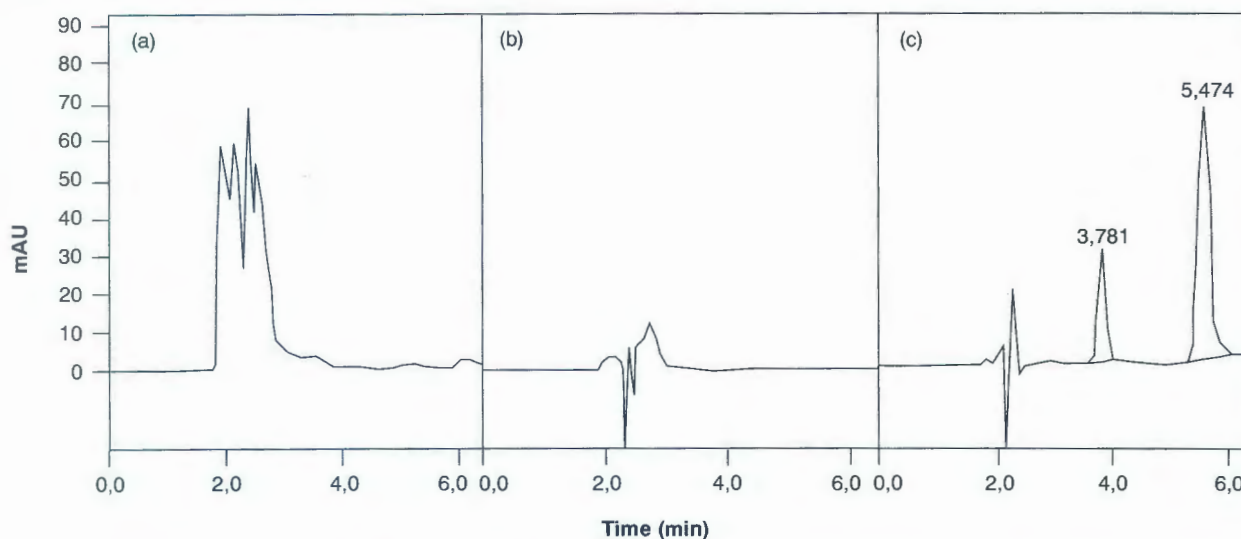


FIG. 2 HPLC chromatograms of blank cattle plasma at 254 nm (a), 370 nm (b) and an extracted plasma sample containing 563,24 ng.mL<sup>-1</sup> of diminazene (c). The chromatograms were plotted on the same scale

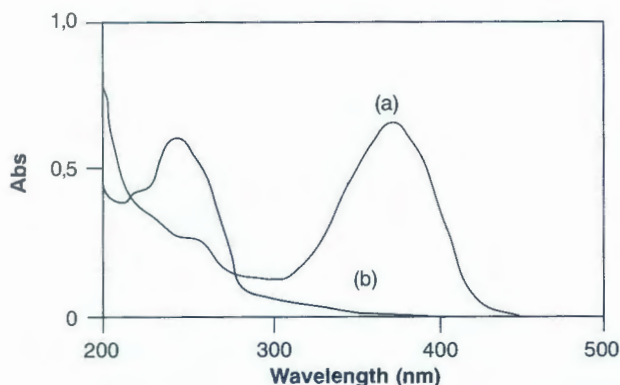


FIG. 3 The UV absorption spectra of diminazene (a) and imidocarb (b) in mobile phase, with mobile phase as blank

TABLE 1 Accuracy and precision of the analytical method

Concentration spiked (ng.mL <sup>-1</sup> )	Concentration found (ng.mL <sup>-1</sup> ) <sup>a</sup>	% found <sup>a</sup>	Coefficient of variation (%) <sup>b</sup>
10	10,23	102,33	14,40
20	20,42	102,08	9,13
50	48,20	96,40	7,47
100	96,02	96,02	5,18
250	244,38	97,75	2,46
500	492,37	98,47	2,55
1000	987,50	98,75	1,57
2500	2466,17	98,65	2,04
5000	4849,33	96,99	0,99

<sup>a</sup> Accuracy

<sup>b</sup> Precision

2% glacial acetic acid in water. The eluent was evaporated by a vacuum centrifuge at 40 °C (Savant Speedvac concentrator, Farminghill, NY) after which the residue was redissolved in 350- $\mu$ l of mobile phase with the aid of a vortex mixer. This sample was then filtered through a 0,45- $\mu$ m fibre syringe filter into 300  $\mu$ l microvials. These were sealed and placed into the HPLC autosampler for analysis.

### Validation procedures

Linearity was determined by extracting six sets of nine standards of diminazene, ranging from 10–5 000 ng.mL<sup>-1</sup>, and performing linear-regression analysis of the peak-area ratios versus standard concentration of diminazene for the spiked plasma. The accuracy and precision were assessed by extracting six sets of spiked samples of known concentrations and measuring their concentration against a linear-regression graph constructed from a different set of standards which were prepared from independent stock solu-

TABLE 2 Recovery of diminazene and imidocarb from plasma

Concentration (ng.mL <sup>-1</sup> )	% recovered	C.V. %
50	90,14	50
250	91,12	250
1000	92,48	1000
Imidocarb*	96,74	Imidocarb <sup>a</sup>

<sup>a</sup> At concentration used for internal standardization

tions. The recovery was assessed by comparing the peak areas obtained for extracted samples ( $n = 6$ ) to those obtained by spiking empty tubes with a similar quantity of diminazene and imidocarb, making it up to a volume of 350  $\mu$ l with mobile phase, and vortexing and injecting it into the HPLC. The limit of quantitation was taken as the lowest concentration, which consistently yields a coefficient of variation of less than 15 %, and the limit of detection was taken as the concentration with a peak height of four times baseline noise.

## RESULTS AND DISCUSSION

The HPLC method resulted in diminazene and imidocarb being well separated with retention times of  $\pm 3,7$  and  $5,4$  min, respectively [Fig. 2(c)].

Wavelength switching improved the detectability of diminazene which absorbs about three times more strongly at  $370$  nm than at  $254$  nm, as illustrated in Fig. 3. Imidocarb cannot be monitored at  $370$  nm because it does not absorb at this wavelength, making wavelength switching the only viable option for optimal detection of both compounds. Monitoring the first  $4,5$  min after injection at  $370$  nm resulted in a much "cleaner" looking chromatogram, due to the fact that fewer co-extracted impurities absorb at  $370$  nm, as opposed to  $254$  nm [Fig. 2 (a) and (b)]. The specificity was also enhanced by monitoring diminazene at a wavelength of  $370$  nm.

Improved chromatographic separation was achieved by using a  $4\text{-}\mu\text{m}$   $C_{18}$  column, and a slightly modified mobile phase. The resolution ( $R$ ) and selectivity factor ( $\alpha$ ) obtained with the modified method was  $4,86$  and  $1,83$ , respectively, compared to  $1,73$  and  $1,48$  for Aliu & Ødegaard's (1983) method.

The response for diminazene was linear over the range studied ( $R^2 > 0,996$ ). Table 1 shows the accuracy and precision of the method, while the recovery from plasma is given in Table 2. The limit of quantitation was  $10\text{ ng}\cdot\text{mL}^{-1}$  (C.V.  $\leq 15\%$ ), and the limit of detection was less than  $2\text{ ng}\cdot\text{mL}^{-1}$ .

## CONCLUSION

By using wavelength switching and concentration of the samples, we were able to refine the method and lower the detection limit fivefold without compromising accuracy. A suitable method for use on cattle plasma was therefore achieved, facilitating more reliable bio-

equivalence studies (Gummow, Swan & Du Preez 1994).

## ACKNOWLEDGEMENTS

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