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Liquid chromatography–tandem mass spectrometry analysis of nitazoxanide and its major metabolites in goat

Zhanzhong Zhao, Lifang Zhang, Feiqun Xue*, Xiaoyang Wang, Wenli Zheng, Tao Zhang, Chenzhong Fei, Keyu Zhang, Minqi Qiu, Ruixiang Xin, Fengkun Yang

Department of Pharmacy, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200232, People's Republic of China

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ABSTRACT

A rapid, sensitive and specific liquid chromatography–electrospray ionization (ESI) tandem mass spectrometry (LC–MS–MS) method has been developed for the identification of nitazoxanide metabolites in goat plasma and urine. The purified samples was separated using an XTerra MS C8 column with the mobile phase consisted of acetonitrile and 10-mM ammonium acetate buffer (pH 2.5) followed a linear gradient elution, and detected by MS–MS. Identification and structural elucidation of the metabolites were performed by comparing their retention-times, full scan, product ion scan, precursor ion scan and neutral loss scan MS–MS spectra with those of the parent drug or other available standard. Four metabolites (tizoxanide, tizoxanide glucuronide, tizoxanide sulfate and hydroxylated tizoxanide sulfate) were found and identified in goat after single oral administration of 200 mg/kg dose of nitazoxanide. In addition, the possible metabolic pathway was proposed for the first time. The results proved that the established method was simple, reliable and sensitive, revealing that it could be used to rapid screen and identify the structures of active metabolites responsible for pharmacological effects of nitazoxanide and to better understand its in vivo metabolism.

1. Introduction

Nitazoxanide is a nitrothiazole benzamide compound that has a wide range of antimicrobial activity against parasites, bacterial and viral pathogens. The broad spectrum of in vivo activity is related to its desacetyl derivative, tizoxanide, and includes intracellular and extracellular protozoa, helminthes, aerobic and anaerobic bacteria, and virus [1–8]. The Food and Drug Administration has approved this drug in December 2002 for treatment of diarrhea caused by *Cryptosporidium parvum* and *Giardia lamblia*, in children 1–11 years of age [9]. Nitazoxanide is used in many areas of the world, especially in Central and South America, as a broad-spectrum parasiticidal agent in adults and children. In addition, it is approved for veterinary use (for the treatment of helminthic infections in cats and dogs) in Switzerland and France.

Although the mechanism of NTZ activity against helminthes has not yet been determined, the antiprotozoal activity of nitazoxanide is believed to be due to interference with the pyruvate–ferredoxin oxidoreductase (PFOR) enzyme dependent electron transfer reaction, which is essential for anaerobic energy metabolism of the parasites [10]. Studies of the anti-viral activity of nitazoxanide suggest that it targets cellular pathways involved in the synthesis of viral proteins [11].

The metabolism and pharmacokinetics of nitazoxanide in healthy subjects have been reported. Following oral administration of a 500 mg nitazoxanide tablet, the drug is partially absorbed from the gastrointestinal tract and rapidly hydrolyzed by esterases to pharmacologically active tizoxanide, and is further reductively metabolized to one major metabolite circulate as glucuronide conjugate, and several minor products [12]. Tizoxanide glucuronide is present in the liver and excreted in urine and bile. Tizoxanide glucuronide is the main metabolite in plasma and urine sample, and it also seems to be entirely hydrolyzed in the intestine, since only non-conjugated tizoxanide could be detected in feces. Approximately two-thirds of an oral dose passes through the intestinal tract and is excreted in feces as tizoxanide [13]. The elimination half-life of tizoxanide from plasma is approximately 1.5 h.

Metabolism of nitazoxanide has been poorly characterized in animals. In mice, rats and dogs, nitazoxanide is quickly hydrolyzed by plasma esterases into tizoxanide. Studies of radio-labelled nitazoxanide in the rat and the dog have shown that approximately two-thirds of the product is excreted in feces of rats and dogs while one-third is excreted in urine. The primary metabolite in the feces of dogs and goats is tizoxanide [14–16].

* Corresponding author. Tel.: +86 21 54084317; fax: +86 21 54081818.
E-mail address: feiqun@gmail.com (F. Xue).

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Drug metabolism experiment has played an important role in the drug discovery, drug design and drug clinical application [17]. Therefore, fast and efficient ways to provide accurate information of drug metabolism on the target compounds and their major metabolites are required [18]. The analysis of metabolites is a challenging task and several different analytical methods have been used in these studies. However, after the introduction of the electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces, liquid chromatography–tandem mass spectrometry (LC–MS–MS) has become a very powerful tool for the identification and structure characterization of drug metabolites owing to its superior specificity and efficiency [19–22]. Because ESI is very soft ionization technique, it is the most suitable for labile conjugates, compared to APCI. The scan modes of product ion scan, selected reactions monitoring, neutral loss scan and precursor ion scan of MS–MS enable rapid acquisition of useful structural information of metabolites [23]. In this study, LC–ESI–MS–MS technique was applied. The superb sensitivity of ESI in combination with tandem mass spectrometry provides information for comprehensive structural characterization of the metabolites of nitazoxanide within a short time.

In the present study, a highly sensitive and specific LC–ESI–MS–MS method was adopted to identify and elucidate the structures of 4 metabolites in goat plasma and urine on the basis of the mass spectra after oral administration of nitazoxanide. Meanwhile, the possible metabolic pathway after oral dosing of nitazoxanide was proposed. This investigation provided a basis from a metabolic point of view for the clarification of action mechanism of nitazoxanide.

2. Materials and methods

2.1. Reagents and chemicals

Nitazoxanide (>98.5%) and Tizoxanide (>99.9%) were synthesized and characterized by NMR, LC–MS and HPLC-UV. Acetonitrile was of high performance liquid chromatography (HPLC) grade (Fisher, Fairl Lawn, NJ, USA). β-Glucuronidase and sulfatase (from *Helix pomatia*), D-saccharic acid 1,4-lactone, and ammonium acetate were purchased from Sigma–Aldrich (St. Louis, USA). The water was purified using a Milli-Q-water purification system (Millipore, Bedford, MA, USA). All other reagents were of HPLC or analytic grade and used without further purification.

2.2. Apparatus

LC–MS–MS experiments were performed on an Waters Alliance 2695 HPLC systems (Waters, Milford, MA, USA) and a Quattro micro API quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface (ESI). The software Masslynx 4.0 (Waters, Milford, MA, USA) was applied for system operation and data collection. Goat plasma and urine samples were extracted on an Waters OASIS™ Solid Phase Extraction Cartridge (60 mg/3 cm^3, Waters, Milford, MA, USA). A high-speed desktop centrifuge (TGL-16G, Shanghai Fulgor Analytical Instruments, Shanghai, China) was used to centrifuge plasma and urine samples.

2.3. Standard samples and mobile phase preparations

Stock solution of nitazoxanide (200 μg/ml) was prepared by dissolving this compound in acetonitrile to yield the target concentration at the time of assay. Tizoxanide stock solution was prepared in acetonitrile with appropriate *N*,*N*-dimethylformamide (DMF) at a concentration of 100 μg/ml. All stock solutions were stored at −76 °C in darkness.

Table 1
The LC gradient program

Time (min)	Flow (ml/min)	Buffer (%)	Acetonitrile (%)
0	0.20	70	30
5	0.20	60	40
15	0.20	30	70

Nitazoxanide and tizoxanide working solutions were prepared by diluting the stock solutions to the desired concentrations with acetonitrile, respectively. All working solutions were kept at −76 °C in darkness until analysis.

The mobile phase (Table 1) was consisted of acetonitrile, and 10 mM ammonium acetate, which were prepared by dissolving ammonium acetate in water and adjusted to the desired pH (2.5) with formic acid (0.5%, v/v).

2.4. Animal and administration

Six healthy cross-breed male goats weighing 25–32 kg, were obtained from commercial farms (Songjiang, Shanghai, China). Following arrival, the goats were kept in our animal facilities for acclimatization for 7 days, during which they had free access to antiparasitic free commercial feed (Guangming Milk Inc., Shanghai, China) and water ad libitum. Before administered 200 mg/kg oral gavage doses of nitazoxanide, the goats were fasted for 24 h but with access to water. After administration, the goats were placed in individual metabolic cages (Suzhou Animal Experimental Apparatus Factory, Suzhou, China) provided with urine–feces separators. All animal work was approved by the Animal Care and Use Committee of the Shanghai Veterinary Research Institute, China Academy of Agricultural Sciences, Shanghai.

2.5. Collection of blood and urinary samples

Blood samples (3–5 ml) were collected by jugular venipuncture into heparinized glass tubes prior to and at 5, 15, 30 min, and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24, 48, 72 and 96 h after drug administration. Blood samples were centrifuged at 10,000 rpm for 5 min to harvest plasma, which were stored at −76 °C until analysis.

Urinary samples were collected over ice for 24 h prior to dosing and for the periods 0–12, 12–24, 24–48, 48–72 and 72–96 h post-administration. Urinary volume and pH were recorded on removal of the sample from the collection vessel and the sample was then centrifuged at 4000 rpm for 10 min. The supernatants were subsequently lyophilized to dryness with a Flexi-Dry™ Freeze-dryer (Stone Ridge, New York, USA), the residues were frozen at −76 °C until analysis.

2.6. Sample preparations

2.6.1. Plasma sample preparation

Free tizoxanide were extracted by acetonitrile (450 μl) with anhydrous sodium sulfate (solid, 0.17 g) from 200 μl plasma. A rotating mixer was used for approximately 2 min to facilitate the extraction. After centrifugation at 12,000 rpm for 10 min, the supernatant was loaded onto an Waters OASIS™ Solid Phase Extraction Cartridge (Waters, Milford, MA, USA), which had been pre-conditioned with 5 ml of methanol, followed by 5 ml of H_2O. After washing the Extraction Cartridge with 2 ml of water, it was dried for 3 min by applying vacuum. The analytes were then eluted (1 drop/s) with 1.25 ml of methanol. The elution solutions were filtered through 0.45-μm film filter (Millipore, Bedford,

MA, USA) and an aliquot of 10 μl was used for LC–MS–MS analysis.

After optimizing the acidity, temperature, enzymatic content and the time of hydrolysis, total tizoxanide were determined by transferring 200 μl of plasma sample into a 1.5-ml eppendorf centrifuge tube with 20-μl acetic acid–sodium acetate buffer (pH 4.3) and treated with 4 μl β-glucuronidase (type HP-3, 101,092 units/ml) at 37 °C for 16 h to hydrolyze glucuronide conjugate of tizoxanide. Acetonitrile (426 μl) was added to stop the reactions and the enzymatic hydrolysis solution was handled followed the same procedures just as the description above.

2.6.2. Urinary samples preparation

The residue (from 10-ml urinary sample) was dissolved in 15 ml acetonitrile and 5 g anhydrous sodium sulfate was added, followed by vortex mix. The mixture was centrifuged for 10 min at 12,000 rpm and the supernatant was used for free extraction and enzymatic hydrolysis.

An aliquot of 1 ml of the supernatant was used for SPE extraction just like the procedure mentioned above. The elution solutions were filtered through 0.45 μm film filter (Millipore, Bedford, MA, USA) and an aliquot of 10 μl was used for LC–MS–MS analysis.

After optimizing the acidity, temperature, enzymatic content and the time of hydrolysis, 1 ml of the supernatant was adjusted to pH 5.0 with a few drops of glacial acetic acid. Then, 0.5 ml of acetate buffer (pH 4.3) was added to the solution prior to enzymatic hydrolysis. β-Glucuronidase hydrolysis was carried out at 37 °C for 12 h. For sulfatase (type HP-2, ≥2000 units/ml) hydrolysis, D-saccharic acid 1, 4-lactone was added in order to inhibit β-glucuronidase activity and the reaction was carried out at 37 °C for 2 h. Acetonitrile was added to stop the reactions and the enzymatic hydrolysis solution was extracted by SPE cartridge immediately, just like the procedure mentioned above.

Free fraction was used for the comprehensive LC–MS–MS analysis of nitazoxanide and its metabolites. The target solutions after enzymatic hydrolysis were only used to assist in the investigation of phase II metabolites.

2.7. Chromatographic conditions

An XTerra MS C8 HPLC column (2.1 mm × 150 mm, 3.5 μm) equipped with a guard column (XTerra C8 2.1 mm × 10 mm, 3.5 μm) (Waters, Milford, MA, USA) was used to separate nitazoxanide and its metabolites in goat urine. The temperature of the column was set at 30 °C. The mobile phase consisted of acetonitrile and 10 mM ammonium acetate buffer (adjusted to pH 2.5 with formic acid) run with a gradient program (Table 1). The flow rate was 0.2 ml/min during the whole run.

2.8. Mass spectrometry conditions

The MS parameters were optimized for sensitivity manually during direct infusion of a solution of tizoxanide standard. Nitrogen was used for desolvation gas (400 l/h) and nebulising gas (50 l/h). For collision induced dissociation (CID) fragmentation, ultra high purity argon was used as the target gas at a pressure of 5×10^{-4} mbar and collision energy was set at 20 or 30 eV depending on compounds of interest. The MS analysis were performed under automatic gain control conditions, using a typical capillary voltage of 4.5 kV, cone voltage of 30 V, and source temperature of 100 °C. The other ESI parameters, including the voltages of extractor and RF, the rates of cone gas flow and desolvation gas, and desolvation temperature were also optimized for maximum abundance of the ions of interest by the automatic tune procedure of the instrument.

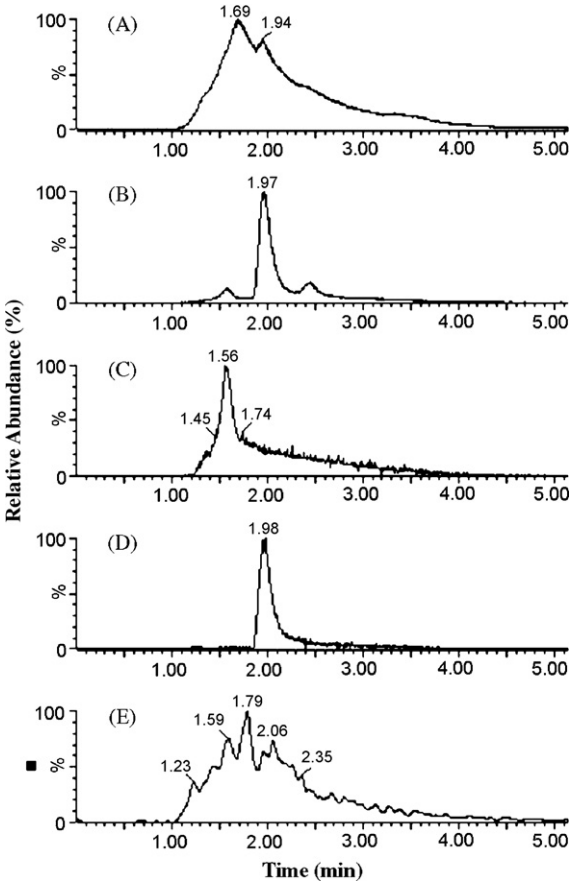

Fig. 1. Total ion chromatogram (TIC) (A) and extracted ion chromatograms (XIC) (m/z = 264, 440, 344 and 360; Rt = 1.97 ± 0.02, 1.56 ± 0.02, 1.98 ± 0.02 and 1.79 ± 0.02) (B, C, D and E) of the extract of medicated urinary sample.

The screening and identification of metabolites by mass spectrometry was based on full-scan, multiple reaction monitoring mode (MRM), neutral loss scan, precursor ion scan, and product ion scan. Full scan was performed over the m/z range 100–500 Da. Neutral loss scan of 80 and 176 Da experiments were done by scanning within the m/z range from 200 to 500 Da. Precursor ion scan experiments were carried out by scanning the characteristic fragment as the product ion between 100 and 500 Da. Product ion scan were obtained by choosing the molecular ion as the precursor ion and scanning MS–MS from m/z 100–500 Da.

The instruments control, data acquisition and data processing by a PC using the software Masslynx 4.0 (Waters, Milford, MA, USA).

3. Results and discussion

3.1. General observation

Clinical examination of all the goats before and after each administration did not reveal any abnormalities. No local or systemic adverse reactions occurred after oral administration of nitazoxanide.

3.2. LC–MS and LC–MS–MS analysis of metabolites

In order to characterize the metabolic profiles of nitazoxanide, the possible structures of metabolites were speculated according to the rule of drug metabolism and the structure of parent drug firstly. Then, the full scan mass spectrum of extraction of

goat biological samples after administration of nitazoxanide was compared with those of blank goat biological samples (data not shown) and nitazoxanide or tizoxanide solution to find out the possible metabolites. Then, these compounds were analyzed by LC–MS–MS. The versatility of MS allows various selective screening strategies to be employed, i.e., full scan mode, neutral loss, precursor ion and product ion scan modes. This allows the identification of possible nitazoxanide metabolites. Spectra taken at the apex of the peaks which appeared in the total ion chromatogram (TIC) revealed the quasi-molecular ions of the parent drug and its metabolites. Structural identification of these compounds can then be achieved by applying product ion, precursor ion and neutral loss scan modes using the identified quasi-molecular ions as parent ion. First, product ion spectra of nitazoxanide and its metabolites were collected at different collision energy. Next, characteristic neutral losses and product ions were selected to set up the constant neutral loss and precursor ion scan modes which were subsequently applied to the various types of biological samples.

Based on the method mentioned above, four metabolites (M1–4) were found in goat urine after administration of nitazoxanide. Their molecular ions $[M-H]^-$ were at m/z 264 (similar to the molecular ion of tizoxanide), 440, 344, 360 (Fig. 1), respectively. The product ions at m/z 114, 144, 190 and 217 corresponding to the characteristic fragment ions of tizoxanide, appeared in the MS–MS spectrum of the molecular ion at m/z 264 (M1, Fig. 2A). Taken together, M1 could be identified as tizoxanide.

MS–MS neutral loss scan analysis enabled determination of glucuronide and sulfate conjugates by monitoring loss of 176 and 80 Da [24], respectively, and facilitated the characterization of metabolic profile of nitazoxanide in goats. Tizoxanide glucuronides and sulfates were sequentially analyzed in neutral loss scan mode using the following program: (1) neutral loss scan of 176 Da for detection of glucuronide conjugates at collision energy of 30 eV; (2) neutral loss scan of 80 Da for screening of sulfate conjugates at 15 eV. Scan events (1) and (2) were set for screening of tizoxanide conjugates. The scan time was set at 0.7 s, and scan events were repeated while the program was running. Under negative ion mode, the glucosidic bond of *O*-glucuronides is easily cleaved in the collision cell to generate product ions of $[M-H-176]^-$, which correspond to the fragments resulted from the neutral loss scan of a gluconic acid from the deprotonated molecule. This opens the possibility that the unknown *O*-glucuronides within a certain m/z range could be sought out from a biological sample by employing a neutral loss scan for the m/z loss of 176 over that range. The neutral loss scan spectrum for 176 of the extract of urinary sample after oral administration of nitazoxanide was shown in Fig. 3B. It could be found that the notable peak at m/z 440 was found within the m/z 100–500 (there was no apparent peak observed after m/z 500). The difference in molecular mass between tizoxanide and the molecular ion at m/z 440, termed M2, i.e. 176, suggested the presence of a glucuronide of tizoxanide. This was further confirmed by LC–MS–MS analysis. The MS–MS spectrum of m/z 440 (M2, Fig. 2B) gave three product ions at m/z 264, 217, 190, and 144 which were the characteristic fragment ions of tizoxanide. So, M2 should be the glucuronide conjugate of tizoxanide conjugated with the hydroxyl group.

The MS–MS spectrum of m/z 344 (M3) (Fig. 3A) gave abundant daughter ion at m/z 264, which was produced by neutral loss of 80 Da, Besides, there was the molecular ion at m/z 344 in the spectrum of the neutral loss scan for 80 of the urine samples, which gave three daughter ions at m/z 264, 217 and 144 in its MS–MS spectrum (Fig. 2C), which were the characteristic fragment ions of tizoxanide. Based on these data, M3 was identified as the sulfate conjugate of tizoxanide. Because phenolic hydroxyl has stronger affinity and higher speed in the sulfate esterifying reactions according to the rule of drug metabolism [25], M3 should be the sulfate conjugate of tizoxanide esterified at its phenolic hydroxyl position.

Precursor ion scan is a very helpful tool to elucidate metabolism of drugs. Typical product ions of nitazoxanide and its metabolite were identified by measurement of reference substances. Using ion m/z 114 as product ion, Three ions m/z 264, 360 and 440 were discovered in urinary sample of goats (Fig. 4). The molecular ion at m/z 360, called M4, was increased by 16 Da compared to that of the M3. So, M4 might be the sulfoconjugate of tizoxanide hydroxylated at benzene ring, although the position of the hydroxy group remains undetermined.

These metabolites can be investigated further by comparing two different extraction procedures. Compared with free fraction, the peak areas of LC–MS–MS chromatograms of M1 increased markedly, and those of M2, M3 and M4 decreased after enzymatic hydrolysis. These results revealed that the deacetyl metabolite of nitazoxanide, tizoxanide (M1), excreted from goat urine as the sulfate conjugated or glucuronide conjugated forms.

The time of excretion of nitazoxanide metabolites in goat urine were detected by determining urine samples collected at different time-points. M3 and M4 were found in 0–12 h urine. M2 could be detected for up 72 h using the tandem MS technique, and M1 could be detected for about 24–48 h.

Similarly, metabolite M1 and M2 were detected in goat plasma after oral administration of nitazoxanide (Fig. 5). The negative electrospray mass spectrum of metabolite M1 showed a $[M-H]^-$ ion at m/z 264 (Fig. 6A). The MS–MS spectrum of m/z 264 (Fig. 7A) generated a series of fragment ions at 114, 144, 190 and 217 corresponded to the characteristic fragment ions of tizoxanide. Therefore, M1 could be confirmed as tizoxanide.

The characteristic product ions at m/z 114, 144, 190, 217 and 264 appeared in the MS–MS spectrum of the molecular ion at m/z 440 (M2) (Figs. 6B and 7B) which was increased by 176 Da compared to that of tizoxanide. We presumed that M2 was the glucuronide conjugate of tizoxanide. This was further confirmed by electrospray LC–ESI–MS–MS using neutral loss scan. The neutral loss scan spectrum for 176 of the extract of plasma sample after oral administration of nitazoxanide was shown in Fig. 8. It could be found that the notable peak at m/z 440 was found within the m/z 100–500 (there was no apparent peak observed after m/z 500). So, M2 should be the glucuronide conjugate of tizoxanide conjugated with the hydroxyl group.

In medicated feces, the parent drug (M0) and metabolite M1 were found after administration of nitazoxanide to goat [16]. In addition, tizoxanide could be detected for up 72–96 h using the tandem MS technique, and the parent drug disappeared in 72 h feces after oral administration of nitazoxanide to goat.

3.3. Metabolic profiles of nitazoxanide in goats

The parent drug (M0), tizoxanide (M1), tizoxanide glucuronide (M2), tizoxanide sulfate (M3) and hydroxylated tizoxanide sulfate (M4) were found in goat biological samples after oral administration of nitazoxanide. Based on the above discussion, the proposed major metabolic pathway of nitazoxanide in goats was shown in Fig. 9. When orally administered, nitazoxanide disappeared rapidly from plasma, and at the same time large numbers of its sulfated and glucuronidated metabolites were observed in goat urine besides tizoxanide. It suggested that quick urinary excretion and extensive metabolism of nitazoxanide, including sulfation and glucuronidation, were two factors causing its rapid elimination from the circulation. The information gained from this study could be very useful for the further pharmacokinetic studies of nitazoxanide and its metabolites.

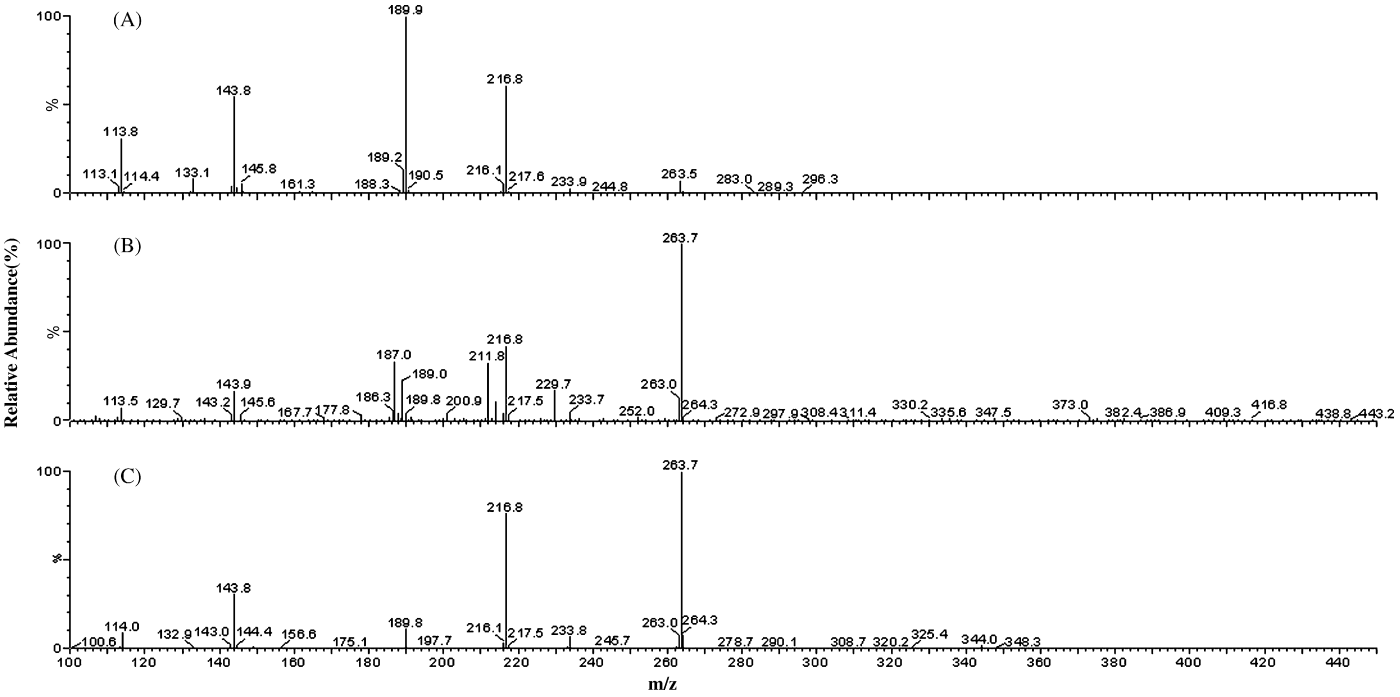

Fig. 2. LC–MS–MS product ion spectrum of M1 (m/z 264, Rt = 1.97 ± 0.02) (A), M2 (m/z 440, Rt = 1.56 ± 0.02) (B) and M3 (m/z 344, Rt = 1.98 ± 0.02) (C).

In plasma, two circulating metabolites of nitazoxanide were identified. Nitazoxanide is first hydrolyzed in plasma to form its active circulating metabolite, tizoxanide, and this metabolite is further glucuronidated to a metabolite called tizoxanide glucuronide. It is worth noting that no trace of nitazoxanide was detected in the plasma of goats that had ingested nitazoxanide. This result supported the well establishment that nitazoxanide is rapidly hydrolyzed by plasma esterases into its desacetyl metabolite, tizoxanide, which was present in goat plasma mainly in conjugated form. This confirms previous studies by Broekhuysen et al. in man. After oral administration of nitazoxanide to man tizoxanide was mainly present as glucuronide in plasma [13]. These two metabolites have shown to have similar pharmacodynamics properties as the parent drug [26] and, therefore, it has been essential to study the mechanism and factors which might contribute to the formation of these metabolites.

No parent drug was detected in urine. It was therefore concluded that metabolism of nitazoxanide was required for urinary elimina-

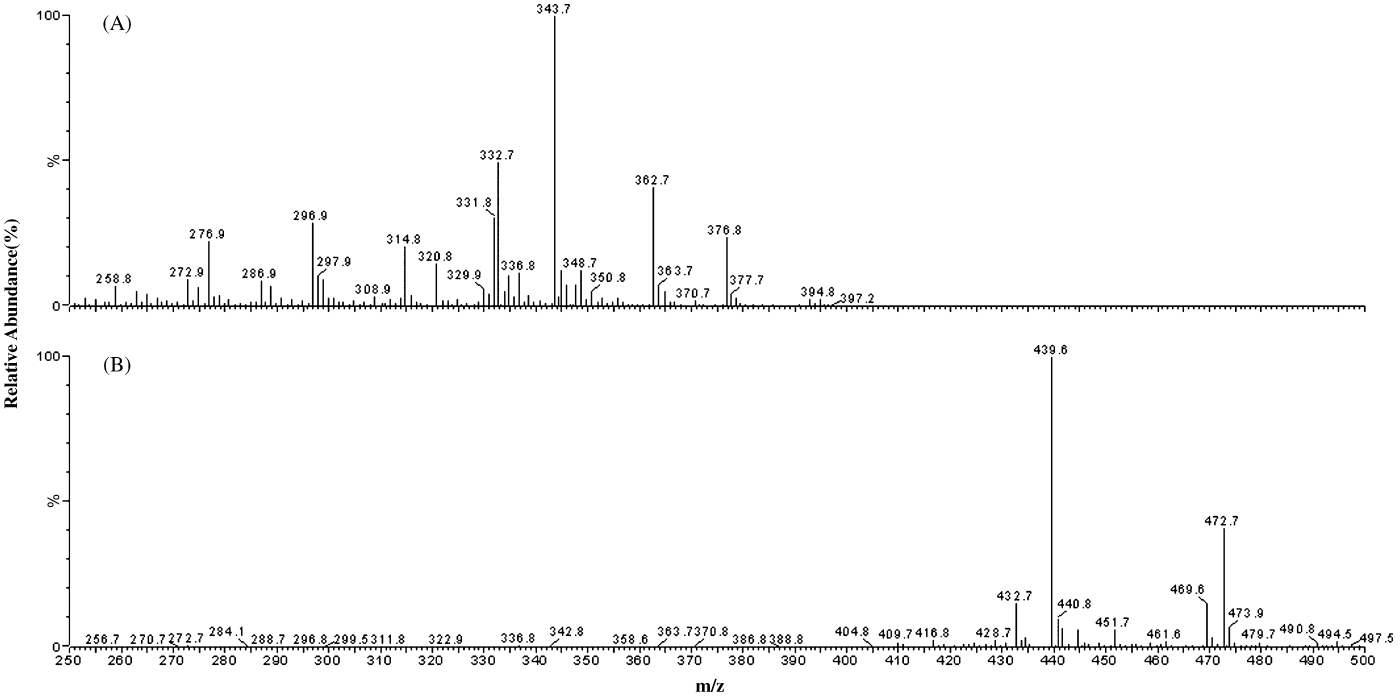

Fig. 3. LC–MS–MS neutral loss spectra for 80 (Rt = 1.98 ± 0.02) (A) and 176 (Rt = 1.56 ± 0.02) (B) of the extract of medicated urinary sample.

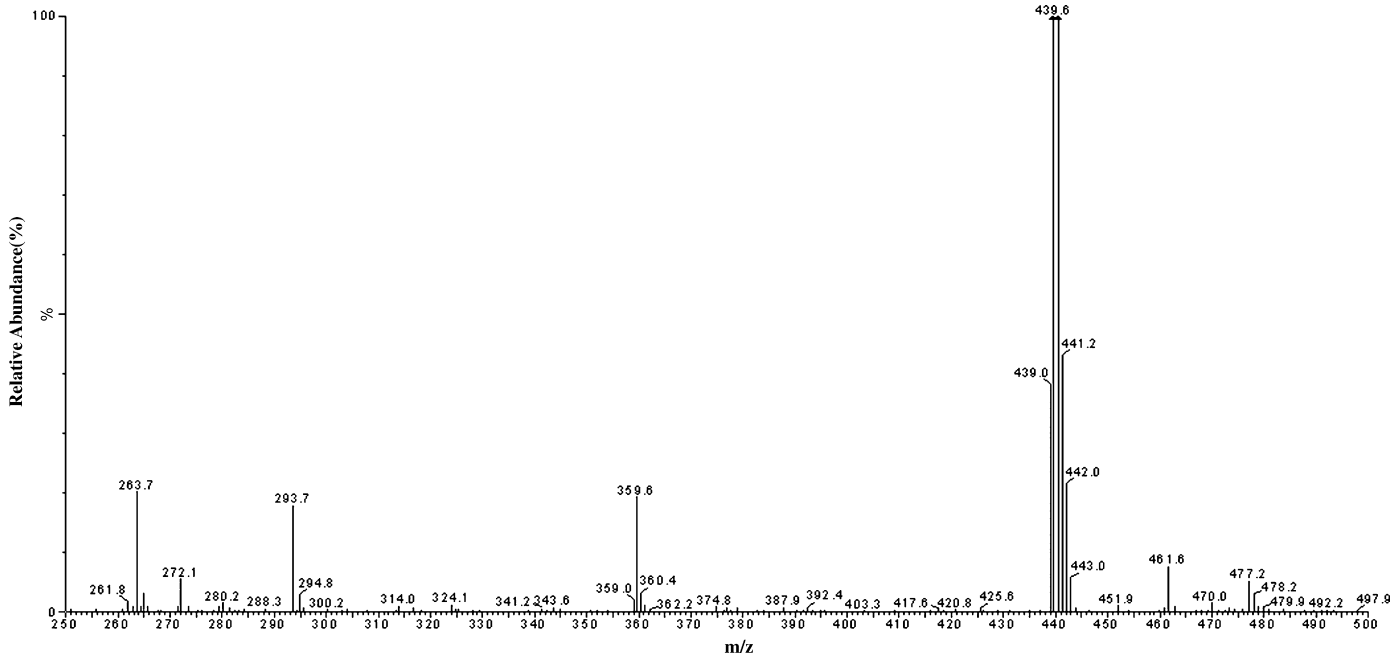

Fig. 4. LC–MS–MS precursor ion spectrum for *m*/*z* 114 (Rt = 1.97 ± 0.02, 1.79 ± 0.02 and 1.56 ± 0.02) of the extract of medicated urinary sample.

tion, and that biotransformation of nitazoxanide in the goat plays the major role in regulating the rate of excretion of this compound.

Unfortunately, it has not been possible to obtain the glucuronide conjugate of tizoxanide, the sulfate conjugate of tizoxanide and hydroxylated tizoxanide for comparison with M2, M3 and M4. These findings suggested that the metabolism of tizoxanide resulting in the formation of sulfate conjugates follows two processes: tizoxanide → sulfate conjugation and tizoxanide → mono-hydroxylation → sulfate conjugation. Although the sulfate conjugate of tizoxanide and hydroxylated tizoxanide might have been detected in the urine, it appears that the glucuronide conjugate of tizoxanide is the main metabolite in plasma and urine. This metabolite seems to be cleared more slowly from the plasma than the free form, perhaps because of its poor solubility [13]. Although, the pharmacological activity of M3 and M4 has not yet been studied, these metabolites may help in understanding the mechanism

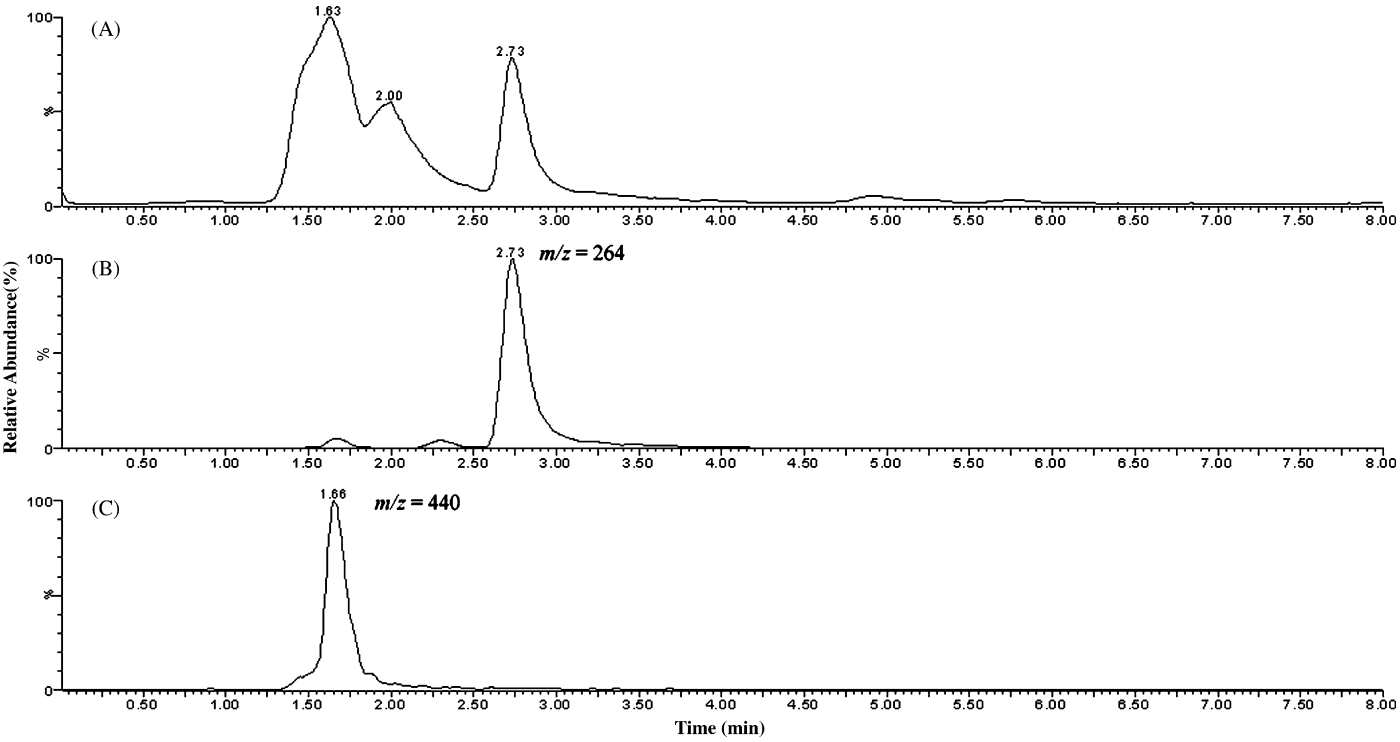

Fig. 5. Total ion chromatogram (TIC) (A) and extracted ion chromatograms (XIC) (*m*/*z* 264, 440; Rt = 2, 73 ± 0.02, 1.66 ± 0.02) (B and C) of the extract of medicated plasma sample.

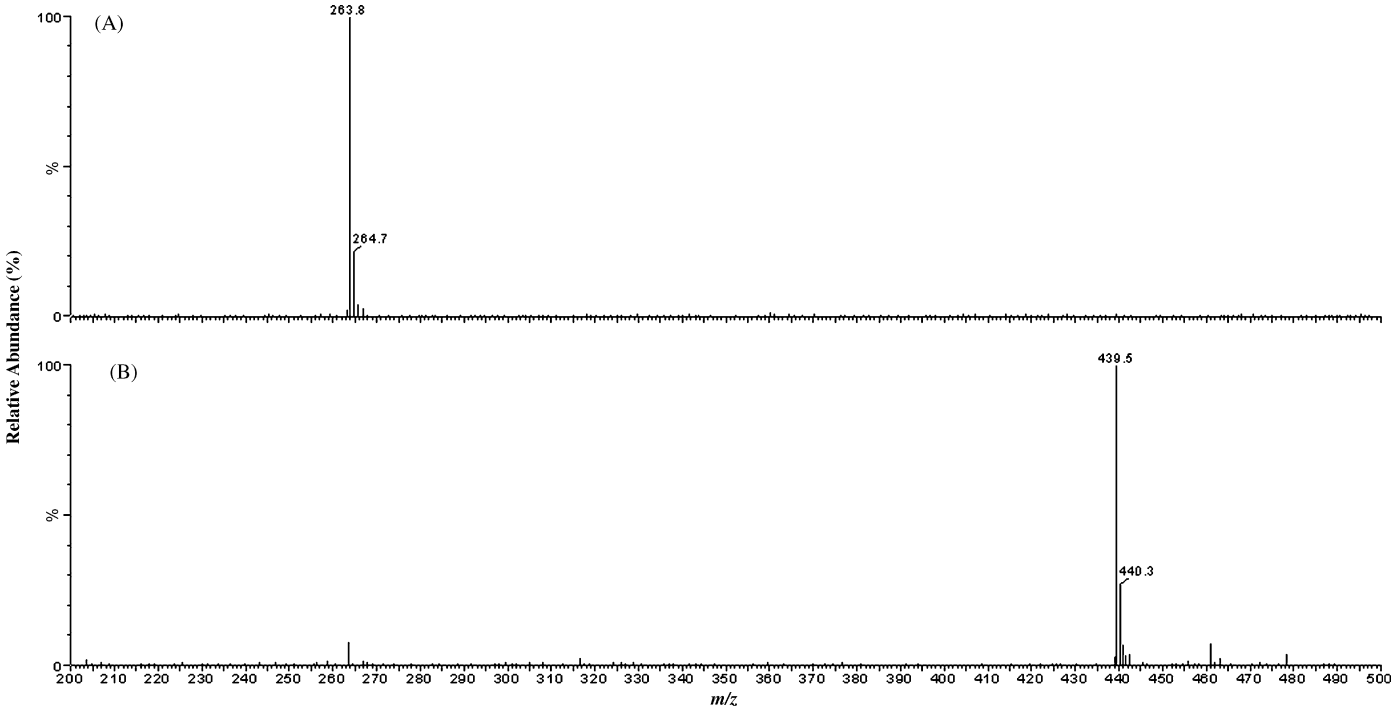

Fig. 6. LC–MS–MS precursor ion spectrum for m/z 114 (m/z 264 and 440; Rt = 2.73 ± 0.02 and 1.66 ± 0.02) (A and B) of the extract of medicated plasma sample.

of these actions and the therapeutic effects of nitazoxanide because these metabolites at least stay in body over 12 h after administration.

The amount of the tizoxanide glucuronide is clearly higher than those of tizoxanide sulfate. This may be due to the fact that the formation of the sulfate conjugate, which is a competitive reaction to the formation of the glucuronide, is limited by the amount of available endogenous sulfur. The rapid elimination of tizoxanide from the plasma is affirmed by the urine concentrations. While tizoxanide is excreted in low amounts, the metabolites, especially the glucuronide is excreted in high amounts with a maximum already at 12 h. This was expected due to the fact that the conjugation reaction of the biotransformation pathway often is a measure to increase the solubility of xenobiotics to promote their elimination by excretion via the urine [27].

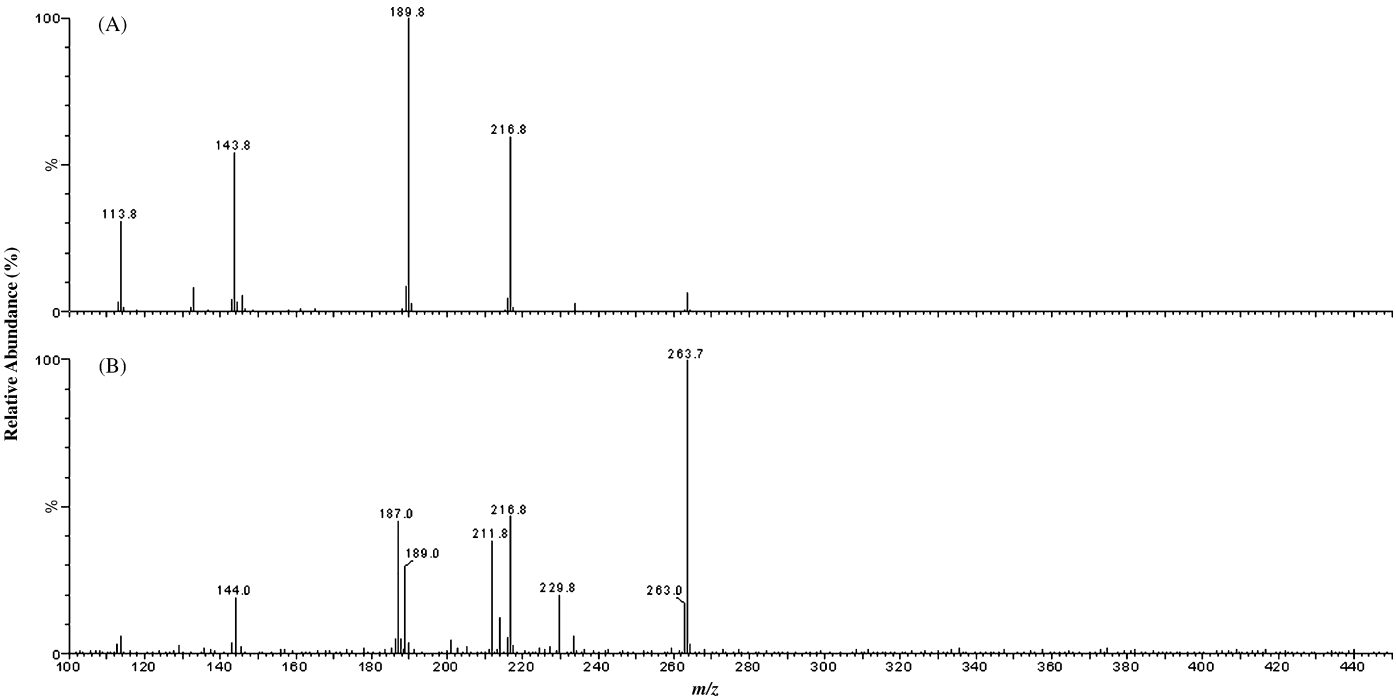

Fig. 7. LC–MS–MS product ion spectrum of (A) M1 (m/z 264, Rt = 2.73 ± 0.02) and (B) M2 (m/z 440, Rt = 1.66 ± 0.02).

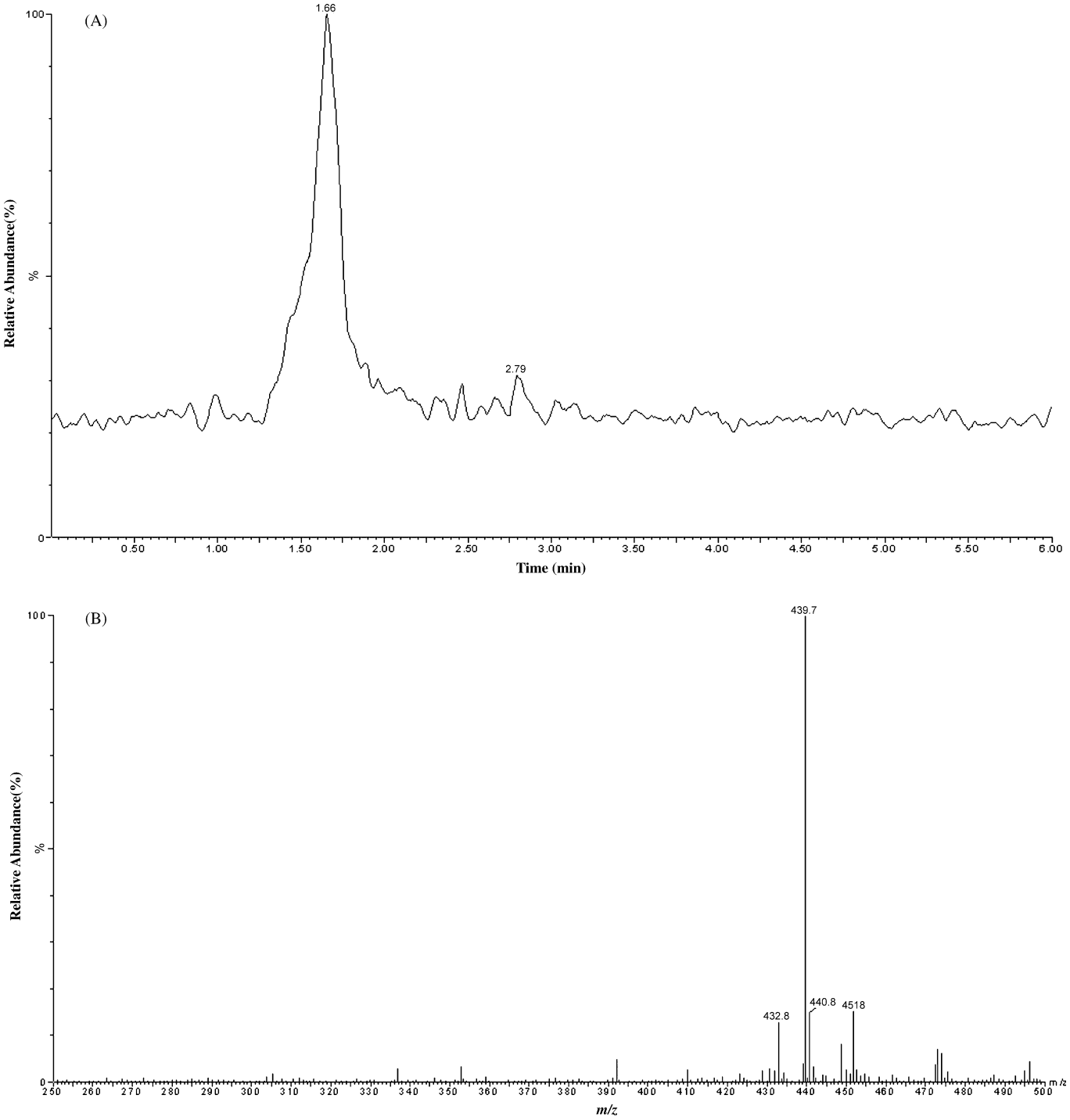

Fig. 8. Neutral loss scan for 176 of the extract of medicated plasma. (A) Chromatographic spectrum; (B) MS–MS spectrum (m/z 440, Rt = 1.66 ± 0.02).

The tizoxanide conjugated products identified in the urine were a mixture of glucuronide ether and sulfate ester conjugates. In contrast, no glucuronide ester of hydroxy tizoxanide (Fig. 9) was identified in the excreta, only the sulfate ether conjugates. These results can be explained on the basis that hydroxy tizoxanide may serve as a less suitable substrate than tizoxanide for UDP-glucuronosyl transferases than for arylsulfotransferases due to steric hindrance from the two hydroxyl groups to the carbonyl group. Additionally, glucuronosyl transferases are microsomal enzymes, as are the cytochrome P450s, which may allow products from Phase I oxidations to efficiently become substrates for Phase II glucuronidation. Arylsulfotransferases, on the other hand, are cytosolic. HPLC chromatographic property of tizoxanide and hydroxy tizoxanide revealed that hydroxy tizoxanide is the more polar molecule. Based on these polarity considerations, hydroxy tizoxanide may preferentially associate with the cytosolic fraction where arylsulfotransferases are located, while tizoxanide may partition between the membrane and cytosolic compartments, thereby being subject to both conjugating enzymes.

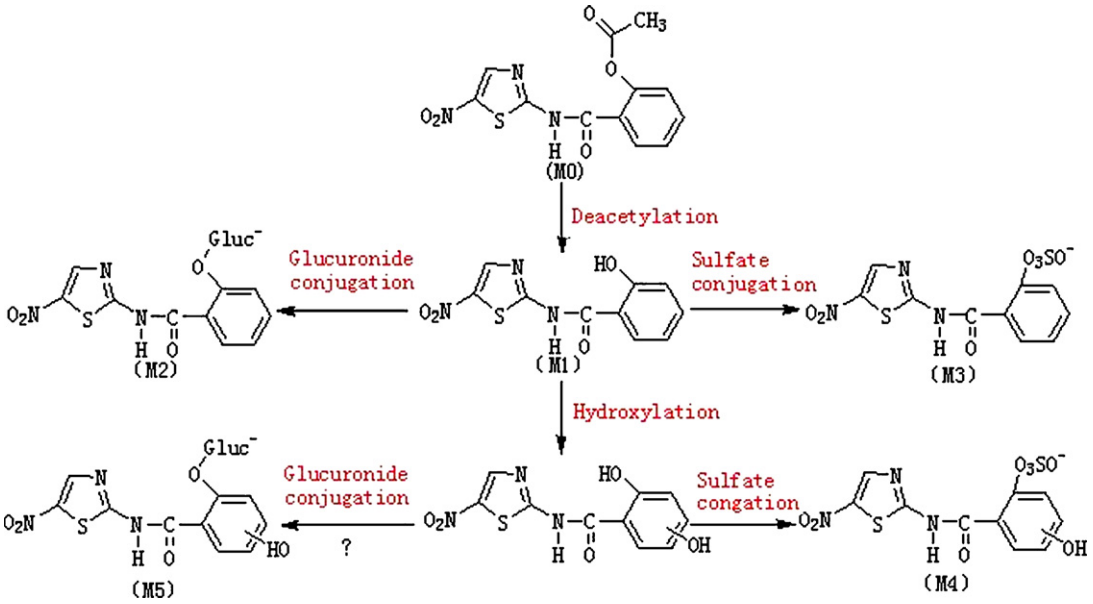

Fig. 9. The proposed major metabolic pathway of nitazoxanide in goat.

Finally, it is known that enzyme kinetics govern the competition of glucuronidation and sulfation of phenolic compounds. Glucuronosyl transferases possess a higher capacity (k_m) for phenolic conjugation than arylsulfotransferases [28], but the opposite is true for the affinity of each enzyme for the phenol substrate [29]. These findings would suggest that a substrate undergoing a slow rate of Phase I hydroxylation would be preferentially sulfated, while for a rapidly metabolized substrate, glucuronidation would be preferred.

No other metabolite could be detected in the urine of goats treated with nitazoxanide in the present study. However, the previous study of Stockis et al., which demonstrated that very small amount of aminonitrothiazole was found in the urine [14]. This difference might be explained by the following facts: the amide bond linking the salicyl moiety of tizoxanide to the thiazole ring seems largely resistant to enzymatic cleavage, or there are different metabolic profiles of nitazoxanide in goat and human, or some other factors such as different analytical methods.

No phase II metabolites were found in feces, and tizoxanide is the predominant fecal metabolite in vivo by goat. Tizoxanide is mainly eliminated in the feces in all species regardless of the route of administration, and fecal excretion accounts for 2/3 of the dose administered with 1/3 of the dose excreted in urine [15]. Although xenobiotic metabolism occurs mainly in the liver, extrahepatic metabolism including gastrointestinal tract are also involved [30]. The reductive environment of the gastrointestinal tract of the ruminants could contribute to the metabolic reduction of the tizoxanide glucuronide. Tizoxanide was mostly excreted from bile as conjugated metabolites and readily hydrolyzed by the intestinal microflora to free tizoxanide. Therefore, tizoxanide had the potential to undergo enterohepatic circulation in the metabolic disposition of tizoxanide. The enterohepatic circulation of tizoxanide may comprise the sequential processes of hepatic uptake from blood, excretion from the liver into the bile, transport of the bile to the duodenum, reabsorption from the intestine, and returning to the liver via the portal circulation. The enterohepatic circulation contributes to the systemic exposures of tizoxanide and its conjugated metabolites in goats. Whether or not enterohepatic circulation of tizoxanide and its conjugated metabolites contribute to the overall pharmacological activity remains to be determined.

3.4. Conclusions

The results of this study have provided, for the first time, detailed metabolic fate of nitazoxanide in goat after oral administration. The superb sensitivity of ESI in combination with tandem mass spectrometry provides information for comprehensive structural characterization of the metabolites of nitazoxanide in a short period of time. The deacetyl product of nitazoxanide, tizoxanide (M1) and its *O*-aryl glucuronide, tizoxanide glucuronide (M2) were the major metabolites of the parent drug (M0) in plasma. The major metabolite in the urine was tizoxanide glucuronide (M2). The absence of unchanged parent drug in urine indicated that nitazoxanide underwent extensive metabolism. In feces, tizoxanide (M1), in a high amount, and the parent drug (M0) were found. So, incomplete absorption of the drug could be assumed. In addition, a metabolic pathway for nitazoxanide in goat was postulated. Nitazoxanide is rapidly hydrolyzed to tizoxanide, which is subsequently conjugated to form *O*-glucuronide and *O*-sulfate metabolites (M2 and M3) respectively, or first hydroxylated at benzene and followed by forming *O*-sulfate metabolite (M4). Tizoxanide (M1) and tizoxanide glucuronide (M2) were two important circulating metabolites in plasma and should be the preferred target for the pharmacokinetics of nitazoxanide administration in goats. Further detailed investigation is required to carry out pharmacokinetic analysis of plasma metabolites and clarify the pharmacological activity of these metabolites, in order to allow better and more relevant studies of the bioactivity and role of nitazoxanide in disease therapy. These studies are now in progress.

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