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Confirmatory method for the determination of nitroimidazoles in milk by liquid chromatography - tandem mass spectrometry

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Abstract

A multiresidue method for the determination of seven nitroimidazoles and their hydroxy metabolites in milk was developed. Milk samples were extracted with acetonitrile and cleaned up on strong cation-exchange solid phase extraction cartridges. Evaporated to dryness first, the extracts obtained were then reconstituted in 0.1% formic acid and injected onto a liquid chromatography-tandem mass spectrometer (LC-MS/MS). The separation of analytes was achieved on gradient mode using a C18 column and a mobile phase consisting of 0.1% formic acid in acetonitrile and 0.1% formic acid in water. Multiple reaction monitoring mode was set for the MS/MS with positive electrospray ionisation. For quantification, the isotope dilution method was used with isotopically labeled analogues of the target analytes. The method was evaluated entirely in accordance with EU Commission Decision 2002/657/EC and all validation criteria were in the required ranges. The method can easily detect and confirm metronidazole, dimetridazole, ronidazole, ipronidazole, and their hydroxy metabolites below the recommended concentration level of 3 µg/kg. The decision limits and detection capabilities ranged from 0.11 µg/kg to 0.22 µg/kg and from 0.19 µg/kg to 0.37 µg/kg respectively. The overall recoveries were between 96.6% and 105.2% with a good coefficient of variation, less than 8.7% under within-laboratory reproducibility conditions.

Keywords: milk, nitroimidazoles, residues, liquid chromatography, tandem mass spectrometry.

Introduction

Nitroimidazoles are antibacterial and antiprotozoal compound drugs. They were used formerly for both prophylactic and therapeutic treatment of diseases such as histomoniasis in poultry, haemorrhagic enteritis in pigs, parasitoses in fish, and genital trichomoniasis in cattle. Metronidazole (MNZ), dimetridazole (DMZ), ronidazole (RNZ), and ipronidazole (IPZ) are examples of these compounds. They are known as 5-nitroimidazoles due to the presence of a nitro (NO_2) group in the fifth position of their ring structure. Nitroimidazoles have been classified as suspect mutagens and carcinogens (8). They are rapidly metabolised in animals within a few hours after their administration. For MNZ the major metabolite formed by hydroxylation is hydroxy-metronidazole (MNZOH), DMZ and RNZ give 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI), and IPZ is metabolised into hydroxyipronidazole (IPZOH). These metabolites still contain the imidazole ring and therefore may have similar mutagenic and carcinogenic potential to the parent compounds. As a consequence, no maximum residue limits (MRLs) could be established (2). Thus, MNZ, DMZ, and RNZ have been banned while the other 5-nitroimidazoles have not been licensed for use in food-producing animals within the European Union (EU). They are also prohibited for the use in the U.S.A, Canada, China, and Japan. For this reason, monitoring of these compounds is now required within the EU as well as by countries exporting to the EU (3). The technical recommendation for the analytical methods has been given by the European Union Reference Laboratory (EURL), and the "recommended concentration" that laboratories should aim to measure for the most important nitroimidazoles and their hydroxy metabolites is 3 µg/kg (4). It has also been recommended that countries with high milk production should perform analysis to detect these

compounds in milk, because their possible illegal use in this matrix cannot be excluded.

To perform official controls, an appropriate analytical method for the determination and confirmation of nitroimidazoles in milk has to be applied. Commission Decision 2002/675/EC defines the requirements which must be met by the analytical methods used in the study of prohibited substances in food of animal origin, including milk (1).

Many procedures for the analysis of nitroimidazoles and their metabolites in various matrices have been published but there are relatively few reports of methodologies for milk (5, 7, 10, 12-14). Most of these methods are screening procedures using optical biosensors (13), capillary electrophoresis (7), tandem mass spectrometry (11), or time of flight mass spectrometry (10, 12). Only two methods have been published for the confirmatory analysis of nitroimidazoles in milk at the recommended concentration (5, 14). As an extraction solvent, trichloroacetic acid (7) or acetonitrile (5, 10-14) have been used, followed either by a hexane wash to remove impurities (5) or by solid-phase extraction (SPE) clean-up with cartridges such as Oasis MCX (7), Strata-X (12), or Strata SDB (14).

The aim of the study was the optimisation and validation of an analytical method for the simultaneous determination of seven nitroimidazoles and their hydroxy metabolites in milk. A method for the analysis of nitroimidazoles in muscle, plasma, and egg developed in a previous study (9) was a starting point for the present study and brought into compliance with European regulations regarding the official controls of chemical residues in food of animal origin.

Material and Methods

Reagents and chemicals. All chemicals and solvents used were of analytical grade and suitable for liquid chromatography. Acetonitrile, acetone, methanol, formic acid, acetic acid and 25% ammonium hydroxide were supplied by J.T. Baker (Deventer, the Netherlands). Anhydrous sodium sulphate was purchased from Sigma–Aldrich (Germany). Pure water 18.2 M cm quality was obtained from a Milli-Q plus system from Millipore (USA). Analytical standards of MNZ, DMZ, and RNZ were from Riedel-de Haën (Germany), whereas IPZ, MNZOH, HMMNI, IPZOH, MNZ-$^{13}C_2$, $^{15}N_2$, DMZ-D_3, RNZ-D_3, IPZ-D_3, MNZOH-D_2, HMMNI-D_3, and IPZOH-D_3 were purchased from Witega Laboratorien Berlin-Adlershof (Germany). All isotopically-labeled nitroimidazoles were of chemical and isotopic purity >98% and were used as internal standards (IS). Strata SCX (strong cation-exchange) SPE cartridges (500 mg, 3 mL) were sourced from Phenomenex (USA).

Standard solutions. Individual stock solutions of analytical standards at 1000 µg/mL were prepared in acetonitrile (stable for at least twelve months at -20°C). Stock solutions of nitroimidazole compounds were combined and diluted in acetonitrile to prepare intermediate standard solutions of 10 µg/mL (stable for at least six months at -20°C). For sample fortification, working standard solutions of nitroimidazoles at 1 and 0.1 µg/mL were prepared in 0.1% formic acid in water and stored at 4°C for three months.

Milk samples. Milk samples used as blanks for method optimisation and validation were of different types including cow, goat, and sheep milk of low and high fat content, and taken from animals never treated with any nitroimidazoles. Raw, pasteurised, and UHT milk was also examined.

Sample preparation. The milk sample (5 g) was weighed into a 100 mL polypropylene centrifugal tube, fortified with IS at the level corresponding to 2 µg/kg by adding 100 µL of 0.1 µg/kg working IS solution, and left for 15 min of equilibration. After adding 50 mL of acetonitrile into the tube the sample was stirred for 1 min and 5 g of anhydrous sodium sulphate was added. The tube was closed and shaken for 1min, then centrifuged at 2200 g for 10 min at 4°C. Next, the upper layer was filtered through anhydrous sodium sulphate (15 g in Whatman No. 4 filter paper) directly into an SCX SPE cartridge fitted with a reservoir and preconditioned with the mixture of acetonitrile and acetic acid (95:5, v/v). To this 5 mL of acetic acid was added and the content was mixed and allowed to run through the SPE cartridge. The cartridge was washed sequentially with 2.5 mL of acetone, 5 mL of methanol, and 5 mL of acetonitrile. After the last wash step a powerful vacuum was applied to dry the cartridge. To elute nitroimidazoles, 5 mL of the mixture of acetonitrile and ammonium hydroxide (95:5, v/v) was used. The eluate was evaporated to dryness at 40°C under a nitrogen stream and the residue was reconstituted in 0.1% formic acid in water (200 µL).

Liquid chromatography-tandem mass spectrometry. A liquid chromatography-tandem mass spectrometer (LC-MS/MS) system was used consisting of an Agilent 1200 series liquid chromatograph (Agilent Technologies, Germany) equipped with a binary pump, a degasser, an autosampler, a column heater, a switching valve, and a QTRAP 5500 linear ion trap quadrupole mass spectrometer with a Turbo V source (AB Sciex, Canada). The instrument was controlled by Analyst software in version 1.5.1 (AB Sciex, Canada). Electrospray ionisation (ESI) was used in the MS with positive ionisation mode, with a spray voltage of 3500 V and a source temperature of 400°C. Nitrogen was used as the nebuliser gas (40 psig), turbo gas (40 psig), and curtain gas (30 psig). Quantification

was done using a multiple reaction monitoring (MRM) mode to monitor precursor → product ion transitions for all standards. The two transitions for each analyte and their respective settings are listed in Table 1. Both Q1 and Q3 quadrupoles were maintained at unit resolution. The chromatographic separation was achieved using a Kinetex XB octadecyl analytical column (2.6 µm, 150 × 2.1 mm) with an octadecyl guard cartridge (Phenomenex, USA). Use was made of a mobile phase A containing 0.1% formic acid in acetonitrile and a mobile phase B containing 0.1% formic acid in water to perform the following gradient elution: 5% of the mobile phase A was held for 0.5 min, decreased to 40% at 1 min, and held for 3 min. From 4.5 to 12 min the system was re-equilibrated with the initial composition of the mobile phase A (5%). The flow rate of the mobile phase was controlled to 250 µL/min, the column temperature was set at 35°C, the injection volume was prescribed at 20 µL.

Method validation. The proposed method was validated according to Commission Decision 657/2002/EC (1) and linearity, specificity, recovery, repeatability, within-laboratory reproducibility, decision limit (CCα), and detection capability (CCβ) were calculated. Quantification was based on peak area and was carried out using an internal standard solvent-based calibration curve with 1/x weighting. For the matrix-matched calibration curve, blank samples fortified with working standard solutions of analytes at eight concentration levels (0, 0.5, 1, 2, 3, 4, 6, and 8 µg/kg) were analysed on three different days. The specificity of the method was determined by analysing twenty blank milk samples originating from different sources. The repeatability was evaluated by fortifying six blank samples with nitroimidazoles, the samples having the three concentration levels of 1, 2, and 3 µg/kg. The samples were analysed on the same day with the same instrument and by the same operators, and the overall CV (%) of the fortified samples were calculated. This was repeated on three separate days with the same instrument but by different operators and the within-laboratory reproducibility was evaluated by calculating the CV of the fortified samples on three different days. The percentage recovery was determined in the same experiment as repeatability by comparing the mean measured concentration with the fortified concentration of the samples. The decision limit and the detection capability were calculated by the matrix-matched calibration curve procedure. CCα was determined with a statistical certainty of 1- (α = 0.01), whereas CCβ was determined with a statistical certainty of 1- (β = 0.05).

Table 1. MS/MS parameters used for LC-MS/MS determination of nitroimidazoles in milk

Analyte	Ion transition (*m/z*)	Declustering potential (V)	Collision energy (eV)	Cell exit potential (V)	Peak area ratio ± tolerance (%)
MNZ	172 → 128 [a]	45	19	16	0.53 ± 20
	172 → 82	45	35	14	
MNZ-$^{13}C_2$,$^{15}N_2$	176 → 132	45	19	16	
MNZOH	188 → 126 [a]	45	25	16	0.98 ± 20
	188 → 123	45	19	16	
MNZOH-D_2	190 → 125	45	19	16	
DMZ	142 → 96 [a]	60	23	10	0.37 ± 25
	142 → 81	60	33	14	
DMZ-D_3	145 → 99	60	33	14	
RNZ	201 → 140 [a]	40	17	16	0.30 ± 25
	201 → 55	40	35	10	
RNZ-D_3	204 → 143	40	17	16	
HMMNI	158 → 140 [a]	50	17	14	0.42 ± 25
	158 → 55	50	24	10	
HMMNI-D_3	161 → 143	50	17	14	
IPZ	170 → 124 [a]	55	25	14	0.99 ± 20
	170 → 109	55	33	16	
IPZ-D_3	173 → 127	55	25	14	
IPZOH	186 → 168 [a]	50	17	16	0.45 ± 25
	186 → 122	50	27	12	
IPZOH-D_3	189 → 171	50	17	16	

[a] ion transition used for quantification

Table 2. Validation results for the LC-MS/MS method for the analysis of nitroimidazoles in milk at the three fortification levels (n = 18)

Analyte	CCα (µg/kg)	CCβ (µg/kg)	Concentration (µg/kg)	Recovery (%)	Repeatability, CV (%)	Within-lab reproducibility, CV (%)
MNZ	0.11	0.19	1	100.6	3.6	4.0
			2	102.9	3.0	3.0
			3	101.1	2.2	2.7
MNZOH	0.18	0.30	1	102.6	5.1	5.3
			2	101.1	2.4	3.4
			3	100.7	3.1	3.1
DMZ	0.20	0.35	1	101.5	3.1	5.6
			2	98.8	5.8	6.6
			3	99.4	4.4	6.1
RNZ	0.22	0.37	1	101.8	4.3	4.5
			2	96.6	6.3	6.4
			3	99.3	3.2	3.6
HMMNI	0.19	0.33	1	97.5	4.0	5.2
			2	97.8	3.8	3.9
			3	97.9	3.1	5.3
IPZ	0.19	0.24	1	102.0	5.1	7.9
			2	103.2	7.4	7.5
			3	105.2	8.1	8.5
IPZOH	0.22	0.28	1	104.5	5.5	8.7
			2	101.8	5.2	5.8
			3	103.1	4.9	5.1

The decision limit and the detection capability were calculated by the matrix-matched calibration curve procedure. CCα was determined with a statistical certainty of 1- (α = 0.01), whereas CCβ was determined with a statistical certainty of 1- (β = 0.05)

Results

The LC-MS/MS parameters used for nitroimidazole confirmation are listed in Table 1, whereas Fig. 1 shows chromatograms of a blank milk sample (A) and a milk sample fortified at the concentration of 1 µg/kg (B). Several method validation parameters were determined including specificity, recovery, repeatability, within-lab reproducibility, CCα, and CCβ (6).

The specificity was evaluated by analysing twenty different blank milk samples (cow milk, goat milk, high fat, and low fat). No interfering peaks from endogenous compounds were found in the retention time of the target analytes. The recovery, repeatability, and within-laboratory reproducibility at three concentration levels (1, 2, and 3 µg/kg) are presented in Table 2. The results show good accuracy ranging from 96.6% to 105.2%, with a good CV of less than 8.7%. The CCα and CCβ were calculated using matrix-matched calibration curves and were from 0.11 µg/kg to 0.22 µg/kg and from 0.19 µg/kg to 0.37 µg/kg respectively.

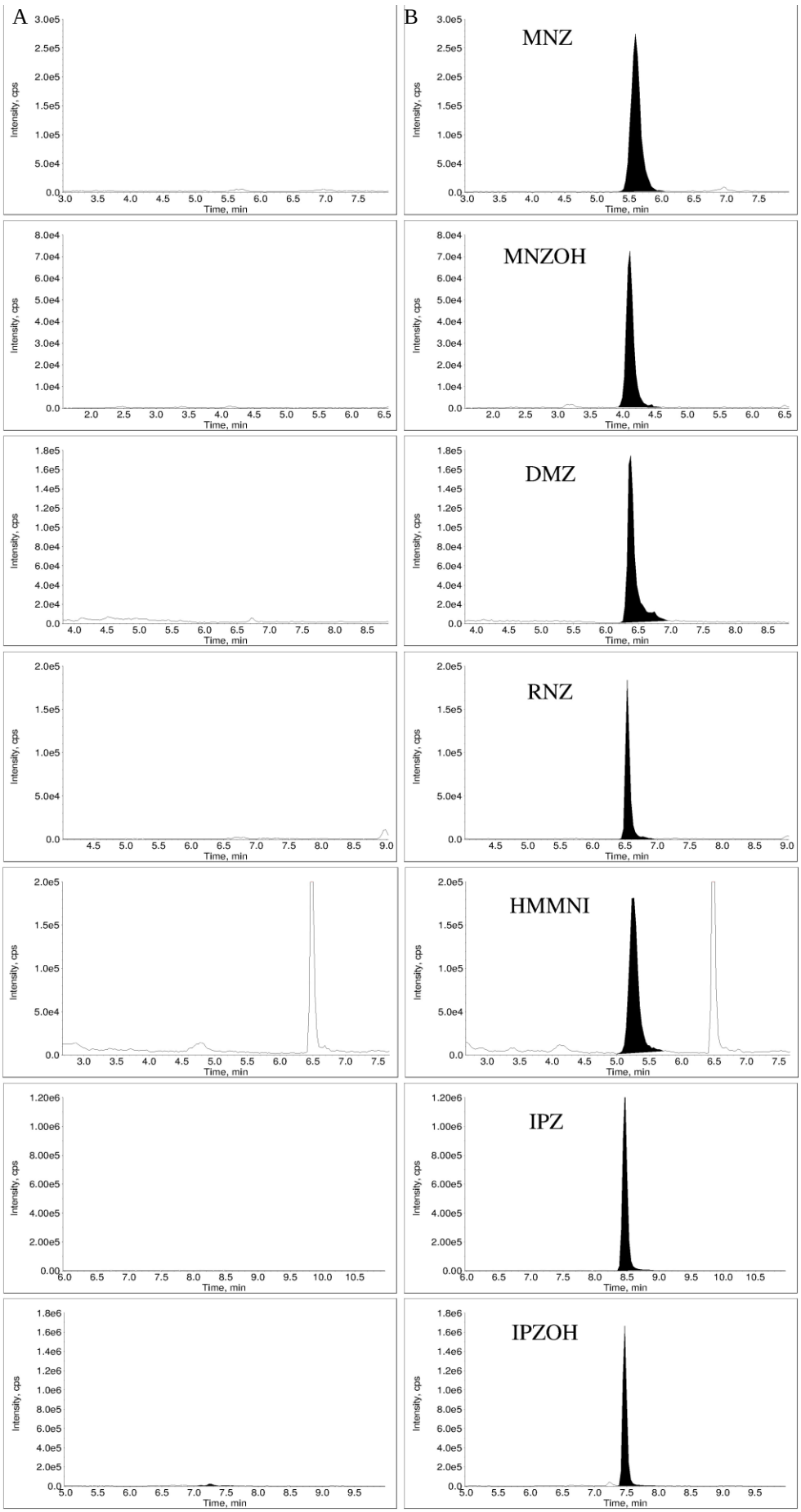


Fig. 1. MRM chromatograms of a blank milk sample (A), and a milk sample fortified with nitroimidazoles at the concentration level of 1 µg/kg (B)

Discussion

The method presented was developed on the basis of the method used in the laboratory of Department of Pharmacology and Toxicology for the analysis of nitroimidazoles in muscle, plasma, and egg (9). The LC-MS/MS system used for this procedure was different than that used previously, so the mass spectrometry parameters required optimisation. Using different collision offset voltages, product ion spectra were measured and two fragmentation reactions were selected for each analyte in MRM mode (Table 1).

The novel column technology of core-shell was adopted for separating the investigated nitroimidazoles and a Kinetex XB C18 core-shell particle column (2.6 µm, 150 × 2.1 mm) was used. Compared to the Luna C18 fully porous particle column (5 µm, 150 × 2.0 mm) used in our previous work (9), the Kinetex XB C18 column with a mixture of 0.1% formic acid in acetonitrile and 0.1% formic acid in water provided better performance and was thus chosen for this study. The chromatographic conditions specified enabled reduction of the analysis time to 12 min and enhanced sensitivity (Fig. 1).

Sample preparation is a fundamental and often the most critical part of the multiresidue method. The nitroimidazole residues are usually extracted from milk samples with acetonitrile (5, 10, 12-14) and this organic solvent was chosen for our study. Moreover, in comparison with solid samples, the residues in milk samples are more uniformly distributed and homogeneity can be easily achieved by mixing or shaking. In order to find optimal conditions for the clean-up of the extracts from milk samples, several SPE sorbents were tested, namely: polymeric reversed phase (Strata-X), polymeric strong cation-exchange (Strata-X-C), and silica based strong cation-exchange (Strata SCX). Compared to the other sorbents examined in this study, the SCX sorbent provided better or similar recoveries of analytes. This cartridge retains the protonated nitroimidazoles on the SCX column by ionic interaction with the benzene sulfonic acid group, and the ionic interactions are much stronger than the non-polar interactions, allowing the use of an extensive wash step and giving clean extracts. An additional advantage of the choosing SCX columns is that they are suitable for the determination of nitroimidazoles from several different kinds of matrices including muscle, plasma, and egg after using the same sample preparation protocol already used in the laboratory of Department of Pharmacology and Toxicology (9). This facilitates the overall analysis and decreases its costs.

To compensate for the signal loss resulting from matrix effect and the analyte loss during sample preparation, the isotope dilution method with isotopically labeled analogues of the target analytes was applied for quantification analysis. The basic assumption with this technique is that relative responses between the analyte and the labeled analogue stay constant. Stable isotopically labeled analogues are commercially available for all tested nitroimidazoles and, as we found, their use limited matrix effect, thus solvent-based calibration curves could be used for quantification of these nitroimidazoles in milk.

The validation results (Table 2) reveal that the confirmatory method as developed meets the required performance of 3 µg/kg set by the EURL. The CCα and CCβ values determined for each tested nitroimidazole are far below this level. The method also performs very well in terms of recovery and repeatability for each of the analytes at three concentration levels (1, 2, and 3 µg/kg) and all the values fall within the acceptable ranges defined in Commission Decision 2002/675/EC (1). The advantage of the proposed method is the possibility of the analysis of seven nitroimidazoles in milk with the same sample preparation protocol as for other matrices like muscle, serum, and egg. Only two methods have been published for the confirmatory analysis of nitroimidazole residues in milk (5, 14). In comparison to them, the method optimised in this study allows quantification using solvent-matched calibration curves and does not require the preparation of matrix-matched calibration samples, which greatly reduces the total analysis time and costs.

Finally, the specificity was evaluated and the proposed method was successfully applied to the determination of nitroimidazoles in milk from various species and of different compositions and qualities. Milk from cows, goats, and sheep, with low and high fat content, raw, pasteurised, and UHT was examined and no differences were found in the performance of the developed assay in these very varied types of milk.

In conclusion, a relatively fast, precise, and low-cost method of determination of seven nitroimidazoles in milk has been developed. The proposed procedure meets the required performance criteria for analytical methods for quantification and confirmation of nitroimidazole residues in food of animal origin and is suitable for official routine milk control.

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