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Short communication

Determination of fumagillin in muscle tissue of rainbow trout using automated ion-pairing liquid chromatography

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Abstract

A high-performance liquid chromatographic assay is described as a routine analytical method for the determination of fumagillin in rainbow trout muscle tissue. Muscle tissue samples (1 g) containing fumagillin were deproteinized with 8 ml of an acetonitrile–water mixture (2:6, v/v). The extracts were purified with a Bond Elut Octyl C_8 cartridge column, washed with a water–methanol mixture (95:5, v/v; 4 ml) and fumagillin was eluted with acetonitrile (1 ml). Analytical separations were performed by reversed-phase HPLC with UV detection at 351 nm under gradient conditions. The mobile phase was acetonitrile–0.005 *M* tetrabutyl ammonium phosphate in water (pH 7.8). The assay is specific and reproducible within the fumagillin range of 20–1000 ng/g and recovery at 20 ng/g was 69.2%. Sample preparation involves the use of a robotic sample preparation system. Gravimetric validation of all operations enabled Good Laboratory Practices to be observed.

1. Introduction

The antibiotic fumagillin (Fig. 1), (2,4,6,8-decatetraenedioic acid mono-[5-methoxy-4-[2-methyl-3-(3-methyl-2-butenyl) oxiranyl]-1-oxaspirol[2.5]oct-6-yl]) is effective in controlling diseases resulting from the action of Microsporidiae such as that caused by *Nosema apis*, a universally occurring protozoan parasite found in the gut of the adult honey bee [1] and Myxosporidiae in different animal species, in particular in fish [2–6]. In order to determine how long it takes

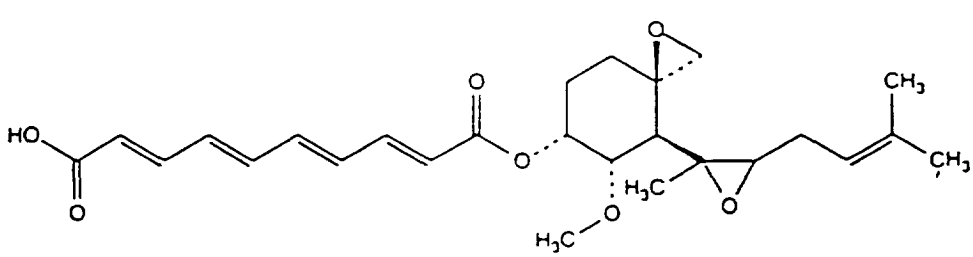

Fig. 1. Structure of fumagillin.

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before the antibiotic has disappeared from the tissues and the treated fish can be safely consumed, the depletion of fumagillin from the tissues must be known and a method to determine fumagillin in muscle tissue is required. Fumagillin has been assayed by thin-layer chromatography [7], spectrophotometry [8] and a microbial assay [9]. More recently a high-performance liquid chromatographic method has been devised, but the procedure describes only the separation of fumagillin from possible impurities and degradation products [10]. None of these methods appear satisfactory for the determination of fumagillin in the tissues of fish. This paper describes a high-performance liquid chromatographic (HPLC) assay method for fumagillin in tissue of rainbow trout (*Oncorhynchus mykiss*) and utilizes a reversed-phase C_8 column and UV detection at 351 nm. Sample preparation involves the use of solid-phase extraction on a C_8 cartridge using a robotic sample preparation system.

2. Experimental

2.1. Chemicals and reagents

Fumagillin as its dicyclohexylamine salt was obtained from Sanofi Santé Nutrition Animale (Libourne, France). Acetonitrile was obtained from Distrilab (Leuven, Belgium), methanol and orthophosphoric acid from Prolabo (Manchester, UK). Potassium dihydrogen phosphate from Merck (Darmstadt, Germany) and 0.005 *M* tetrabutyl ammonium phosphate from Millipore (Milford, MA, USA). The water was purified using a Milli-Q system (Millipore).

2.2. Preparation of standards

The stock solution of fumagillin (1 mg/ml) was prepared in methanol–acetonitrile (6:94, v/v). This solution was kept at 4°C, protected from the light and stored in the dark. Working solutions were prepared immediately before use by diluting this stock solution with distilled water. Drug-free muscle tissue was prepared from freshly killed trout and samples were stored at −20°C. A series of drug-free muscle tissue was spiked with fumagillin to give final concentrations of 20, 50, 100, 250, 500 and 1000 ng/g. The overall volume of drug solution added to muscle tissue was 100 μl.

2.3. Chromatography

The chromatographic system consisted of a Varian 9010 gradient pump (Walnut Creek, CA, USA), a Varian 2550 UV spectrophotometric detector set at 351 nm, an Ultrabase C_8 Octyl analytical column (particle size 5 μm, 250 × 4.6 mm I.D.; Shandon, UK), a Varian 9090 automated injector and a Perkin-Elmer Nelson 1020 integrator (Norwalk, CT, USA) for recording of the peak areas. The mobile phase was a mixture of acetonitrile (eluent A) and 0.005 *M* tetrabutyl ammonium phosphate in water (pH 7.8) (eluent B). At $t = 0$, the mixture consisted of 10% A and 90% B, which changed linearly in 17 min to 90% A and 10% B. Then, the system returned to its initial state in 5 min. The flow-rate was 0.7 ml/min, and fumagillin was detected at 351 nm by measuring the peak area.

2.4. Robotic sample preparation system

The Zymark centre benchmate system (Hopkinton, MA, USA) consisted of a controller, a microprocessor-based command center designed to manage the operations of the Benchmate automat system and a master laboratory system which provided automated liquid handling. A liquid–solid extraction section was used to separate sample components from a solvent matrix using a C_8 Octyl Varian Bond-Elut (100 mg/1 ml) solid-phase extraction column (Cat No. Al-121 020-02, Harbor City, CA, USA). Gravimetric verification was performed of all liquid volumes. [All operating parameters and work sequences were recorded on a floppy disk using a PC-type computer. This floppy was then loaded in the station disk drive to start the programmed manipulations.]

2.5. Sample preparation procedure

A 1-g quantity of minced trout muscle tissue was introduced into a 15-ml disposable screw-cap culture tube, and 6 ml of distilled water were added followed by 2 ml of acetonitrile. The mixture was stirred laterally for 10 min and centrifuged at 3000 *g* for 10 min. Next, 1 ml of 0.01 *M* potassium dihydrogen phosphate solution pH 2 (standardized with phosphoric acid) was added to the supernatant, the mixture was vortex-mixed for 10 min and centrifuged at 3000 *g* for 10 min. The supernatant was separated by specific absorbance to a bonded reversed-phase packing material, according to the following procedure:

–step 1: the extraction column was conditioned with 1 ml of methanol.

–step 2: the extraction column was conditioned with 1 ml of distilled water.

–step 3: 9 ml of the supernatant to be assayed was loaded onto the extraction column.

–step 4: the syringe was washed with 10 ml of distilled water.

–step 5: the extraction column was flushed with 4 ml of distilled water–methanol (95:5, v/v).

–step 6: fumagillin was eluted with 1 ml of acetonitrile.

–step 7: the extraction column was washed with 1 ml of methanol.

–step 8: 0.2 ml of eluate was injected onto the chromatographic system.

2.6. Calibration and calculation

Analyses of drug-free trout muscle tissue spiked with known amounts of fumagillin were carried out applying the above-described procedure. Linearity was evaluated from six calibration graphs prepared and run on six different days within the fumagillin range 20–1000 ng/g. Precision was evaluated by repeated analyses of fumagillin at three concentrations on the same day. The accuracy and reproducibility of the method was tested by repeatedly injecting a set of standard samples on six different days. To evaluate the extraction recovery, the calculated concentration of fumagillin in trout muscle tissue was compared with that obtained with a working solution injected directly onto the chromatographic system.

All chromatograms obtained were evaluated by peak-area measurement. The concentrations of fumagillin were calculated with the calibration curve generated on each day by weighted linear regression of the peak-area measurements against the theoretical concentrations.

2.7. Animal study

A study of the depletion of fumagillin (as dicyclohexylamine salt) in rainbow trout was carried out at two dosages: 3 mg/kg/day and 15 mg/kg/day. The trout (>50 g) were held in tanks at constant temperature (16–17°C) and a water flow of approximately 150 l/min. Fumagillin was distributed twice a day for 10 consecutive days as a medicated diet (5 g of fumagillin as dicyclohexylamine per 100 g of feed). The quantity of feed was 1.5% of the weight of the fish. Twelve fish were slaughtered at 1 h, and at 4, 10 and 21 days after the end of the treatment. The muscle tissue of rainbow trout was assayed by using the present method.

3. Results and discussion

3.1. Sample preparation and HPLC separation

The structure of fumagillin is characterised by the following functions: a carboxylic acid function which is a moderately strong acid, an unsaturated hydrocarbonated chain carrying four conjugated ethylene bonds which absorb in the UV range, a carboxylic ester bond and hydrophobic peripheral groups, methyl and methoxy. Fumagillin is soluble in polar organic solvents, especially ethanol and chloroform. Its octanol–water partitioning coefficient is close to 0.5, indicating that this molecule will not a priori tend to accumulate in highly lipidic areas, but rather remains localised in hydrophillic areas. The molecule is thermostable when dry, but heat-unstable in aqueous and acidic media and

can be degraded by light and by oxygen, with possible formation of "neofumagillin", a cycled rearrangement product of dioic acid [11]. As a moderately polar compound, fumagillin can usually be extracted with organic solvents such as chloroform. Thus, a simple and rapid liquid–solid extraction was performed from a solid adsorbent packed with C_8 octyl in a small cartridge with 100 mg of bonded-phase silica. This was preceded by a simple protein precipitation with water–acetonitrile (6:2, v/v) and the supernatant was acidified by potassium dihydrogen phosphate solution at pH 2 and loaded onto the cartridge.

Chromatograms obtained after extraction of muscle tissue of rainbow trout spiked with 50 and 1000 ng/g of fumagillin are shown in Fig. 2. The drug-free sample did not contain substances that would interfere with the detection of fumagillin at 351 nm. Several stationary phases have been tried for the determination of fumagillin in muscle tissue. Kromasil C_{18} and Ultrabase C_{18} and C_8 were found to be generally usable. However, an appropriate system proved to be an Ultrabase C_8 column with acetonitrile–0.005 *M* tetrabutylammonium phosphate in water as mobile phase. In addition, this column made it possible to work at extreme pHs, ranging from 2 to 8, and to improve the separation of the chromatographic peaks. Tetrabutylammonium phosphate was added to the mobile phase in order to form a non-polar ion with the carboxylic acid function of fumagillin, which is then retained on the analytical column and better resolved.

3.2. Limit of quantitation

The limit of quantitation in muscle tissue of trout was the lowest concentration which is measured within the limits of precision and accuracy set for the method (15% of precision, 20% of percentage difference). This limit was determined to be 20 ng/g at the detection wavelength of 351 nm. The intra-assay ($n = 12$) and inter-assay coefficients of variation (C.V.) at these concentrations were 6.9% and 2.5%, with deviations from the theoretical value of 1.7%. For the determination of fumagillin depletion in muscle tissue of rainbow trout, a quantitation limit of 20 ng/g is considered satisfactory.

3.3. Limit of detection

The limit of detection in muscle tissue of trout was equal to the mean of the measured content of representative blank samples ($n = 20$) plus 3 times the standard deviation of the mean. This limit was determined to be 7 ng/g at the detection wavelength of 351 nm.

3.4. Assay validation

Analyte concentrations were determined from their peak-area ratio, using calibration standards made from the same biological material as the samples. The calibration standards (20–1000 ng/g) gave correlation coefficients of 0.999. The results in Table 1 show that fumagillin is determined with good precision.

The day-to-day variation was determined by analysing one calibration curve for six days over a period of two weeks. The day-to-day reproducibility was determined over the concentration range 20–1000 ng/g in muscle tissue. The results show good reproducibility (Table 2).

Mean recovery of fumagillin from muscle tissue was calculated at three concentration levels, and gave a recovery of 69.2% at the limit of quantitation (Table 3).

3.5. Animal study

The fumagillin depletion study carried out in rainbow trout shows that oral administration of 3 mg/kg at 12-h intervals for 10 consecutive days resulted in low drug concentrations in the muscle tissue. The results are given in Table 4. Each result is the mean of twelve analyzed trout. Fumagillin is very rapidly eliminated, and at the low dose, practically no fumagillin is measured (i.e. lower than 20 ng/g) from the 4th day onwards.

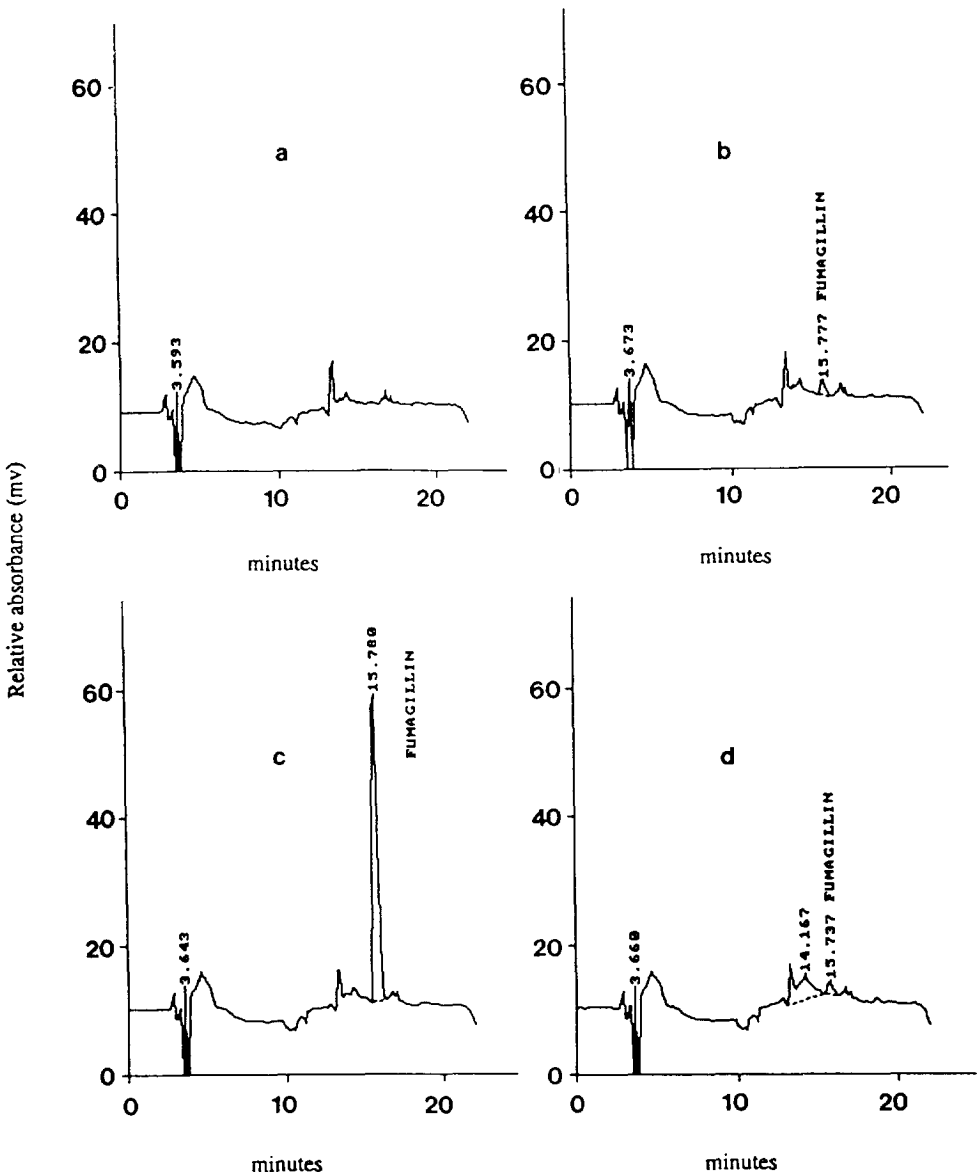

Fig. 2. Chromatograms after addition of (a) drug-free trout muscle tissue, (b) trout muscle tissue spiked with 50 ng/g fumagillin, (c) trout muscle tissue spiked with 1000 ng/g fumagillin, and (d) trout muscle tissue from one animal obtained on day 10 of treatment (15 mk/kg), one hour after the last dose of fumagillin.

4. Conclusions

Using the combination of an automated system and a simple HPLC system, fumagillin can be determined with high precision; liquid–solid automated separation gives reproducible recovery. The method is easy to perform; no manual liquid–solid treatment of the samples is necessary. Four samples can be prepared and analysed per hour, as the benchmate system can be run with minimal manual labor, the capacity of the system is high. The simplicity of the method, the high sample throughput and the quantitation limit of 20 ng/g enable this system

Table 1
Within-day precision

Concentration added (ng/g)	Peak area (mean ± S.D., n = 12) (mV)	C.V. (%)
20	62 289 ± 4281	6.9
100	251 587 ± 5253	2.1
1000	2 879 863 ± 53 365	1.9

Table 2
Day-to-day reproducibility and accuracy

Concentration added (ng/g)	Concentration found (mean ± S.D., n = 6) (ng/g)	C.V. (%)	S.E. (%)
20	20 ± 1	2.5	1.7
50	47 ± 5	9.8	−6.7
100	104 ± 13	12.1	3.8
250	249 ± 32	12.7	−0.4
500	522 ± 60	11.4	4.4
1000	1029 ± 110	10.7	2.9

Table 3
Recovery

Concentration added (ng/g)	Concentration found (mean ± S.D., n = 12) (ng/g)	Recovery (%)
20	14 ± 0	69.2
100	56 ± 0	55.8
1000	640 ± 6	64.0

Table 4
Fumagillin concentration in muscle tissue of trout at various slaughtering times after the last administration

Dose (mg/kg)	Concentration (mean ± S.D., n = 12) (ng/g)			
	1 h	4 days	10 days	21 days
3	30 ± 8	<LOD	<LOD	<LOD
15	46 ± 24	<LOD	<LOD	<LOD

< LOD = lower than the limit of detection (7 ng/g of tissue).

to be used as a suitable analytical method for the determination of fumagillin in muscle tissue of trout.

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