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Solid-phase extraction and ion-pair reversed-phase HPLC of isometamidium in bovine serum and tissues

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Summary

An analytical method has been developed for the determination of isometamidium in bovine serum and tissues. Samples were enzymatically hydrolysed and cleaned up on a solid-phase system (C8 Bond Elut column). The drug was chromatographed by an ion-pair reversed-phase technique using heptane sulphonate as a pairing-ion and triethylamine as a counter-ion reagent. Detection was by fluorescence at 593 nm (excitation = 380 nm). The method is more sensitive and specific than existing methods and it is currently being used in evaluating the pharmacokinetics of isometamidium in cattle.

Key words: ion-pair HPLC; isometamidium; serum; tissues.

Introduction

Although isometamidium (ISMM) (Samorin, RMB Animal Health Ltd.) has been employed in the treatment and prophylaxis of bovine trypanosomiasis for nearly 30 years, its pharmacokinetic behaviour is still unknown due to lack of suitable analytical methods. The spectrophotometric method of Philips et al. (1967) used in studies in goats (Braide and Eghianruwa, 1980) and camels (Ali and Hassan, 1984) is not sufficiently specific and sensitive to detect ISMM at concentrations less than 1 μg/ml of plasma or 2.3 μg/g of tissue. The HPLC method of Perschke and Vollner (1985) is indirect in that ISMM is converted to homidium before quantitation and therefore it is not a specific assay. In addition, it requires processing of large volumes of plasma of up to 10 ml and injection of sample volumes of up to 1 ml on to the chromatographic column. The main problems associated with the assay of ISMM are due to its low concentrations in plasma; its binding to plasma and tissue macromolecules and its instability at elevated temperatures and in extreme pH conditions.

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We have developed a method for the determination of ISMM in bovine serum and tissues which has considerable advantages over previously reported methods. The properties of the drug which have been exploited are its native fluorescence and ionic character which permitted the use of heptane sulphonic acid as an ion-pair reagent to optimise reversed-phase chromatography of the intact molecule.

Materials and Methods

Chemicals and reagents

ISMM chloride (Samorin) was obtained from RMB Animal Health Ltd. Protease Type VIII (Subtilisin Carlsberg) and Protease Type XI (Proteinase K) were purchased from Sigma Chemical Company Ltd., Poole, England. Heptane sulphonic acid was purchased from Fisons, Loughborough, England. The other reagents were of analytical grade, purchased from the British Drug House. Methanol was re-distilled before use.

Sample preparation

To 4 ml of serum, 2 ml of borate buffer pH 8.0 was added, followed by 4 mg of Subtilisin Carlsberg and incubated at 37 °C for 1 h with occasional shaking. The incubated samples were then applied to C8 Bond Elut (1 ml) columns (Analytichem International, Harbor City, USA) which had been pre-conditioned with 1 ml of methanol and 1 ml of distilled water. The columns were then washed with 1 ml of distilled water, followed by 1 ml of methanol-water (50:50, v/v). The drug was eluted with 0.5 ml of methanol-0.1 M heptane sulphonic acid-triethylamine (92:8:0.1, v/v/v) (apparent pH 7.5). The effluent was vortexed briefly and an aliquot (50 µl) was injected on to the chromatograph.

Tissue samples were prepared as 10% homogenates in borate buffer pH 8.0 using an Ultra-Turrax homogenizer (Janke and Kinkel, IKA-Werk, Staufen). To 5 ml of tissue homogenate, 5 mg of Subtilisin Carlsberg were added, incubated for 1 h at 37 °C and filtered through loosely packed glass wool to remove any undigested material. Four ml of the filtrate was passed through the extraction column and washed with 1 ml of distilled water. The drug was eluted as described for serum.

As a test for improving recoveries of the extraction procedure, the effect of varying the enzyme and chemical functionality of the extraction columns was investigated. Sorbents tested include ethyl (C2), octadecyl (C18), phenyl (PH), cyanopropyl (CN), carboxymethyl (CBA) and benzesulphonyl-propyl (SCX). Also, different methods of protein precipitation including use of acetone, methanol and acetonitrile-propanol were tested on both serum and tissues.

Chromatography

The HPLC system comprised an Altex pump Model 110A and a fluorescence detector, Perkin-Elmer-Model LS-4. The excitation wavelength was 380 nm and emission wavelength was 593 nm. Injections were performed via a 100 µl sample loop fitted to a Negretti 342 syringe loading injection valve (Southampton, England). The column (16 cm × 5 mm, i.d.) used was packed with ODS-Hypersil (5 µm) (Shandon Southern Products Ltd., Cheshire, UK) and the mobile phase consisted of methanol – 0.1 M heptane sulphonic acid-triethylamine (80:20:0.14, v/v/v) (apparent pH 6.6) pumped at a flow rate of 1.2 ml/min.

Calibration

Stock standard solutions were prepared in water. Working standard solutions were made in the eluent used for drug extraction and they were stable for up to 5 days. Control drug-free serum and tissue homogenate samples were spiked with standard solutions prepared in water and subjected to the extraction and chromatographic procedures described above. Recoveries were determined by reference to peak heights resulting from direct injection of the working standard solutions.

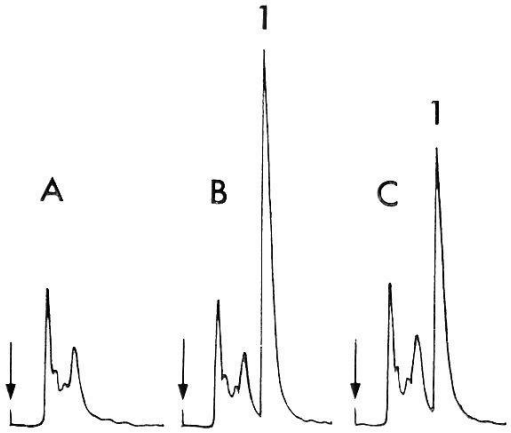

Fig. 1

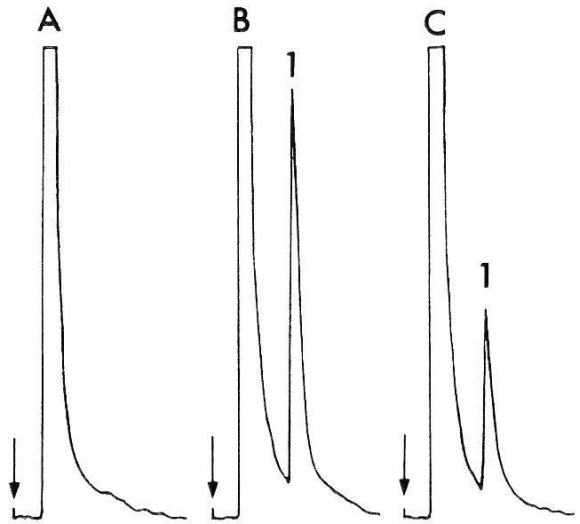

Fig. 2

Fig. 1. Typical chromatograms for isometamidium in serum. A: drug-free serum; B: drug-free serum spiked at a concentration of 0.25 µg/ml; C: calf serum 1 h after intravenous administration of Samorin at a dose of 0.5 mg/kg body weight. Peak: 1 = isometamidium.

Fig. 2. Typical chromatograms for isometamidium in kidney cortex. A: drug free kidney homogenate; B: drug free kidney homogenate spiked at a concentration of 1 µg/ml; C: calf kidney 8 days after intravenous administration of Samorin at a dose of 0.5 mg/kg body weight. Peak: 1 = isometamidium.

Animal experiment

This was performed to test the suitability of the method. A clinically healthy calf weighing 150 kg was dosed with Samorin (1% w/v aqueous solution) via the jugular vein at a dose rate of 0.5 mg/kg body weight. Blood was collected from the contralateral jugular vein over the next 4 days and allowed to clot. Serum was separated and kept at –20 °C until analysed. After 8 days, the calf was sacrificed and tissue samples were collected and kept at –20 °C until analysed. The concentrations of ISMM in the samples were determined by comparison with the drug-free serum or tissue samples spiked with the drug and taken through the analytical procedure.

Results

Chromatograms

Using the procedure described above, ISMM was well chromatographed with a retention time of 3.7 min. Typical chromatograms are shown in Figs. 1 and 2 for serum and tissue samples, respectively. In both cases, there were no interferences from coextracted endogenous compounds.

Recovery and precision

The recovery varied according to the matrix, with serum having a much higher recovery of 82.8 ± 2.86% (mean ± SEM, n = 28) than tissues in which it varied from about 25 to 33% (Table 1). Enzymatic hydrolysis was found to improve recoveries and to facilitate percolation of the samples through the extraction columns. No differences in recoveries were observed between Subtilisin Carlsberg and Proteinase K.

Table 1. Recovery of isometamidium from serum and tissue homogenates after hydrolysis with Subtilisin Carlsberg and precision of the HPLC method

Sample	Added concentration (μg/ml)	Number of assays	Recovery (%)	Inter-day coefficient of variation (%)
Serum	0.10	5	73.9	7.71
	0.25	6	80.5	8.60
	0.50	5	82.0	6.74
	1.0	6	87.1	8.11
	2.0	6	90.6	5.62
Liver	1.0	3	32.9	12.10
Kidney	1.0	3	25.1	11.11
Muscle	1.0	3	28.0	13.21

Of the different preparative column support materials tested for sample extraction, C8 (Octyl) showed a better retention of the drug from which it could be readily eluted after washing out coextractives. Ion exchange sorbents such as benzenesulphonylpropyl showed a very high retention for ISMM and it could be eluted only with acidic methanol which was found to degrade the drug. None of the protein precipitation methods tested gave recoveries higher than those achieved after enzymatic hydrolysis.

Precision of the method was evaluated by inter-day coefficient of variation on spiked replicate samples as shown in Table 1. The mean inter-day coefficient of variation for serum was low, averaging 7.36%. The corresponding values in tissues were 11.11, 12.10 and 13.21 for kidney, liver and muscle, respectively.

Linearity

Linearity of peak height versus concentration was assessed on the standard solutions. Linearity was excellent over the concentration range studied (r^2 = 0.9998).

Limit of detection

The limit of detection corresponding to 3 times the noise level was 10 ng/ml of serum and about 500 ng/g of tissue.

Applicability

The concentrations of ISMM in serum from the calf as determined using this method are shown in Fig. 3. The concentration of the drug in serum could be measured and found to decline biexponentially to levels below detection after 24 h. Kinetic analysis of the serum concentration versus time data revealed a terminal phase elimination half life of 1.53 h and a volume of distribution of 1.2 l/kg. Eight days after drug administration, the concentrations in the kidney, liver, heart and muscle were 4.4; 2.6; 0.8 and 0.7 μg/g, respectively.

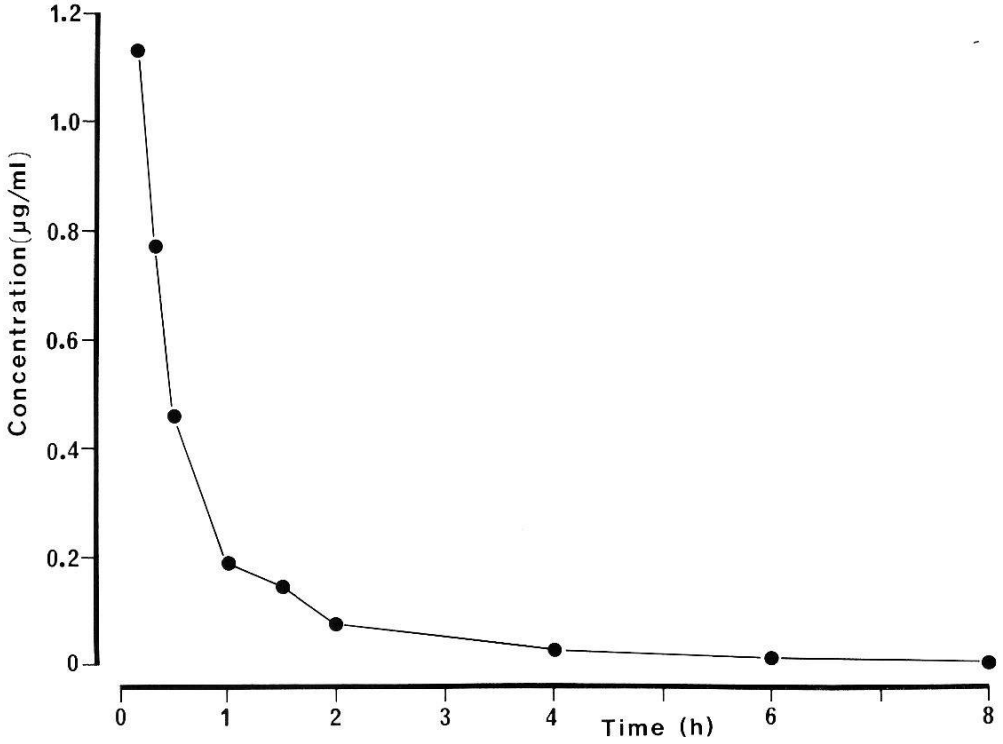

Fig. 3. Serum concentration versus time profile of isometamidium in a calf after intravenous administration at a dose of 0.5 mg/kg bodyweight.

Discussion

From the results presented here and from theoretical considerations (Hung et al., 1982), ion-pair chromatography should be the most suitable separation technique for ISMM since it exists as a cation at all pH ranges. Neither of its phenanthridinium analogues, homidium nor pyrithidium, was found to interfere with its detection when tested during method development. Similarly the other commonly used trypanocidal drugs, diminazene and quinapyramine, did not give any interference. However, none of these compounds was found to be a suitable internal standard because the excitation and emission wavelengths used were optimized for the detection of ISMM only to achieve the highest sensitivity. Moreover, the metabolic pattern of ISMM is unknown and it was therefore envisaged that use of any of its analogues as an internal standard might interfere with the detection of putative metabolites either by UV or fluorescence.

The high extraction recoveries of ISMM from serum shows that the solid-phase system used is a suitable method. Inclusion of the enzymatic hydrolysis step improved recoveries due to protein degradation, thereby releasing the bound drug (Osselton et al., 1977, 1978; Werkhoven-Goewie et al., 1983). Although Proteinase K and Subtilisin Carlsberg gave similar recoveries, the latter was chosen for further use because it was cheaper. The low recoveries of the drug from tissues, unlike those from serum, may be attributed to strong binding to other macromolecules such as mucopolysaccharides, nucleic acids and lipids (Philips et al., 1967; Kinabo and Bogan, 1987).

The sensitivity of the present method is high primarily because of the fluorimetric detection and the concentration step of extraction. These factors have made possible the detection of ISMM in serum even at concentrations lower than those reported to be effective against trypanosomes (Hill and McFadzean, 1963; Hill, 1965; Jefferies and Jenni, 1987).

No specific conclusions on the kinetics of ISMM can be drawn from the one animal experiment. However, it may be seen that the high concentrations of the drug measured in tissues, unlike those in serum, are well in agreement with the volume of distribution (> 1 l/kg) which indicates that the drug is accumulated in tissues.

In conclusion, this method provides a sensitive and specific assay for ISMM in biological matrices. The extraction and chromatographic steps are simple and reproducible. With slight modifications, it can be adapted to assay its analogues. It is currently being used in determining the kinetics and tissue residues of ISMM in cattle.

Acknowledgments

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