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Short Communication

High-performance liquid chromatographic determination of the anthelmintic mebendazole in eel muscle tissue

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ABSTRACT

A simple high-performance liquid chromatographic method for the determination of mebendazole in eel muscle tissue was developed. It is applicable for concentrations of 10 μg/kg and higher. Muscle tissue was extracted with ethyl acetate, and the extract, after addition of hexane, was concentrated and cleaned up on a silica gel solid-phase extraction column. Mebendazole was eluted from the column with 3% acetic acid in methanol. The eluate, after reconstitution in the chromatographic mobile phase, was analysed on a LiChrosorb RP-8 column with acetonitrile–0.05 *M* ammonium phosphate buffer (pH 6.2) (3:7, v/v) as the mobile phase. Detection was performed at 311 nm. The average mebendazole recovery over the concentration range 10–5000 μg/kg eel muscle tissue was 72 ± 6.4%. The calibration curve for spiked samples was linear throughout the range 10–10 000 μg/kg (r = 0.999).

INTRODUCTION

As a result of the intensive systems applied in aquaculture, health problems may arise which need application of drugs. In eel farming, one of the most important problems is infection with the internal worm parasite *Pseudodactylogyrus* spp. [1]. This can be effectively treated with mebendazole (5-benzoyl-2-benzimidazole carbamic acid methyl ester) [2], a broad-spectrum anthelmintic drug that belongs to the group of the benzimidazoles. When fish are treated with mebendazole, residues in muscle tissues and organs intended for human consumption may occur. Therefore the determination of residues of mebendazole in these tissues is of major interest.

Only a few methods have been described for the determination of mebendazole residues in animal tissues, whether or not in combination with other benzimidazoles. These are muscle tissues of swine [3], ovine, bovine and swine muscle and liver tissues [4] and eel tissues [5]. These procedures, however, require laborious liquid–liquid extractions and often involve the repeated evaporation of organic solvents. In some cases a solid-phase extraction (SPE) step was introduced for clean-up only. Long *et al.* [6] used matrix solid-phase dispersion (MSPD) for the isolation of five benzimidazole anthelmintics from beef liver. In all procedures mebendazole was determined by

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high-performance liquid chromatography (HPLC) with UV detection.

Some methods have also been described for the determination of mebendazole in human plasma. These consist of liquid–liquid extraction followed by HPLC quantitation using UV detection [7–9] or electrochemical detection [10], or SPE followed by HPLC quantitation using UV detection [11] or electrochemical detection [12].

This paper describes a rapid sample preparation method for the determination of mebendazole in eel tissue using SPE for clean-up and concentration. Quantitation is performed by HPLC with UV detection.

EXPERIMENTAL

Reagents and chemicals

Water was purified via a Milli-Q system (Millipore, Bedford, MA, USA). Methanol, acetonitrile, formic acid and anhydrous sodium sulphate were from Merck (Darmstadt, Germany). Acetic acid (99–100%), ammonium phosphate and potassium carbonate were from J.T. Baker (Phillipsburg, NJ, USA). Ethyl acetate and *n*-hexane were from Rathburn (Walkerburn, UK). All organic solvents were HPLC grade.

Filter paper circles (S&S 589.1, diameter 90 mm) were from Schleicher and Schüll (Dassel, Germany). Mebendazole was from Sigma (St. Louis, MO, USA)

A mebendazole stock solution (2 mg/ml) was prepared by dissolving 100 mg of mebendazole and 0.4 ml of formic acid in chloroform and adjusting to 50 ml with chloroform. Working standards for HPLC were prepared in the range 0.05–2.5 μg/ml by diluting the stock solution in the HPLC mobile phase. A spiking solution containing 1 μg/ml mebendazole was prepared by diluting the stock solution in ethyl acetate.

Silica gel disposable SPE columns (3 ml; 500 mg) were from J.T. Baker. Just before use, the column was pretreated by passing *ca.* 6 ml of ethyl acetate–*n*-hexane (1:2.5, v/v). After the pretreatment the column should not be allowed to run dry. The SPE elution solvent was prepared by dissolving 3 ml of acetic acid in methanol and adjusting to 100 ml with methanol.

An ammonium phosphate buffer was prepared by dissolving 0.05 mol of ammonium phosphate ($NH_4H_2PO_4$) in 950 ml of water, adjusting the pH to 6.2 with 10 *M* NaOH, and adjusting to 1000 ml with water. The mobile phase for HPLC was acetonitrile–0.05 *M* ammonium phosphate buffer (30:70, v/v), with an apparent pH of 6.7.

Samples

For spiking studies, blank eel muscle tissue was used. Ground tissue samples were spiked with the spiking solution at levels of 10, 20, 50, 100, 200, 500 and 5000 μg/kg at least 15 min before extraction by the procedure described below.

Apparatus and chromatographic conditions

The instruments used were a Moulinette homogenizer (Moulinex, Gouda, Netherlands), a KS 500 mechanical shaker (IKA-Labortechnik, Janke and Kunkel, Staufen, Germany), a Vibrofix VF 1 vortex mixer (IKA Labortechnik), a Centra-8R centrifuge (IEC, Needham, MA, USA), a Reacti-Therm III heating module and a Reacti-Vap III evaporator (Pierce, Rockford, IL, USA) and a SPE-21 column processor (J.T. Baker).

The HPLC system consisted of a 2150 HPLC pump (Pharmacia-LKB, Uppsala, Sweden), a 2153 autosampler (Pharmacia-LKB) equipped with a 7010 air-actuated sampling valve (Rheodyne, Cotati, CA, USA) and a 50-μl sample loop, and a 783A HPLC monitor with a 10-μl HPLC flow-cell (Applied Biosystems, Foster City, CA, USA) operated at 311 nm. A ChromSep cartridge holder system contained a stainless-steel guard column (10 mm × 2.1 mm I.D.) packed with pellicular 40-μm reversed-phase particles and an analytical glass column (100 mm × 3.0 mm I.D.) packed with 5-μm LiChrosorb RP-8 (Chrompack, Bergen op Zoom, Netherlands). The system was operated at ambient temperature. Peak areas were quantitated with an SP 4270 integrator (Spectra-Physics, San Jose, CA, USA). An LC-235 diode-array detector (Perkin-Elmer, Norwalk, CT, USA) was used to obtain the UV spectrum of mebendazole, which was plotted on a GP-100 graphics printer (Perkin-Elmer).

Sample preparation

Extraction. Ground eel muscle tissue (*ca.* 5 g) was accurately weighed into a 50-ml disposable plastic centrifuge tube, and 5 g of sodium sulphate, 0.5 ml of 4 *M* potassium carbonate solution and 10 ml of ethyl acetate were added. The tube was capped, shaken on a vortex mixer for 10 s to break up tissue lumps, and placed on a mechanical shaker for 10 min (500 rpm). The suspension was centrifuged for 5 min at 1300 *g*. The supernatant was decanted. The extraction procedure was repeated with another 10 ml of ethyl acetate.

Clean-up. To the combined extracts, 50 ml of hexane (the ethyl acetate-to-hexane ratio should be *ca.* 1:2.5, v/v) and 5 g of anhydrous sodium sulphate were added. The solution was shaken and allowed to stand until it had become transparent. The solution was filtered over an S & S 589.1 filter paper circle, and the filtrate was passed through the pretreated silica gel column via a 75-ml reservoir. The flask and filter were rinsed with 5 ml of ethyl acetate–hexane (1:2.5) and the rinse liquids were also passed through the SPE column. The column was dried in a stream of nitrogen for 10 min. Immediately after drying, mebendazole was eluted with 3 ml of 3% acetic acid in methanol. The eluate was evaporated to dryness in a stream of nitrogen at 37°C. The residue was dissolved in 1.0 ml of the mobile phase. This solution was used for HPLC analysis.

Chromatography. Aliquots of the sample and standard solutions (50 μl) were injected. Samples were eluted isocratically at a flow-rate of 0.5 ml/min.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms from standard, blank and spiked eel muscle tissue extracts are shown in Fig. 1. The chromatographic conditions used are based on those described by Allan *et al.* [11]. These authors used methanol–0.0165 *M* ammonium phosphate (pH 5.5) (55:45, v/v) as the mobile phase for isocratic elution. In our hands this resulted in a somewhat broad and tailing mebendazole peak. Replacing methanol in the mobile phase by acetonitrile resulted in a sharper and more symmetrical peak. Moreover, small changes were made to the proportions of the components of the mobile phase, the column dimensions, the particle size of the stationary phase and the flow-rate.

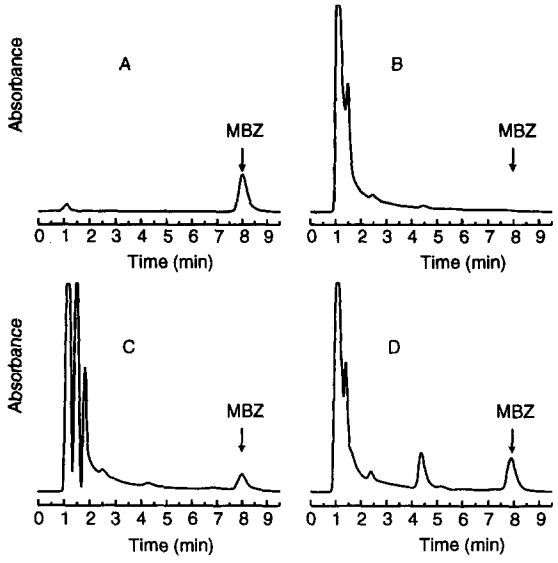

Fig. 1. Typical chromatograms of (A) a standard solution of mebendazole (MBZ) (150 μg/l), (B) a blank eel muscle tissue extract, (C) a spiked (20 μg/kg) eel muscle tissue extract and (D) a muscle tissue extract of a mebendazole-treated eel (32 μg/kg). For chromatographic conditions, see text. Attenuation = 8.

Under the conditions that were finally chosen, a retention time for mebendazole of *ca.* 8–9 min was obtained. It has to be remarked that the pH of the mobile phase is very critical: a small deviation from an apparent value of 6.7 resulted in a sharp shift in the retention time of mebendazole.

Although the molar extinction coefficient for mebendazole is lower at 311 nm (see also Fig. 2), mebendazole was detected at 311 nm instead of at 254 nm, which was the wavelength used by Allen *et al.* [11]. Fewer interferences from endogenous compounds were encountered at 311 nm.

For identification purposes, a photodiode-array UV detector was coupled to the analytical column. It was possible to obtain UV identification for mebendazole in a spiked eel muscle ex-

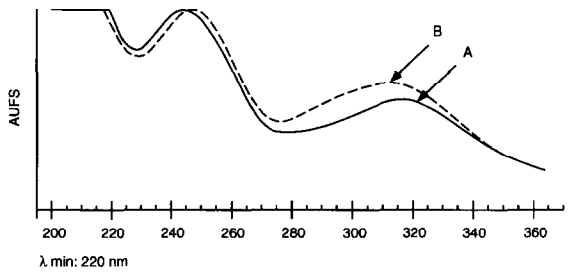

Fig. 2. Photodiode-array UV overlay spectra of mebendazole in (A) a spiked muscle tissue extract (100 μg/kg) and (B) a mebendazole standard (4000 μg/l). For chromatographic parameters, see text. Calculated purity index = 1.6.

tract at a level of 100 μg/kg or higher via calculation of the purity index of overlay spectra (200–360 nm) of the mebendazole peaks in the chromatograms of a spiked muscle tissue extract and of a mebendazole standard. Two spectra were considered to be from the same compound if the purity index had a value between 1 and 1.6. The overlay spectra are shown in Fig. 2.

Spiking studies

Recovery experiments were carried out on eel muscle tissues spiked at 20, 200 and 5000 μg/kg. In six replicates each amount was added to ground muscle tissue. In addition, some recovery experiments were carried out in duplicate. All samples were submitted to HPLC analysis in duplicate according to the procedure described. The results are presented in Table I. A good recovery at all the levels investigated and a low standard deviation for repeatability were attained.

The absolute limit of detection of mebendazole was found to be 0.5 ng on-column, at a signal-to-noise of 3.0. As no interferences of endogenous compounds from the matrix on the retention time of mebendazole were observed, a mebendazole level of 10 μg/kg in eel tissue can be easily quantitated. The calibration curve for spiked samples was linear throughout the range 10–10 000 μg/kg (r = 0.999).

Sample pretreatment

The starting point in the development of the analytical procedure was the extraction method described by Wilson *et al.* [4], in which ethyl acetate is used as an organic extractant. The SPE procedure was developed for clean-up and concentration of the ethyl acetate extract. A silica gel SPE column proved to be the most suitable. However, *n*-hexane has to be added to the ethyl acetate extract to improve the retention of mebendazole on the silica gel SPE column, as was also the case for the retention of chloramphenicol [13].

TABLE I

MEAN RECOVERY OF MEBENDAZOLE FROM SPIKED EEL MUSCLE TISSUE

Mebendazole added (μg/kg)	Mean recovery (%)	Coefficient of variation (%)	n
10	61.8	–	2
20	67.9	2.4	6
50	83.1	–	2
100	70.9	–	2
200	71.7	5.0	5[a]
500	73.5	–	2
5000	73.3	3.5	6

[a] One sample lost.

Originally, an SPE procedure starting from the acidic aqueous extract as used by Mellergaard *et al.* [5] was investigated. In this case, mebendazole was extracted from the tissue with a 0.01 *M* HCl solution and the extract was applied to a C_{18} SPE column. The retention of mebendazole on the C_{18} column was nearly quantitative. Mebendazole could be fully eluted from the SPE column with 3 ml of methanol. However, this procedure proved to be unsuitable because mebendazole could not be extracted in high yield from eel muscle tissue with an acidic aqueous solution, not even when concentrated HCl solutions (of up to 4 *M*) were used. This procedure may, however, be suitable for the determination of mebendazole in aquarium and pond water or in hatchery effluents.

Treated eels

As an illustration of the applicability of the described method, muscle tissue of three meben-

dazole-treated eels were analysed. To obtain real samples, three eels with a mean weight of 30 g were kept in an aquarium containing 4 mg/l mebendazole for 12 h. Thereafter the eels were kept in an aquarium without mebendazole for 15 h, and killed.

A typical chromatogram of a muscle tissue extract of a treated eel is shown in Fig. 1. The peak with a retention time of *ca.* 4.5 min in the chromatogram is possibly a metabolite of mebendazole. The amounts of mebendazole found in muscle tissue of treated eels were 24, 26 and 32 μg/kg. These amounts are too low to allow UV identification with the diode-array detector used.

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