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Short communication

# Liquid chromatographic determination of the antibiotic fumagillin in fish meat samples

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**Abstract**

A procedure for the determination of fumagillin, an antibiotic of *Aspergillus fumigatus* in fish meat samples, using reversed-phase high-performance liquid chromatography is described. Liquid chromatography was performed on an octadecylsilane column using acetonitrile–water–phosphoric acid solution as mobile phase, with detection at 350 nm. Two different types of sample preparation were developed, clean-up and enrichment, and the limits of quantification were 100 ng/g and 5 ng/g, respectively, in fish meat. The recovery was 75 ± 3% in the concentration range 100–500 ng/g. To introduce the methodology and demonstrate its usefulness, a practical experiment was performed. Trouts fed with fumagillin were examined for elimination of fumagillin. After 24 h, the concentration was shown to decrease to below 100 ng/g.

## 1. Introduction

Fumagillin (Fig. 1) is a metabolite of *Aspergillus fumigatus* and has a potent amoebicidal property. This activity led to large-scale production and clinical studies of its therapeutic potential. At present, however, because of toxic side-effects encountered in human clinical trials, medical applications of fumagillin are confined to its use by apiarists and to the veterinary profession. Fumagillin may be effective in the treatment of angiogenesis-related diseases [1] and may be effective in suppressing tumor growth, is a potent antibiotic that inhibits *Entamoeba histolytica* [2], may be used in the honey industry to protect bees from *Nosema apis* [3] and has been used against microsporodian in fish [4–6]. Several assay methods have been developed for fumagillin, e.g., thin-layer chromatography [7], microbial assay [8] and spectrophotometric assay [9]. Each of these techniques has its disadvan-

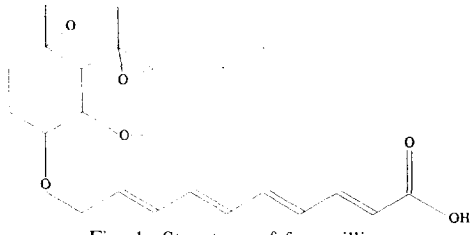


Fig. 1. Structure of fumagillin.

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tages, mainly due to interference caused by degradation products of fumagillin and impurities or poor reproduciblity. High-performance liquid chromatography (HPLC) is rapid and accurate and separates compound from possible impurities and degradation products. Brackett et al. [10] developed an HPLC assay for fumagillin and Assil and Sporns [3] developed a sensitive HPLC method for the determination of fumagillin in honey.

## 2. Experimental

### 2.1. Instrumentation

Solutions obtained from the extraction procedure were injected through a 100-$\mu$l loop (Labor MIM, Budapest, Hungary) for analysis on a reversed-phase silica column ($\mu$Bondapak $C_{18}$ 300 mm × 3.9 mm I.D.) (Waters, Milford, MA, USA) at ambient temperature. The mobile phase was acetonitrile–water–phosphoric acid (600:400:1, v/v/v) pumped by a Waters Model 510 HPLC pump at a flow-rate of 1 ml/min. Detection was carried out using a Bio-Rad (Richmond, CA, USA) Model 1306 variable-wavelength UV monitor at 350 nm. A Waters Model 740 data module integrator was used to record chromatograms and to calculate the peak area of fumagillin.

### 2.2. Chemicals and materials

Acetonitrile for the mobile phase and the extraction procedure was of chromatographic grade (Reanal, Budapest, Hungary). Deionized water was distilled then deionized through a Milli-Q water-purification system. Phosphoric acid was of analytical-reagent grade (Reanal).

Fumagillin DCH (62.1% fumagillin acid content) was supplied by Chinoin Pharmaceutical and Chemical Works (Budapest, Hungary).

For the development of the extraction process, fumagillin-free fish were purchased. Fish fed with fumagillin for the study of fumagillin concentration in muscular tissues were supplied by the University of Veterinary Sciences (Budapest, Hungary). As fumagillin is unstable towards light [9] and heat [11], and to prevent biotic decomposition, samples were stored deep frozen at −20°C packed in aluminium foil.

### 2.3. Procedures

*Standard solution*

To prepare a standard solution, fumagillin was dissolved in acetonitrile–water solution corresponding to the eluent excluded phosphoric acid.

For the recovery study, meat samples were spiked with various amounts of fumagillin in the range 1–1000 ng/g meat.

*Sample preparation*

Two methods of sample preparation were applied according to the fumagillin concentration in the sample. Based on the expected fumagillin concentration in solutions obtained from the extraction process, clean-up was used for the 0.1–5 $\mu$g/ml and enrichment for the 0.01–0.1 $\mu$g/ml range of fumagillin concentration in the extraction solution.

In order to optimize both clean-up and enrichment, we studied the adsorption of fumagillin on a Sep-Pak $C_{18}$ column. Before sample application, the Sep-Pak $C_{18}$ column was washed with acetonitrile.

*Clean-up*

Acetonitrile (4 ml) was added to 1.0 g of meat (pulped by passing through a plastic syringe, measured water content 0.7 ml/g). The suspension was homogenized, then the cells were disrupted with a 10-min treatment in an ultrasonic bath. After centrifugation for 10 min at 10 000 *g*, the supernatant was passed through a Sep-Pak $C_{18}$ column, discarding the first 0.4 ml, then 100 $\mu$l were injected on to the reversed-phase silica column for analysis.

*Enrichment*

Acetonitrile (10 ml) was added to 5.0 g of meat (prepared as above). The suspension was homogenized, then the cells were disrupted with a 10-min treatment in ultrasonic bath. After centrifugation for 10 min at 10 000 *g*, 100 ml of

water were added to the supernatant and the solution was filtered through a Sep-Pak $C_{18}$ column. Fumagillin was eluted with 2 ml of acetonitrile–water eluent (90:10), discarding the first 0.2 ml, then 100 $\mu$l were injected on to the reversed-phase silica column for analysis.

## 3. Results and discussion

No reports on the determination of fumagillin in muscular tissue of fish or on the solid-phase extraction of fumagillin were found. This work involved not only the study of an HPLC assay for fumagillin; our primary aim was to develop a method for the determination of fumagillin in biological samples, including solid-phase extraction, clean-up and enrichment.

Fumagillin is highly sensitive to light and oxidation; however, our studies showed than the ultrasonic bath we utilized for the extraction of fumagillin caused no decomposition of the analyte.

The eluent used for the determination of fumagillin in meat samples is among those not recommended by Brackett et al. [10], as according to their studies fumagillin eluted with its impurities. In the method developed, the use of phosphoric acid instead of acetic acid allowed the separation of fumagillin and its impurities. Moreover, the veterinary fumagillin we used may not contain more than 0.1% of unidentified impurities as prescribed by standards, and the deviation caused by possible impurities fell below the standard margin of the method in the measured concentration range.

Under the chosen conditions (eluent composition, flow-rate), the retention time of fumagillin was about 7.45 min. In the blank sample, no disturbing peaks were detected near the retention time of fumagillin (Fig. 2).

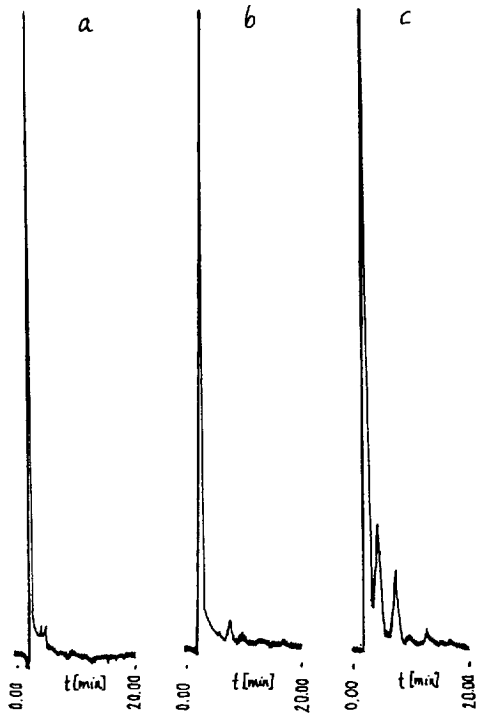


Fig. 2. Chromatograms showing (a) blank muscle sample, (b) limit of detection (muscle spiked with 1 ng/g of fumagillin), (c) sample from muscular tissue of fumagillin-fed trout 9 h after feeding. Loop volume, 100 $\mu$l; column, $\mu$Bondapak $C_{18}$ (300 mm × 3.9 mm I.D.); mobile phase, acetonitrile–water–phosphoric acid (600:400:1, v/v/v); temperature, ambient; flow-rate, 1 ml/min; detection wavelength, 350 nm. $A$ = absorbance ($8 \times 10^{-3}$ AUFS).

### 3.1. Analytical data

*Linearity*

In the examined concentration range (0.01–10 $\mu$g/ml), the detector response was found to be linear for both peak-area and peak-height measurements.

*Limit of detection*

With enrichment, the least possible detectable concentration was found to be 1 ng/g of fumagillin, calculated as three times the standard deviation of the noise.

*Limit of quantitation*

With the clean-up method the limit of quantification was 100 ng/g and for the enrichment method 5 ng/g.

*Recovery*

The recovery was found to be 75 ± 3% in the 100–500 ng/g concentration range for the whole

process. The loss due to the adsorption on the apolar surface of the Sep-Pak $C_{18}$ column was $5 \pm 1\%$. According to our investigations, the 5–10-min ultrasonic treatment caused no degradation of fumagillin.

*Precision*

The intra-day reproducibility was studied. The results showed good agreement, with a relative standard deviation of only 0.67% at a 1 mg/l fumagillin concentration.

### 3.2. Practical application

To illustrate the usefulness of the method, trouts fed with fumagillin were examined. During the feeding experiment, trouts were fed with 20 mg of fumagillin per kilogram body mass. The change in fumagillin concentration was studied in muscular tissue samples obtained from fish bled 3, 9 and 24 h after feeding. The standard deviation was relatively high owing to differences between specimens, such as differences in digestion, metabolism and secretion of drugs. From 3 to 9 h after feeding the fumagillin concentration showed a rapid decrease of more than 70%; 24 h after feeding the fumagillin concentration was less than 100 ng/g. These data indicate that at the beginning the fumagillin concentration is high in muscular tissues and then it is relatively rapidly eliminated.

## 4. Conclusion

The present results demonstrate that HPLC is suitable for the determination of fumagillin in fish meat samples. The method is rapid and accurate and may be used to investigate the pharmacokinetic effects of fumagillin.

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