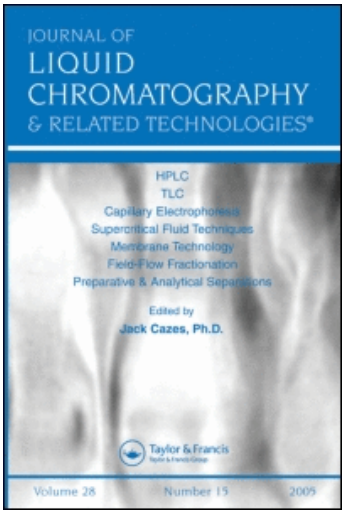

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Determination of Rafoxanide in Plasma Using High Performance Liquid Chromatography (HPLC) and in Tissue Using HPLC-Thermospray Mass Spectrometry

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DETERMINATION OF RAFOXANIDE IN PLASMA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AND IN TISSUE USING HPLC-THERMOSPRAY MASS SPECTROMETRY

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ABSTRACT

Methods are described for the determination in plasma and tissue samples, of the drug rafoxanide which is used to control liver fluke in sheep and cattle.

HPLC with UV detection is used to measure levels of the drug in plasma and combined HPLC-thermospray mass spectrometry is used to measure residue levels in tissue. The methods are both specific and sensitive with detection limits of 0.1 ug ml^{-1} for plasma and 0.02 ug g^{-1} for tissue.

INTRODUCTION

Rafoxanide (3,5-diiodo-3′-chloro-4′-[p-chlorophenoxy] salicylanilide) is one of a group of salicylanilide drugs widely used to control liver fluke (Fasciola hepatica) in sheep and cattle. Although the drug has been in use over a long period, there have been few reports on its pharmacokinetics or methods of assay. Mohammed-Ali and Bogan (1) recently reported that rafoxanide had a half life of 17 days in the plasma of sheep and that it was extensively bound to plasma proteins. They determined the half life value from plasma rafoxanide levels following oral administration of the normal therapeutic dose of 7.5 mg kg^{-1} bodyweight. The assay was described as using high performance liquid chromatography (HPLC) but no details of the method were given. Protein binding was determined using ultrafiltration. They also reported that rafoxanide was detectable in plasma for up to 112 days after this normal therapeutic dose.

These findings, combined with public demand for meat free from harmful drug residues, dictate that methods should be available to measure residues of rafoxanide in plasma and in the carcases of slaughtered animals. A gas chromatographic (GC) method has been published (2) for the determination of rafoxanide in plasma. This method involved extraction, hydrolysis of the extract, liquid/liquid partition, derivatization and detection using electron capture GC, and we did not consider it suitable for routine use. A simple

spectrophotometric assay has also been pubished (3) but plasma from untreated animals showed a high level of background interference. To the best of our knowledge no methods have been published for the determination of rafoxanide in tissues.

The present study describes the development of an HPLC method for the quantitation of rafoxanide in plasma and an HPLC/thermospray mass spectrometric (LC/MS) method for the detection and quantitation of residue levels in tissues.

EXPERIMENTAL

Apparatus

The HPLC system for the plasma consisted of a 1050 series isocratic pump, programmable wavelength detector set at 282 nm, autosampler, and a model 3396A integrator (Hewlett Packard Ltd, Stockport, UK). The HPLC column was a 100 x 4 mm Nova-pak C18 reverse phase column (Millipore) Waters, Harrow, Middlesex, UK).

The HPLC system for the tissue assay consisted of a Merck-Hitachi model L-6000 pump (BDH Ltd, Romford, Essex), a Rheodyne model 7125 injector fitted with a 50 ul sample loop (BDH Ltd), and a Lichrosorb RP18, 125 x 4 mm reverse phase cartridge with holder (BDH Ltd). This system was coupled to a Vestec model 201A Thermospray LC/MS (Vestec Comp, Houston, Texas, USA), complete with a Technivent workstation.

The instrument was operated in the negative ion chemical ionisation (CI) mode using filament initiated

ionisation with an electron beam current of 250 uA. The electron multiplier voltage was 2400 V. The temperatures of the vaporiser, block, tip heater and lens assembly were set at 150, 280, 250 and 150°C respectively. The tuning parameters were checked weekly according to the manufacturers' instructions using a 50 $mg.l^{-1}$ solution of polyethylene glycol 300 in 50% acetonitrile, containing 0.1 M ammonium acetate. The instrument was operated either in the full scan mode to collect spectra or in the selected ion monitoring mode (SIM) for maximum sensitivity in analysing samples. For the former, a dwell time of 5 m.sec. was used, and for the latter the dwell time was 100 m.sec. In each case the sweep window was set at 0.5 AMU.

Reagents

Acetonitrile and petroleum spirit (40-60°C boiling range) were HPLC grade, other reagents were Analar grade. Rafoxanide was obtained from Chanelle Veterinary Products, Loughrea, Co. Galway, Ireland. A stock standard (1 $mg.ml^{-1}$) was prepared by dissolving 50 mg in 5 ml acetone in a 50 ml volumetric flask, and diluting to the mark using acetonitrile. This standard was stable for at least 1 month if stored at 4°C in the dark. Dilute standards (1 and 5 $ug.ml^{-1}$) were prepared daily by dilution of the stock standard in mobile phase. The mobile phase for the HPLC plasma assay consisted of acetonitrile/0.1 M ammonium acetate ratio 55:45. For the LC/MS tissue assay, the mobile phase consisted of acetonitrile/tetrahydrofuran/0.1 M ammonium acetate,

ratio 60:5:35. Both solutions were de-gassed and filtered under vacuum through a 0.45 u filter using a solvent filtration system (Millipore-Waters Ltd, Harrow, Middlesex, UK). The flow rate for each mobile phase was 1 $ml.min^{-1}$.

Plasma Method

Aliquots of plasma (1 ml) were pipetted into 100 x 15 mm glass centrifuge tubes. Acetone (3 ml) was added while vortexing and the tubes were centrifuged at 2000 g and 4°C for 10 min. Aliquots of the supernatants (2.5 ml) were transferred to 100 x 15 mm glass tubes fitted with ground glass stoppers and water (4 ml) was added to each. The solutions were extracted with diethyl ether (3 ml) by shaking gently for 20 sec. and centrifuging. The ether extracts were transferred to clean tubes and the extractions were repeated once with further aliquots of diethyl ether (3 ml). The ether extracts were combined and evaporated to dryness at 40°C under nitrogen in a fume cupboard. The residues were dissolved in 70% acetonitrile (500 ul) by placing the tubes in an ultrasonic bath for 5 min and vortexing. Aliquots of the extracts (50 ul) and rafoxanide standards (50 ul, 5 ug ml^{-1}) were injected on to the HPLC system using the autosampler. Peaks were recorded on the integrator and results were calculated from peak area measurements.

Tissue Method

Tissue samples were cut into small cubes and stored at -20°C until frozen or until required. The

frozen cubes were pulverised in a domestic food blender to form a fine powder. Aliquots (3g) were weighed into 100 x 25 mm centrifuge tubes fitted with ground glass necks. Water (6 ml) was added and the tissues were homogenised for 1 min using a Silverson homogeniser. Disodium hydrogen orthophosphate (2 ml, 0.5 M) was added followed by acetone (25 ml). The homogenates were mixed and the tubes placed in an ultrasonic bath for 15 min and centrifuged at 2000 g and 4°C for 10 min. Aliquots of supernatants (12 ml) were transferred to clean tubes and water (12 ml) was added. The solutions were extracted with petroleum spirit (7 ml) by shaking gently for 20 sec and centrifuging for 10 min. The petroleum spirit extracts were transferred to 100 x 15 mm glass tubes and evaporated to near dryness under nitrogen at 40°C in a fume cupboard. The extractions were repeated once with further aliquots of petroleum spirit (5 ml). These extracts were combined with those of the first extractions and evaporated to dryness under nitrogen. The residues were dissolved in mobile phase (200 ul) by placing the tubes in an ultrasonic bath for 5 min and vortexing. Aliquots of the extracts (50 ul) and standards (50 ul, 1 ug ml^{-1}) were injected into the LC/MS system using the Rheodyne injector. Peak data was collected in the SIM mode for the negative ion at M/Z 626 using the workstation. Results were calculated by comparing the peak areas of samples with those of the standard.

RESULTS

Plasma

HPLC chromatograms for a 10 ug.ml^{-1} rafoxanide standard, a blank plasma extract, and an extract of plasma containing 3.5 ug.ml^{-1} rafoxanide are shown in Fig 1. Rafoxanide eluted at 6.3 min. The peak eluting at 4 min was found in only a few of the plasma samples tested and is of unknown origin. It did not interfere with the rafoxanide peak. Similarly closantel and oxyclosanide which are the other two salicylanilides commonly used in animals had different chromatographic retention times to rafoxanide.

The linearity and recovery values for assay were checked by spiking duplicate aliquots of blank plasma with rafoxanide. The results are shown in table 1. Least squares linear regression analyses of the data gave values for slope, intercept (ug.ml^{-1}) and correlation coefficient (R^2) of 0.94, 0.18 and 1.00, respectively. The assay therefore exhibited good linearity up to at least 20 ug.ml^{-1} in plasma. Plasma samples containing higher levels of rafoxanide were diluted in isotonic saline before analyses. Recovery values ranged from 93 to 104%.

The precision of the assay was checked by assaying two plasma samples containing rafoxanide, five times each using the described procedure. The results for mean, (standard deviation) and coefficient of variation (CV) were 1.96 (0.206) ug.ml^{-1} and 10.5% for sample 1 and 10.95 (0.485) ug.ml^{-1} and 4.4% for sample 2.

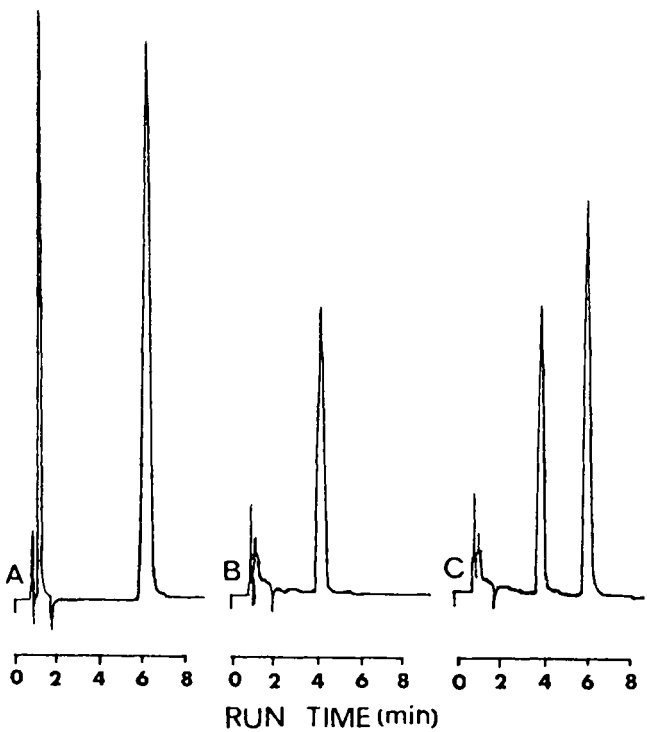

Fig 1 HPLC chromatogram at 282 nm of 50 ul injections of (A) a 10 ug ml^{-1} rafoxanide standard equivalent to 5 ug ml^{-1} in plasma, (B) a blank plasma extact and (C) an extract from plasma estimated to contain 3.5 $ug.ml^{-1}$ rafoxanide. Rafoxanide elutes at 6.3 min.

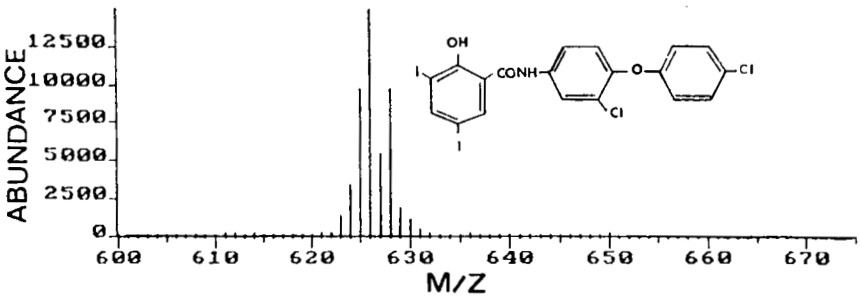

Fig 2 The negative ion thermospray CI spectra of a 50 ul injection of a 10 ug.ml^{-1} standard of rafoxanide from M/Z 600 to 670. The structure of rafoxanide (MW 626) is also shown.

Table 1

Linearity and recovery of rafoxanide in plasma.

Added (ug .ml^{-1})	Found (ug.ml^{-1})	% Recovery
2.0	2.0	100
5.0	5.2	104
7.5	7.0	93
10.0	10.1	101
15.0	13.9	93
20.0	19.1	95

Results are the mean of duplicates.

The limit of detection for rafoxanide in plasma (signal to noise ratio = 5) was 0.1 ug ml^{-1}.

Tissue

The thermospray LC/MS spectra for a 50 ul injection of a 10 $ug.ml^{-1}$ rafoxanide standard is shown in Fig 2. The most abundant ion was the molecular ion at M/Z 626, surrounded by a cluster of isotopic ions. For optimum sensitivity in determining rafoxanide levels in tissue, the molecular ion is monitored with the LC/MS operating in the SIM mode. Alternatively, for more definitive proof as to the presence of rafoxanide, several of the isotopic ions could be monitored and ion-ratio measurements used, although overall sensitivity would be reduced.

Ion chromatograms at M/Z 626 for 50 ul injections of (A) a 1 $ug.ml^{-1}$ rafoxanide standard, (B) a blank muscle extract and (C) an extract of muscle estimated to contain 0.2 $ug.g^{-1}$ rafoxanide are shown in Fig 3. Rafoxanide elutes at 2.15 min.

The linearity and recovery values for the tissue assay were determined by spiking duplicate aliquots of blank muscle with rafoxanide and carrying them through the extraction procedure. The results are shown in table 2. Least squares linear regression analyses of the data gave values for slope, intercept ($ug.g^{-1}$) and correlation coefficient (R^2) of 0.89, 0.008 and 1.000 respectively. Recovery values ranged from 66 to 89%.

The precision of the tissue assay was determined by assaying two tissue samples containing rafoxanide

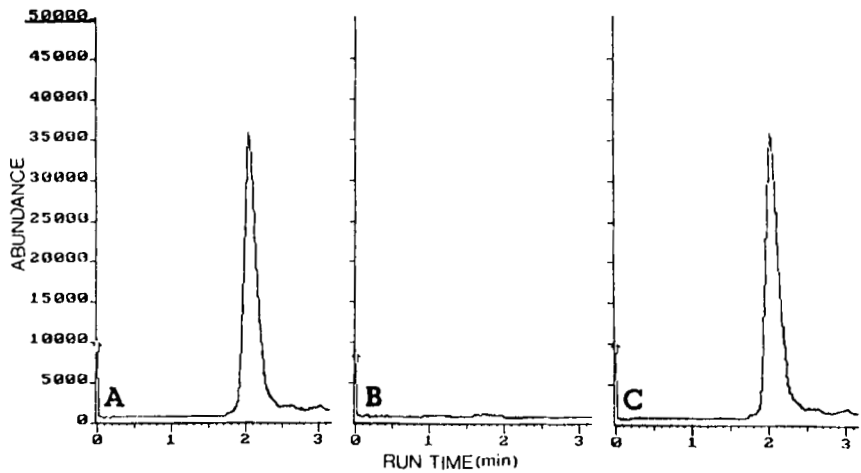

Fig 3 Ion chromatograms at M/Z 626 for 50 ul injections of (A) a 1 ug ml^{-1} rafoxanide standard, (B) a blank muscle extract and (C) an extract of muscle estimated to contain 0.2 ug g^{-1} rafoxanide.

Table 2

Linearity and recovery of rafoxanide in tissue.

Added ug.g^{-1}	Found ug.g^{-1}	% Recovery
0.05	0.033	66
0.10	0.840	83
0.20	0.156	78
0.50	0.445	89
1.00	0.880	88

Results are the mean of duplicates.

five times each using the described procedure. The values for mean (standard deviation) and CV were 0.078 (0.0098 $ug.g^{-1}$) and 12.6% for sample 1 and 0.332 (0.024 $ug.g^{-1}$) and 7.2% for sample 2.

The limit of detection for rafoxanide in tissue (signal to noise ratio = 5) was 0.02 ug g^{-1}.

DISCUSSION

Rafoxanide is extensively bound to protein in biological samples. In order to determine the level of rafoxanide present, it is therefore necessary to first release it from the protein matrix and then extract it from the sample. Attempts to carry out a direct extraction from plasma by altering the pH and extracting with diethyl ether proved unsuccessful due to poor recovery values. The most effective way we found of carrying this out was to denature the protein and solubilise the rafoxanide using a three fold excess of acetone. Following centrifugation, the supernatant was diluted with water and any rafoxanide present was extracted into diethyl ether. The concentrated extract was then applied directly to the HPLC system using UV detection. Recoveries ranging from 93 to 104% showed that the technique was effective.

A similar extraction procedure was found to be effective for tissue samples, following initial homogenisation with water. Rafoxanide was finally extracted into petroleum spirit rather than ether since

this gave a cleaner extract following concentration. No further clean-up was required and the ion chromatograms at M/Z 626 (Fig 3) showed no evidence of any interfering peaks. In contrast when the same extracts were applied to the HPLC system for plasma, using UV detection, we found evidence of interference, particularly at low levels of rafoxanide, and some further clean-up would have been necessary.

In general we have found CI thermospray LC/MS using the system described to be an excellent technique for the analyses of a number of drug residues. The sensitivity of technique is somewhat compound dependent and for a drug such as rafoxanide which shows good sensitivity, this compound dependency is useful in reducing overall background interference. The relatively high mass ion (626) of rafoxanide and the choice of either positive or negative ion mode also contribute to the low background levels in crude extracts. Furthermore CI LC/MS provides mass related data, a feature which is preferable in statutory residue analyses.

Modern thermospray techniques are also capable of producing good quantative data, as shown by the precision figures for the assay. This is in contrast to older LC/MS techniques where data could only be used qualitatively.

The described assay for plasma has been used for a number of pharmacokinetic trials of different formulations of rafoxanide. Fig 4 demonstrates the use

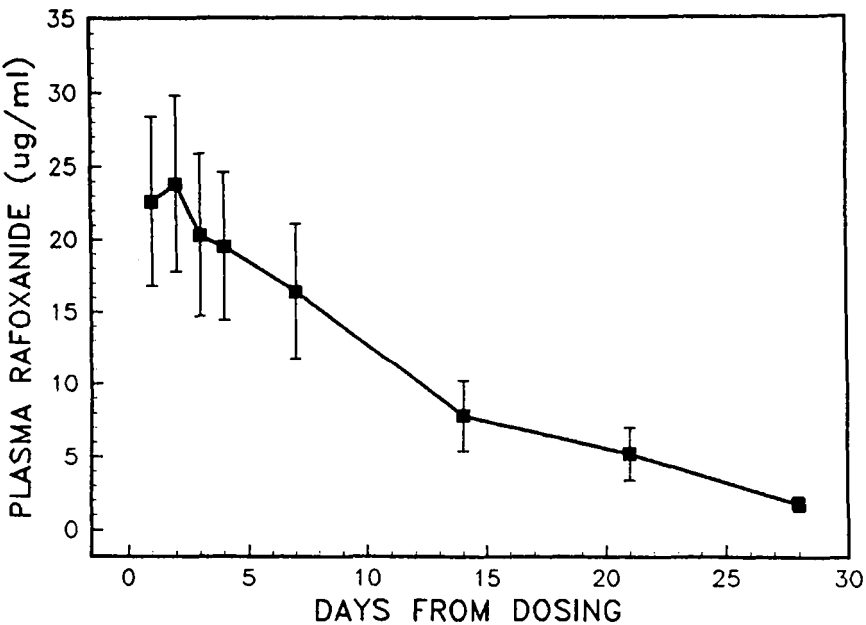

Fig 4 Rafoxanide levels found in the plasma of sheep using the described assay, following an oral dose of a commercial preparation of rafoxanide. Each point represents the mean plasma levels of 10 different sheep with the bars representing standard deviation.

of the assay for one such trial, where the plasma levels of rafoxanide were followed for 30 days after oral dosing with 7.5 mg kg^{-1} bodyweight in sheep.

The tissue assay has been used to determine the levels of rafoxanide in liver, muscle and kidney samples from sheep and calves which were slaughtered at various times after dosing. Table 3 demonstrates the applicability of the assay for this purpose.

Table 3

Levels of rafoxanide in muscle and liver of 3 sheep slaughtered up to 28 days after oral dosing with a commercial formulation of rafoxanide.

		Days from dosing		
		11	20	28
Rafoxanide ($ug.g^{-1}$)	Liver	0.413	0.122	0.093
Rafoxanide ($ug.g^{-1}$)	Muscle	0.101	0.047	0.051

Results are the mean of duplicate analyses.

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