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Short communication

Determination of five nitroimidazoles in water by liquid chromatography–mass spectrometry

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Abstract

A rapid liquid chromatography–mass spectrometric method for the determination of five nitroimidazoles in water from different sources is described. The extraction procedure was based on HLB solid-phase extraction with acetonitrile followed by an evaporation step. Ternidazol, another nitroimidazole, was used as the internal standard. The liquid chromatographic separation was made on a C_{18} bonded silica column applying a gradient with an ammonium acetate buffer solution and acetonitrile. Following electrospray ionisation, the protonated molecular ions $[M+H]^+$ were obtained. Quantification for each nitroimidazole was carried out by monitoring its molecular ion. Calibration functions, quantification and detection limits, intra- and inter-day reproducibility and accuracy were estimated. For the confirmatory assay, several fragment ions from each nitroimidazole were obtained and monitored. The method was applied successfully to determine and identify nitroimidazoles in water from different sources at a level of 0.2 $\mu g\ l^{-1}$.

Keywords: Water analysis; Nitroimidazoles

1. Introduction

Nitroimidazoles are a class of veterinary drugs used for the treatment and prevention of certain bacterial and protozoal diseases in poultry as well as for swine dysentery [1,2]. To this end, they are placed into coccidiostat substances. In the European Union (EU), coccidiostats are incorporated into feed products as feed additives. The most popular nitroimidazoles used as additives are metronidazole (MNZ, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazol), dimetridazole (DMZ, 1,2-dimethyl-5-nitroimidazole), ipronidazole (IPZ, 2-isopropyl-1-methyl-5-nitroimidazol) and ronidazole (RNZ, 1-methyl-2-(carbamoylmethyl)-5-nitroimidazole). But it has been reported that these compounds show mutagenic, carcinogenic and toxic properties [3,4]. For this reason, the EU has prohibited their use as additives in feed for food-producing species and all of them are currently in Annexe IV of the Council Regulation (EC) 2377/90 [5], meaning that any residue of these compounds found in food-producing animals or in products intended for human consumption has to be considered as a violation of the EU regulation.

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Investigation up to now has focused principally on proposed methods to detect or analyse residues from one or more nitroimidazoles in muscle and egg [6–13]. For several of these methods, the use of gas [6] or liquid [7–12] chromatography coupled with a mass spectrometer, gives them their principal advantage as techniques of confirmation and identification. Recently, determination of DMZ, RNZ and MNZ in poultry and meat by gas chromatography with nitrogen–phosphorus detection has been proposed [13].

Nevertheless, to the best of our knowledge, no method to determine nitroimidazoles in water samples has been reported. However, this type of procedure is needed, since one of the ways to administer these drugs to animals is via drinking water and it is thus possible to find potential residues in water when these drugs been used fraudulently. Additionally, nitroimidazoles have been used for oral treatment in several trout diseases [14–16]. Thus, it is possible to find residues of these drugs in water samples from fish farms located both in rivers and the sea. The purpose of this work was to propose a method to determine MNZ, DMZ, IPZ, RNZ, and the metabolite 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI) in water samples using liquid chromatography–mass spectrometry with electrospray ionisation following a solid-phase extraction. The method was applied successfully to drinking water samples as well as river and seawater samples.

2. Experimental

2.1. Apparatus and software

The HPLC system was a 2690 Waters coupled with a 996 photodiode array detector and a Z-Spray mass detector micromass platform LCZ-4000 with an electrospray interface. The equipment was connected to a Pentium 200 fitted with a MassLynx workstation. The automatic syringe bomb used to inject the samples directly into the mass detector was from Harvard Apparatus (Holliston, USA). The multiplace-heating block used to evaporate the samples was from Selecta (Barcelona, Spain).

Software programs used for the statistical treatment of the data were the STATGRAPHICS PLUS 6.0 software package (Statistical Graphics System, USA, 1992) and EXCEL software package (Microsoft Office 97, ver. 8.0, 1997).

The lack-of-fit test was applied to check the linearity of the calibration graphs according to guidelines of the Analytical Methods Committee [17].

2.2. Reagents

All reagents were of analytical reagent grade unless stated otherwise. Reverse-osmosis type quality water (purified with a Milli-RO plus Milli-Q station from Millipore) and HPLC quality were used throughout. Methanol, acetonitrile, dichloromethane, phosphoric acid and ammonium were supplied by Panreac (Barcelona, Spain). Nitrogen gas (99.999% of purity) for the mass spectrometer was purchased from Praxair (Malaga, Spain).

DNZ, MNZ and RNZ were supplied by Sigma (Barcelona, Spain); HMMNI, IPZ and TNZ, used as an internal standard, were kindly supplied by the EU Reference Laboratory for Residues of Veterinary Drugs (Berlin, Germany).

An individual stock standard solution of every nitroimidazol containing 1.0 g l^{-1} was prepared by dissolving each pure compound in ethanol. These solutions were stored in dark bottles at −20 °C and warmed to room temperature before use. Intermediate standard solutions of 10.0 and 1.0 mg l^{-1} were prepared by dilution with methanol and stored in dark bottles at 4 °C. The working standard solutions were prepared daily in amber coloured vials by diluting the intermediate standard solutions in 5.0 m*M* ammonium acetate buffer solution, pH 4.3. The calibration function was estimated between 0.1 and 5.0 mg l^{-1} of each nitroimidazole, using seven concentration levels and four replicas for each level.

2.3. Sample treatment procedure

Water samples were filtered through a cellulose acetate filter with a pore size of 0.45 μm (Millipore HAWP 04700) and collected in a glass bottle. The pH of this filtered water was adjusted to 2 with 0.1 *M* phosphate buffer solution, pH 2, and stored at 4 °C in darkness until analysis.

Solid-phase extraction was performed using Oasis HLB (Waters) 60 mg (or 500 mg) cartridges fitted

with a reservoir. The cartridges were preconditioned by treatment with 1 ml of dichloromethane, 1 ml of methanol and 1 ml of 0.1 *M* phosphate buffer solution, pH 2. Volumes of 5 ml (or 50 ml for the 500-mg cartridges) of the filtered samples were transferred to the reservoir and passed through the HLB cartridges at a flow-rate of 1 ml min^{-1}. The nitroimidazoles were then eluted with 1 ml of acetonitrile and collected in a glass tube. The eluate was evaporated to dryness under a gentle stream of nitrogen on a multiplace-heating block at a temperature not exceeding 30 °C. The residue was dissolved in 0.1 ml of 5.0 m*M* ammonium acetate buffer solution, pH 4.3, and injected into the chromatograph for analysis.

2.4. Chromatographic and mass detection procedure

A 10-μl volume of the sample was injected by the autosampler. Separation was performed on a C_{18} column 150×2.1 mm I.D.; particle size 5 μm. A C_{18} precolumn 10×2.1 mm I.D.; particle size 5 μm, was used to protect the chromatographic column. A gradient was applied with 5.0 m*M* aqueous ammonium acetate buffer, pH 4.3 (A) and acetonitrile (B); the gradient conditions were as follows: from 0 to 10 min, holding 6% B; from 10 to 20 min, ramp to 30% B and from 20 to 23 min, holding 30% B, from 23 to 23.1 min ramp to 6% B. The total run time was then 23 min. The flow-rate was 0.3 ml min^{-1}. The HPLC system was coupled to the mass spectrometer detector with an electrospray interface. No split was necessary to introduce the eluent into the mass spectrometer.

The mass spectrometer was operated in the electrospray ionisation (ESI) mode with positive ion detection. High-purity nitrogen was used as the drying gas and as the ESI nebulising gas, setting its pressure at 200.0 p.s.i. (gas flow 300 l/h). No auxiliary gas was used. The source block and the desolvation temperature were set at 100 and 400 °C, respectively. The capillary voltage was set at 2.00 kV. The instrument was operated in selected ion recording (SIR) mode with a dwell time of 0.50 ms per ion and applying the cone voltages for each molecular ion as summarised in Table 1. The tube lens voltage and the ion energy were set at 0.40 and 0.5 V, respectively.

All the mass spectrometer parameters cited above, except the nitrogen flow, the source block and desolvation temperatures that are related to the flow and composition of the mobile phase, were optimised by direct injection of the individual standard solutions of 1.0 μg l^{-1} of each nitroimidazole in methanol by the syringe bomb.

3. Results and discussion

3.1. LC–MS analysis

The chromatographic conditions were optimised to achieve a complete separation of the five drugs and

Table 1
SIR conditions

Nitroimidazole	Cone voltage (V)	Molecular peak $[M+H]^+$	Mass for confirmation	Chemical species
HMMNI	30.0	158	199 140	HMMNI+AcCN HMMNI−H_2O
MNZ	25.0	172	128	MNZ−C_2H_4O
RNZ	20.0	201	140 126	RNZ−$HOC(O)NH_2$ 140−CH_2
DMZ	30.0	142	183 112	DMZ+AcCN DMZ−NO
IPZ	35.0	170	124	IPZ−NO_2
TNZ	25.0	186	–	–

the internal standard. With a mobile phase comprising 6% of eluent phase B it was possible to separate HMMNI, MNZ, RNZ, DMZ and the internal standard TNZ in 10 min; a gradient up to 30% of eluent B was necessary for another 10 min to elute IPZ, the most nonpolar of the six compounds.

The full-scan spectra of each nitroimidazole showed an intense $[M+H]^+$ and some reproducible fragment ions that permit a confirmatory analysis. The presence of nitroimidazoles in a water sample could be based on the following criteria proposed by Ref. [7]: (1) correct retention time (Fig. 1 shows that the retention time from a standard sample and from a spiked sea water sample are very similar) (2) presence of several specific ions of the nitroimidazole investigated (those cited in Table 1), (3) and the same relative abundance of each ion in the sample and in the standard.

The analytical parameters were obtained from the registered chromatograms monitoring the molecular peak of each nitroimidazole using a single-ion recording mode (SIR). The calibration curves were established using the internal standard methodology. To check the linearity of the calibration function, the lack-of-fit test was applied. The results for the intercepts (a), slopes (b) and correlation coefficients (R^2) and the probability levels of the lack-of-fit test (P%) are summarised in Table 2. The results are indicative of good linearity within the range under study with a correlation coefficient higher than 0.999 and probability levels up to 70%, demonstrating good linearity of the response of the detector under the selected conditions for each compound discussed above. The limits of detection (LODs) and the limits of quantification (LOQs) were calculated from the calibration function [18], the LOD being below 30.0 ng l^{-1} for all the nitroimidazoles.

3.2. Extraction procedure and application to real samples

A concentration step is needed because the expected levels of nitroimidazoles in water samples are low due to their poor solubility despite the fact that they are polar compounds. Solid-phase extraction was carried out using HBL cartridges containing 60 and 500 mg. The influence of the pH of the water

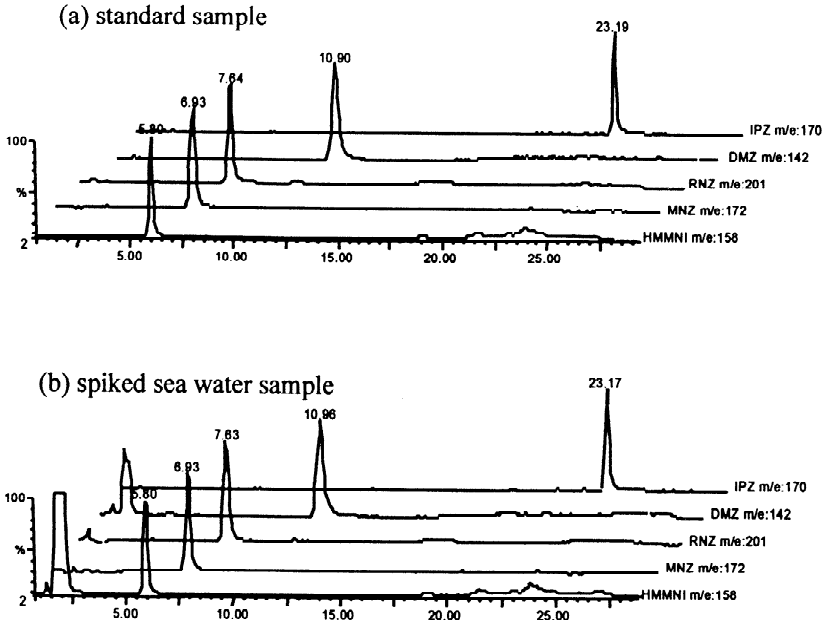

Fig. 1. LC–MS chromatograms: (a) standard sample, 0.1 mg l^{-1} of each nitroimidazole; (b) sea water sample spiked with 2.0 μg l^{-1} of each nitroimidazole after applying the extraction procedure to 5 ml of sample.

Table 2
Analytical parameters

	HMMNI	MNZ	RNZ	DMZ	IPZ
Intercept (*a*)	−547	7113	3316	−524	2291
Slope (*b*)	426479	742940	415179	475889	2301990
R^{2}[a] (%)	99.97	99.98	99.97	99.97	99.98
P value (%)[b]	90.18	76.77	97.50	91.97	9365
Linear dynamic range (ng ml^{-1})	99–5000	66–5000	77–5000	85–5000	66–5000
Detection limit[c] (ng ml^{-1})	30	20	23	25	20

[a] Correlation coefficient.
[b] Probability of lack of fit test.
[c] Calculated as indicated in Ref. [18].

sample on the SPE was studied from 1 to 12 and pH 2 was found to be optimal for all the analytes with recovery values up to 90%.

The retention capacity of the cartridge and the water sample volume that can be extracted were 10.5 μg (including the five nitroimidazoles and the internal standard) and 5.0 ml for the 60-mg cartridge, and 100.5 μg and 50 ml for the 500 mg cartridge. This indicated that he critical point in the SPE is not the retention capacity, but the sample volume, because the analytes can be washed by their own water sample. The recovery values fall (<90%) when higher sample volume are used.

The method was applied to drinking, mineral, river and seawater samples, in order to validate its use in water samples of different compositions. Drinking water samples were from the cities of Granada and Santa Fe. The river water samples were taken from two points of the Genil river (Loja, Spain): near the entrance and directly after the fish farm located on this river. The seawater samples were taken around a fish farm located on the coast of the province of Granada (Spain).

After confirming the lack of nitroimidazoles by applying the method, intra-day repeatability and accuracy were assessed in terms of precision (relative standard deviation, RSD) and recovery (%), respectively by analysing 5 ml (using cartridges of 50 mg) of six spiked water sample from each source at 10.0 μg l^{-1} (in each one of them). The recovery values were independent of the water sample source and they were close, 96–102% for HMMNI, 97–103% for MNZ, 97–104% for RNZ, 94–103% for DMZ and 92–102% for IPZ; the RSD was <4.5% in all cases.

Inter-day reproducibility and accuracy (5 days) were also tested, at the same level of concentration, by analysing four spiked samples from each source. The accuracy was 99.6% for HMMNI, 100.3% for MNZ, 101.1 for RNZ, 99.1 for DMZ and 97.4 for IPZ and the RSD was not >5% in any case.

Taking into account the quantification limit of each nitroimidazole, and the preconcentration factors achieved with the SPE procedure, the minimum concentration that can be analysed is 2.00 and 0.20 μg l^{-1} for HMMNI, 1.6 and 0.16 μg l^{-1} for RNZ, 1.7 and 0.17 μg l^{-1} for DMZ and 1.4 and 0.14 μg l^{-1} for MNZ and IPZ using the 50- and 500-mg cartridges, respectively. Thus, three replicates from each source spiked with 2.0 and 0.2 μg l^{-1} of each nitroimidazole were analysed. The recovery values were close to 94 and 109% and between 93 and 111%, respectively with RSD values <7.5% in both cases (Table 3).

4. Conclusion

The LC–MS method described here allows for the simultaneous determination of five nitroimidazoles. The extraction procedure from the water samples using HLB cartridges is simple and suitable for routine analyses of a large number of samples. In addition, the low levels analysed are appropriate for the detection of fraudulent animal treatment. This study serves as an initial step in a longer-term research project focusing on the veterinary field in which different means of analysing nitroimidazoles will be studied.

Table 3
Study of the limit of application of the method

		HMMNI	MNZ	RNZ	DMZ	IPZ
2 μg l^{-1} [a]	Recovery[b] (%)	101	98	102	104	100
	RSD[c] (%)	5.3	7.5	5.6	4.0	4.4
0.2 μg l^{-1} [a]	Recovery[b] (%)	102	103	101	100	99
	RSD[c] (%)	4.4	6.7	5.2	6.3	7.2

[a] Level of concentration of each nitroimidazole in the spiked water samples.
[b] Mean recovery of 12 independent analyses.
[c] Relative standard deviation of 12 independent analyses.

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