# DETERMINATION OF METRONIDAZOLE RESIDUES IN WATER AND SEDIMENT SAMPLES

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## ABSTRACT

Metronidazole (MET) is an antibacterial and antiprotozoal drug widely used in animal husbandry, including fish farming. It belongs to a class of antibiotics known as nitroimidazoles. MET has a minimum inhibitory concentration of 1 μg. $mL^{-1}$ against *S. vortens in vitro*, with the recommended dose in ornamental fish equal 10 mg per 1 g fish food. For the treatment of fish food, 2.5 g $kg^{-1}$ or 4 mg $L^{-1}$ is recommended. However, in 1998, due to its environmental side effects, cytotoxicity and genotoxicity European Council Regulation 613/98/EEC announced that any residue of this drug found in food-producing animals or in products intended for human consumption must be considered as a violation of the EU regulation. Addition of metronidazole to animal food is also banned in the USA. In surface waters, this substance may be accumulated in sediments and/or undergo abiotic (photodegradation and/or hydrolysis) and biotic degradation. Investigations, of its fate and distribution in aquatic and edaphic environments are still very few. One of the main reasons is limited number of methods adopted for determination of metronidazole in aquatic and sediment samples.

In this work, development of new analytical methods for determination of MET in surface waters and sediments samples using HPLC-UV and LC-MS techniques has been carried out. Isolation and enrichment of MET from surface waters was performed using solid phase extraction procedure. Several conditions such as type of SPE cartridges, type of washing solvents, type of eluents were tested. Application of Strata X-C cartridge with elution of MET using a mixture of 28% $NH_4OH$ and acetonitrile (5:95, v/v) was found as optimal. Next, isolation of MET from sediments samples was optimized. Two extraction methods: QuECheRS and shaking with 0.1 M HCl solution were tested. The obtained extracts were purified by SPE. It was found that the second procedure allowed to achieve higher recovery of MET (92.8%). Next, validation of whole analytical procedures was performed. Such parameters as: linearity, sensitivity, repeatability and reproducibility of the proposed methods for determination of metronidazole in waters and sediments were established. In the end, the proposed methods were applied for analysis of surface waters and sediments collected from rivers in Northern Poland.

**Keywords**: metronidazole, water, sediment, determination, HPLC-UV, LC-MS

## 1. INTRODUCTION

The presence of pharmaceuticals in environmental matrices (in the pg $L^{-1}$ to μg $L^{-1}$ range) is well documented (e.g. Aga, 2008; Reemtsma and Jekel, 2007). Their continual entry into the environment, and their physicochemical properties, may have significant, long-term effects on the stability of ecosystems (e.g. Santos, 2010). Special attention is put on the residues of antibiotics since it was proven, that due to the formation of the dangerous

phenomenon of bacteria resistance, these substances may pose a real threat not only to ecosystem, but also human health.

The objective of this study – metronidazole (MET) (Figure 1) - belongs to a class of antibiotics known as nitroimidazoles. This antibacterial (for anaerobic bacteria) and antiprotozoal drug is widely used in animal husbandry, including fish farming. It is well absorbed and serum concentrations reach high levels, with tissue concentrations being generally similar to or slightly lower than those measured in the serum (Sagan *et al*., 2005). Metronidazole is metabolized to several derivatives. Its most important metabolites include an alcohol and an acid derivative.

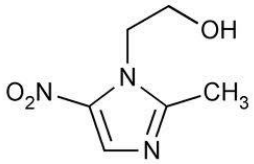


**Figure 1.** Chemical structure of metronidazole.

It has been reported that MET shows mutagenic, carcinogenic and toxic properties (Voogd, 1981). In mammalian cells DNA damage seems to be related to the production of reactive oxygen species. Its metabolites are also carcinogenic and mutagenic in some animal species because the original nitroimidazole ring is retained (Sanco/3400/2005). The use of MET in food-producing species is prohibited within the EU (L82/14 CRE, 1998). Similarly, it is prohibited from using in animal food in the USA (FARAD, 2010). To ensure human food safety, a requirement to have robust and reliable screening and confirmatory tests capable of low level detection of MET residues has been introduced to monitor illegal use. Thus, the development of methods for determination of MET in biological and environmental samples is still an important analytical task in environmental and food science (Mahugo-Santana *et al.*, 2010). This work is focused on the evaluation of the methods for determination of MET in surface waters and sediments samples using HPLC-UV and LC-MS techniques.

## 2. EXPERIMENTAL PART

### 2.1. Chemicals and standard solutions

Metronidazole (purity (> 98%) and acetonitrile (ACN) (LC–MS Chromasolv®) were provided by Sigma-Aldrich (Steinheim, Germany). Methanol (MeOH) (HPLC – grade) was obtained from POCH S.A. (Gliwice, Poland), acetone was supplied by Stanlab (Lublin, Poland). 37% hydrochloric acid (HCl) of analytical grade was provided by Chempur (Piekary Śląskie, Poland). Ammonium acetate, ethyl acetate, dichloromethane, $KH_2PO_4$, and acetic acid (all of analytical reagent grade) were purchased from Chempur (Piekary Śląskie, Poland). Deionized water was produced by the HYDROLAB System (Gdańsk, Poland). Standard stock solution of the target compound (0.5 mg/mL) was prepared in acetonitrile and stored at –18 °C. Working calibration standard solutions were prepared by diluting standard stock solution in the appropriate amounts in acetonitrile and stored in the dark at < 4 °C. Water and sediment samples spiked with the known amount of MET were also prepared.

### 2.2. Isolation and enrichment of MET from surface waters

Isolation and enrichment of MET from surface waters was performed using solid phase extraction procedure. Several conditions such as type of SPE cartridges (Oasis HLB (Waters, USA), Strata X-C (Phenomenex, USA)), type of washing solvents (water, acetone, MeOH, ACN), type of eluents (ACN, MeOH, a mixture of 28% $NH_4OH$ and ACN (5:95, v/v)) were tested.

### 2.2. Isolation and enrichment of MET from sediments

Two extraction methods: QuECheRS (Anastassiades *et. al.,* 2003) and shaking for 15 min with 0.1 M HCl solution were investigated. The obtained extracts were purified by SPE procedure.

### 2.3. HPLC-UV and LC-MS/MS measurements

In the experiments a high performance liquid chromatograph (Perkin Elmer Series 200) consisting of a binary pump, a UV/VIS detector, a vacuum degasser and a Rheodyne injection valve was used. The HPLC–MS/MS system equipped with an Agilent 1200 Series LC system (Agilent Technologies Inc., Santa Clara, USA) and an HCT Ultra ion trap mass spectrometer (Brucker Daltonics, Bremen, Germany) with an electrospray ionization source was applied. For data acquisition EsquireControl software was used. Such factors as: the composition of the mobile phase, elution program, flow rate of the mobile phase, the type of column packing, time of analysis and detection parameters were optimized. Next, validation procedure for the methods of determination of MET in water and sediment samples were carried out.

### 2.4. Application of the proposed methods to the analysis of environmental samples

The proposed methods were used to analyse metronidazole residues in river waters and sediments collected in Northern Poland.

## 3. RESULTS

### 3.1. Optimization of HPLC-UV and LC-MS/MS analyses

Among all tested conditions for determination of MET by HPLC-UV and LC-MS/MS the application of a column C18 (150 x 4,6 mm, 5 µm, Phenomenex) and the mobile phase: (A) ACN; (B) $H_2O$ : ACN [%] (90:10, v/v) + 1 mM $NH_4Ac$, pH = 3,56 (gradient from 0% A to 50% A for 6 min and from 50% A to 0 % A for 5 min) was found as optimal. In case of HPLC-UV the detection of MET was performed at $\lambda$ = 315 nm, whereas during LC-MS/MS measurements using the ions: m/z 172, m/z 128 and m/z 111.

### 3.2. Extraction methods

Application of Strata X-C cartridge with elution of MET using a mixture of 28% $NH_4OH$ and acetonitrile (5:95, v/v) was found as optimal. The absolute recovery (AR) of the target compound from the water samples was 98.4%. Optimization of the procedure of isolation of MET from sediments samples confirmed that the shaking of sediments samples with 0.1 M HCl solution allowed to achieve satisfactory AR value for metronidazole (92.8%).

### 3.3. Method validation

Validation of the analytical protocols was performed according to validation guidelines (Konieczka and Namieśnik, 2009; Ravichandran et al., 2010). Such parameters as: linearity, sensitivity, repeatability and reproducibility of the proposed methods for determination of metronidazole in waters and sediments were found to be satisfactory (for example, the accuracies for water samples were ranging from 88% to 106%, for sediments from 95.5 to 113.0%, RSD < 7.95%).

### 3.4. Analysis of the environmental samples

The proposed methods were applied for screening of MET residues in surface waters and sediments collected from rivers in Northern Poland. The obtained results confirmed the presence of MET in some investigated samples.

## 4. CONCLUSIONS

A rapid LC–MS/MS and HPLC-UV methods for the identification, confirmation and quantitation of metronidazole in waters and sediments samples have been developed and validated. Water samples were extracted using SPE procedure based on Strata X-C cartridge, whereas MET from sediments was isolated by shaking with 0.1 M HCl solution. The usefulness of these methods for determination of MET in environmental samples was fully confirmed. Thus, the proposed methods can be successful by applied for the monitoring of the level of metronidazole in surface waters and sediments.

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