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Simultaneous determination of seven nitroimidazole residues in meat by using HPLC-UV detection with solid-phase extraction

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Abstract

A method was developed for the determination of the seven nitroimidazoles including metronidazole (MNZ), ronidazole (RNZ), dimetridazole (DMZ), tinidazole (TNZ), ornidazole (ONZ), secnidazole (SNZ) and the common metabolite of RNZ and hydroxydimetridazole (DMOHZ) in poultry and pork muscles by high-performance liquid chromatography (HPLC) with ultraviolet detection (UV). After extraction with ethyl acetate and evaporation, the nitroimidazoles were redissolved in ethyl acetate and purified using strong cation exchange (SCX) solid-phase extraction (SPE) column. The HPLC separation was carried through on a C_{18} bonded silica column with a deionized water–methanol–acetonitrile mobile phase using a gradient elution procedure. The limit of detection of all the seven nitroimidazoles was 0.2 μg/kg. The recoveries of the seven nitroimidazoles for chicken, pork and bacon samples spiked with 1–20 μg/kg were in the range of 71.4–99.5%. The linearity is satisfactory with a correlation coefficient of >0.998 at concentrations ranging from 0.7 to 60 μg/kg. The relative standard deviations of 10 measurements for spiked chicken, pork and bacon samples at the concentration of 1 and 20 μg/kg were in the range of 6.2–13.9% and 4.0–8.7%, respectively. The intra-day precision ($n=5$) for nitroimidazoles residues in chicken spiked at 20 μg/kg is 6.9%, and the inter-day precision for 5 days ($n=25$) is 11%. The method is capable of identifying nitroimidazole residues at ≥0.7 μg/kg levels and was applied in the determination of nitroimidazole residues in meat sample.

Keywords: Solid-phase extraction; High-performance liquid chromatography; Nitroimidazoles; Multi-residue analysis; Meat sample

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. Their main compounds include metronidazole (MNZ), ronidazole (RNZ), dimetridazole (DMZ), tinidazole (MNZ), ornidazole (ONZ) and secnidazole (SNZ). Their molecular structures and the structure of the common metabolite of RNZ and hydroxydimetridazole (DMOHZ) are shown in Fig. 1.

It has been reported that RNZ, DMZ and MNZ show mutagenic, carcinogenic and toxic properties [1–3]. In mammalian cells DNA damage seems to be related to the production of reactive oxygen species. It has been shown that treatment with nitromegazol entails the production of reactive oxygen species that are highly damaging for cellular structures [4–6]. Their use in food-producing species is prohibited within the EU [7–9]. Similarly, DMZ and MNZ are prohibited from using in food animals in China [10]. TNZ, ONZ and SNZ are the derivatives of DMZ with similar molecular structure. Although these compounds have not been prohibited in food-producing animals, but there is a potential risk. To ensure human food safety, the development or improvement of multi-residue analysis in animal tissues is an important analytical task.

Several analytical methods have been reported for the determination of nitroimidazole either in pure, dosage or in biological fluids and animal tissues, including spectrophotometry [11,12], electrophoresis [13], gas chromatography (GC) [14–16], GC–mass spectrometry (MS) [17,18], thin-layer chromatographic (TLC) [19,20], HPLC [21–26], and liquid chromatography (LC)–MS [10,27–30]. Among them, chromatography has been utilized extensively for the determination

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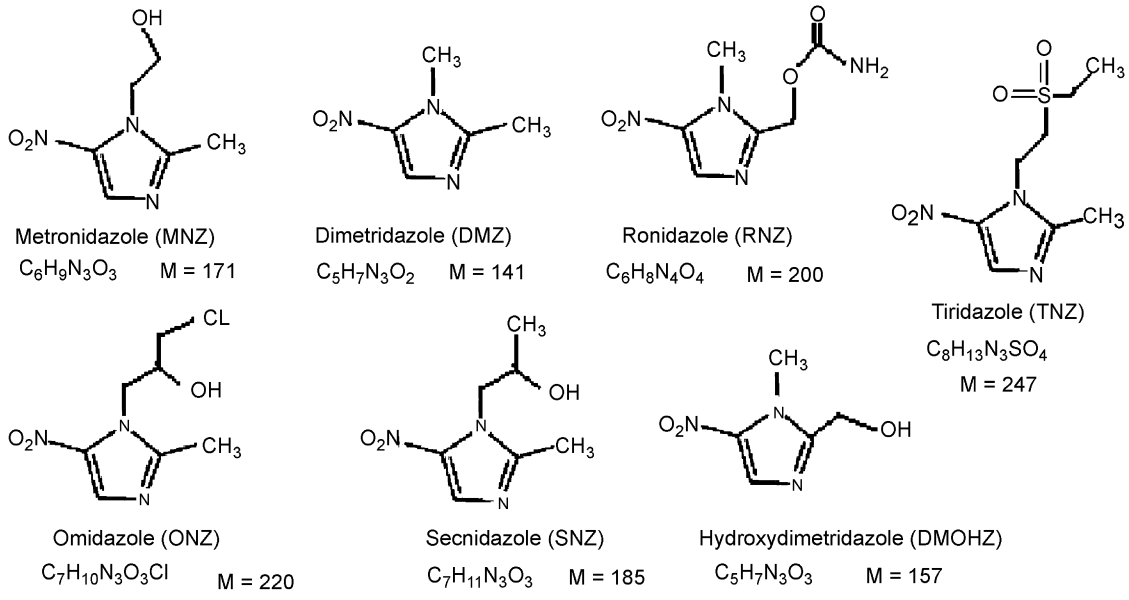

Fig. 1. Structural formula of the seven nitroimidazoles.

of nitroimidazoles. In comparison to biological fluids, residue analysis of nitroimidazoles in animal tissues is generally more difficult [31]. Wang developed a method for determination of the three nitroimidazole residues in poultry meat by gas chromatography with the detection limits of 0.2 μg/kg for DMZ and MNZ and 0.5 μg/kg for RNZ [16]. Gaugain and Abjean reported a TLC method for the detection of RNZ, DMZ and DMOHZ in animal muscle with the detection limits of 2–5 μg/kg [20]. Several HPLC methods were described for the detection and identification of DMZ residues in muscle tissue with a confirmation limit of 5 μg/kg [21], as well as DMZ, MNZ in poultry tissue, serum and eggs [22]. A solid-phase extraction–HPLC method was reported for the determination of MNZ in vaginal tissue with a detection limit of 100 μg/kg [24]. The LC–MS methods were presented for the determination of DMZ in poultry tissues and eggs [27], and the four nitroimidazoles in poultry muscle, which is suitable for statutory residue testing and identifying nitroimidazole residues at levels below 5 μg/kg [28]. A sensitive HPLC–atmospheric pressure chemical ionisation-MS method was developed for the determination of three nitroimidazoles with a detection limit of 0.1 μg/kg for DMZ and RNZ and 0.5 μg/kg for DMOHZ [29]. A HPLC–tandem mass spectrometry (APLC-MS/MS) method was developed for the analysis of three residues in royal jelly with detection limits of 1.0 μg/kg for DMZ and 0.5 μg/kg for MTZ and RNZ [30].

Up to now, the most of published HPLC methods were used only for the residues analysis of one kind or 2–4 kinds of nitroimidazoles in animal tissue with lower sensitivity. The use of LC–MS/MS can provide the mass spectrum of the compound to identify the compound in biological matrices and its quantitation, but expensive instrument is to be required. The purpose of this study was to develop a simple and effective extraction and clean-up procedure for simultaneous determination of six nitroimidazoles and one-metabolite residues in meats by using SPE–HPLC method. The extraction and clean-up conditions were investigated and optimized. A new procedure with ethyl acetate as extraction reagent has been proposed. SCX SPE cartridge is more suitable for purification of the seven analytes than C_{18} and silica cartridge. The proposed SPE–HPLC method has some excellencies, such as higher recovery, lower detection limit, multi-residue analysis, effective baseline separation and lesser reagent to be required. The detection limit for all the seven analytes was 0.2 μg/kg. The intra-day precision ($n = 5$) for nitroimidazoles residues in chicken sample spiked at 20 μg/kg is 6.9%, and the inter-day precision for 5 days ($n = 25$) is 11%. The proposed method has been applied for simultaneous determination of the six nitroimidazoles and one-metabolite residues in spiked chicken, pork and bacon samples at ≥0.7 μg/kg.

2. Experimental

2.1. Chemicals and reagents

Ethyl acetate, acetone, methanol and acetonitrile were of HPLC grade (Dikma, USA). Acetic acid and ammonium hydroxide were of commercial analytical reagent grade. The water was deionized by a water purification system (Human power III plus, Korea). The solvents for HPLC were filtered through 0.45-μm nylon membrane filter and degassed in an ultrasonic bath. Strong cation exchange SCX SPE cartridges (250 mg, 3 mL) were purchased from Varian.

2.2. Instrumentation

The chromatography was carried out using an LC-2010C instrument (Shimadzu, Japan). The entire system included a tetra-member pump, an autosampler and a UV spectrophotometric detector connected to Shimadzu CLASS-VP 6.0 workstation.

Table 1
The time procedure of gradient elution

Time (min)	Water (%)	Acetonitrile (%)	Methanol (%)
0.0	77	4	19
5.0	77	4	19
12.0	71	10	19
20.0	71	10	19
23.0	77	4	19
28.0	77	4	19

A Diamonsil^{TM} C_{18} (5 μm particle size, 250 mm × 4.6 mm i.d.) from Dikma was used as the analytical column. The mobile phase was consisted of acetonitrile, methanol and water. A tri-member gradient elution procedure is shown in Table 1.

2.3. Standards

Nitroimidazoles standards were obtained from Sigma (St. Louis, MO, USA). Stock standards were prepared at 100 μg/mL in methanol. These solutions were stored in dark at −18 °C. A mixed intermediate standard at 1 μg/mL was prepared by combining 1 mL each stock standard and diluting to 100 mL with purified water. Working standards (0.005, 0.01, 0.05, 0.10 μg/mL) were prepared by serial dilution of the intermediate standard with water. The standards were stored at 1–4 °C in dark.

2.4. Sample preparation and extraction

Poultry muscle and pork were purchased fresh, cut into small pieces and ground into a homogeneous sample using a mincer. This material was then kept frozen at −18 °C.

A 1 mL volume of 0.01 μg/mL standards was spiked into each of the samples, obtaining 2 μg/L in the final sample solution for each nitroimidazole. A 5-g amount of homogenized fortified sample was extracted with 20 mL ethyl acetate. The mixture was allowed to stand for 5 min in ultrasonic bath, followed by centrifugation at 3000 rpm for 10 min. The residue was extracted again with 20 mL ethyl acetate. The ethyl acetate extraction obtained twice was combined and evaporated just to dryness with a vacuum evaporator (water bath at 35 °C). The residue was dissolved in 5 mL of acetic acid–ethyl acetate (5:95, v/v). The obtained solution was used for SPE clean-up.

2.5. SPE clean-up

Using a vacuum manifold, SCX SPE columns (3 mL, 250 mg, Varian, USA) were conditioned by washing sequentially with 4 mL of methanol and 4 mL of acetic acid–ethyl acetate (5:95, v/v). The solution obtained above was applied to the columns via a 30 mL reservoir with polyethylene frits at a flow rate of approximately 45 drops per minute. The residues were dissolved twice and passed through the SCX cartridges. The cartridges were washed sequentially with 5 mL of acetone, 3 mL of methanol and 3 mL of acetonitrile, and were eluted with 5 mL of ammonium hydroxide (28%)–acetonitrile (5:95, v/v). The eluant was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved in 1.0 mL of water and vortex mixed. All samples were analyzed soon after preparation.

2.6. HPLC-UV analysis

A 50 μL aliquots of the sample extracts were injected into the Diamonsil^{TM} C_{18} column, and the mobile phase given in Table 1 was pumped at a flow rate of 1 mL/min. Injections of a mixed standard containing all seven analytes were made at regular intervals during the run. The absorption of all the seven analytes was detected at 320 nm. Recovery of the analytes was calculated by comparison of standards with extracts of fortified blank tissue at a concentration of 1–20 μg/kg. All calculations were performed using peak areas.

3. Result and discussion

3.1. Extraction and clean-up

The use of acetonitrile as extraction reagent provided lower detection limit for the simultaneous determinations of DMZ, RNZ and MNZ [16], and DMZ, RNZ, MNZ and DMOHZ [28], as well as DMZ, RNZ and DMOHZ [29]. Our test showed that low recovery was obtained for extraction of TNZ, ONZ and SNZ with acetonitrile as extraction reagent. When ethyl acetate and hexane were used for extracting RNZ, MNZ and DMZ from poultry meat in sequence, more reagents were to be required [25]. Three different SPE procedures have been described for clean-up of spiked samples after solvent extraction [25,27,29]. A C_{18} SPE was used for clean-up of MNZ, RNZ and DMZ, the detection limit was 0.5, 1.0 and 1.0 μg/kg, respectively [25]. Although a silica cartridge was used for the purification of DMZ using methylene chloride acetone as elution reagent [27], but this procedure is not suitable for multi-residue analysis because of much interferences. Our test discovered that when SCX SPE cartridge was used for clean-up after extraction with acetonitrile, the recoveries of TNZ, ONZ and SNZ were lower than 40%, so this procedure was also not suitable for analysis of all the seven nitroimidazoles.

We have developed a new extraction–clean-up procedure, which uses SCX SPE cartridge and ammonium hydroxide (18%)–acetonitrile (5:95, v/v) as elution reagent for purification after extraction with ethyl acetate and followed by evaporation. An accumulative elution curve of 2 mL 50 μg/L standard is shown in Fig. 2. The result showed that the seven analytes can be completely isolated from SPE column with 4.5 mL elution reagent.

The recoveries of seven nitroimidazoles were more than 70.5% and there was no interference at the retention times of the compounds of interest. SCX cartridge for SPE is more suitable than C_{18} and silica cartridge, the reasons are listed as follows: The protonated nitroimidazoles is retained on SCX column by ionic interaction with benzenesulfonicacid group. The ionic interactions are much stronger than the non-polar interactions, allowing the use of extensive wash sequence and giving clean extracts. The absorption ability of C_{18} cartridge

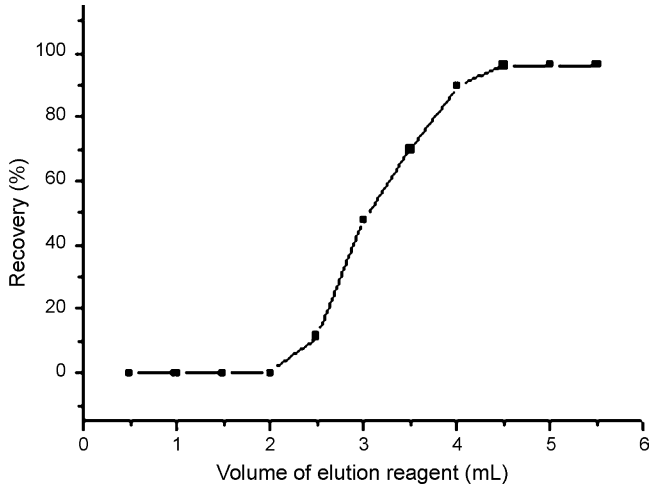

Fig. 2. The accumulative elution curve of standard solution of nitroimidazoles with ammonium hydroxide (18%)–acetonitrile (5:95, v/v) as elution reagent.

and silica cartridge to the polar compounds is weaker than SCX cartridge.

3.2. HPLC characteristics

For the HPLC separation, several analytical columns were tested, such as Cloversil ODS-U (5 μm particle size, 250 × 4.6 mm i.d.) from Clover, ZORBAX Eclipse XDB-C_{18} (5 μm particle size, 250 × 4.6 mm i.d.) from Agilent and Diamonsil^{TM} C_{18} (5 μm particle size, 250 mm × 4.6 mm i.d.) from Dikma. The last column with the gradient elution procedure gave the best chromatograms with Gaussian peaks and no interferences. In order to separate the seven analytes, a gradient elution procedure was used. A weak acid buffer was usually chosen as a mobile phase. Considering that the column working life was shortened in a long-term acid medium and the gradient elution was suitable for multi-component separation, a time tri-member gradient elution procedure was used and the mobile phase included methanol, acetonitrile and water. We discovered that the acetonitrile remarkably affected the retentions of the seven analytes. Low concentration of acetonitrile was useful for the separation of DMOHZ, RNZ and MNZ, and high concentration of acetonitrile can make the separations easy for TNZ, SNZ and DMZ, and larger volume of acetonitrile was needed to elute ONZ. The use of methanol could improve the peaks and also shorten the analysis time. So percentage of methanol in elution solution was kept at 19%, and the percentage of acetonitrile was changed in gradient elution procedure.

The maximum UV-absorption wavelengths of RNZ, DM, MNZ, TNZ, ONZ, SNZ and DMOHZ were from 310 to 340 nm. Their UV absorptions were detected at λ = 320 nm.

3.3. Validation of the method

3.3.1. Specificity

The chromatograms of seven analytes for standards, spiked blank sample and blank sample were investigated. The effective baseline separation of the seven analytes was observed for spiked chicken, pork and bacon samples.

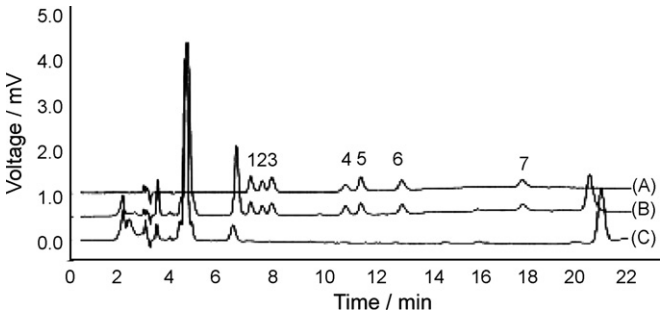

Fig. 3. Chromatograms obtained by UV detection at 320 nm for standards, spiked blank sample and blank sample. (a) Standards at 2 μg/kg for each nitroimidazole; (b) spiked blank chicken muscle at 1 μg/kg for each nitroimidazole; (c) blank chicken sample extraction. (1) DMOHZ, (2) RNZ, (3) MNZ, (4) SNZ, (5) DMZ, (6) TNZ, (7) ONZ.

The chromatograms for spiked chicken samples are shown in Fig. 3. No interfering peak was observed in the chromatogram.

3.3.2. Linearity and detection limit

Under the optimal conditions, the linearity for analysis of spiked meat samples was evaluated with the measured peak area of spiked standard for each nitroimidazole against their concentrations. The obtained linear regression equations for spiked chicken sample were as follows: $A = 143.714C - 111.915$ for DMOHZ, $A = 129.034C + 37.741$ for RNZ, $A = 185.370C - 157.847$ for MNZ, $A = 112.352C - 466.868$ for SNZ, $A = 197.441C - 731.080$ for DMZ, $A = 153.376C - 613.624$ for TNZ, and $A = 108.058C - 333.081$ for ONZ, where A is peak area of spiked standards based on three parallel measurements and C is the concentration (μg/kg) of spiked standards for each nitroimidazole. The linearity was proved to be satisfactory in the range of 0.7–60 μg/kg in meat sample with a correlation coefficient of >0.998.

The limit of detection (LOD) was determined as the sample concentration that produces a peak with a height three times the level of the baseline noise, and the limit of quantification (LOQ) was calculated as the sample concentration that produces a peak with a height 10 times the ratio of signal to noise [32,33]. The LOD value for all nitroimidazoles and metabolites was 0.2 μg/kg, and the LOQ value was 0.7 μg/kg. The obtained detection limit for this method is very lower than that for the methods reported in literature [19–25,27,28,30]. The proposed method can permit the detection of the seven nitroimidazoles in chiken and porcine tissue samples at level of ≥0.7 μg/kg.

3.4. Spiked sample analysis

Recovery and relative standard deviation (RSD) were performed in the range of 1–20 μg/kg. The results are listed in Table 2.

The recovery of the seven nitroimidazoles in chicken, pork and bacon samples spiked with 1–20 μg/kg was in the range of 72.3–99.0%, 72.2–99.5% and 71.4–99.5%, respectively. There was a variation between the recoveries for different analytes, it is because the recovery is possibly related with the structure

Table 2
Mean recovery and precision of the seven analytes assay ($n = 10$)

Analyte	Matrix	Chicken			Pork			Bacon		
	Spiked (μg/kg)	1.0	5.0	20.	1.0	5.0	20	1.0	5.0	20
DMOHZ	Recovery (%)	72.7	83.8	83.8	81.1	85.6	85.6	71.8	76.2	85.0
	RSD (%)	7.5	7.3	7.3	11.3	8.0.	7.9	8.1	6.8	4.7
RNZ	Recovery (%)	81.3	86.4	86.4	72.2	82.4	82.4	79.2	77.0	80.3
	RSD (%)	7.4	7.1	7.0	12.8	6.6	6.6	6.9	7.4	5.0
MNZ	Recovery (%)	72.3	85.2	85.2	76.8	88.6	88.6	80.9	73.2	89.4
	RSD (%)	6.2	4.0	4.0	13.9	11.1	11.1	7.5	5.7	4.7
SNZ	Recovery (%)	82.2	99.0	99.0	73.4	97.2	97.2	74.5	71.4	89.5
	RSD (%)	11.1	5.7	5.7	10.3	5. 1	5. 0	6.6	5.6	5.6
DMZ	Recovery (%)	75.8	91.2	91.2	78.6	83.4	83.4	79.8	73.0	85.1
	RSD (%)	13.8	7.0	6.9	9.5	9.1	8.7	8.7	7.9	6.8
TNZ	Recovery (%)	81.0	88.4	88.4	99.5	96.0	96.0	83.9	84.9	87.3
	RSD (%)	9.1	4.5	4.5	13.5	7.0	6.9	9.3	5.4	5.4
ONZ	Recovery (%)	98.5	94.4	94.4	87.4	94.0	94.0	89.6	94.3	99.5
	RSD (%)	13.9	5.9	5.8	11.3	9.7	8.7	9.7	8.3	6.8

of analyte and its combinative intensity with chicken, pork and bacon tissues.

From Table 2, it is indicated that the RSD of 10 measurements for chicken, pork and bacon samples spiked with 5 μg/kg of each nitroimidazole was in the range of 4.0–7.5%, 5.1–11.1% and 5.4–8.3.7%, respectively. The intra-day precision ($n = 5$) for nitroimidazoles residues in chicken sample spiked at 20 μg/kg is 6.9%, and the inter-day precision for 5 days ($n = 25$) is 11%.

4. Conclusion

The use of ethyl acetate as extraction reagent is very effective for extraction of nitroimidazoles residues. SCX SPE cartridge is more suitable for purification of the seven analytes than C_{18} and silica cartridge. The presented SPE–HPLC method has some excellencies, such as higher recovery, lower detection limit, multi-residue analysis, effective baseline separation, lesser reagent to be required. The detection limit for all the seven analytes was 0.2 μg/kg, with good intra- and inter-day precision. The proposed method can be applied for simultaneous determination of the seven nitroimidazoles residues in spiked chicken, pork and bacon samples at ≥0.7 μg/kg.

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