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Identification of *in vitro* metabolites of a new anticoccidial drug nitromezuril using HepG2 cells, rat S9 and primary hepatocytes by liquid chromatography/tandem mass spectrometry

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RATIONALE: Nitromezuril is a novel triazine compound possessing remarkable anticoccidial activity that could have possible future use in the prevention of coccidiosis; however, its metabolic characteristics have still not been revealed.
METHODS: In the present study, the *in vitro* metabolism of nitromezuril in HepG2 cells, rat S9 and primary hepatocytes was investigated using high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. The structures of metabolites and their product ions were easily and reliably characterized based on the accurate MS^2 spectra and known structure of nitromezuril.
RESULTS: As expected, three metabolites (M1–M3) were detected in a HepG2 cells system, one metabolite was respectively detected and identified as M1 in rat S9 and M2 in rat primary hepatocytes. M1 and M2 were confirmed respectively based on comparing their retention times, full scan, product ion scan with available authentic standards and M3 was tentatively identified as hydroxyl compound of M2.
CONCLUSIONS: Pathways of nitromezuril were reported for the first time and no obvious species difference was shown. The proposed metabolic pathways of nitromezuril can be expected to play a key role in pharmacodynamics and food safety evaluations. Copyright © 2014 John Wiley & Sons, Ltd.

Coccidiosis is one of the most important diseases affecting the poultry industry. The estimated total annual cost derived directly or indirectly from diseases caused by *Eimeria* species in raising fowl reaches up to US $800 millions.[1] Prophylactic anticoccidial drugs as feed additives are widely used to control coccidiosis.[2] However, the development of resistance to most drugs has prompted a constant search for products that could replace drugs against which organisms have acquired resistance.[3] Since the 1980s, triazine coccidiostats, including diclazuril and toltrazuril, have been widely used in chickens and turkeys for their remarkable clinical effects on the members of the genus *Eimeria* but have generated resistance problems in recent years. The structure of 2-(3-methyl-4-(4-nitrophenoxy)phenyl)-1,2,4-triazine-3,5(2*H*,4*H*)-dione, also known as nitromezuril (CAS:1352755-63-5, Scheme 1), is similar to that of diclazuril and toltrazuril. It is a novel anticoccidial triazine compound developed using systematic structure–activity relationship studies by the Shanghai Veterinary Research Institute of the Chinese Academy of Agricultural Sciences in recent years.[4] The chemical synthesis of this compound has been published in China Patent No. CN 102285930. Relevant preclinical research indicated good safety and high effect of anticoccidiosis, anticoccidia indices (ACI) of which were 185–190.[4] Furthermore, nitromezuril can exert effects on coccidia that resist diclazuril and toltrazuril, which illustrates its future wide application prospective.[4]

Owing to its selectivity, sensitivity, and speed of analysis with minimal sample processing, liquid chromatography/tandem mass spectrometry (LC/MS/MS) has been proven to be a powerful modern analytical tool for the identification and structural characterization of drug metabolites in biological matrices and has become the preferred method for metabolite identification in the fast paced environment of drug discovery and development.[5] Complex metabolite samples from biological matrices can be separated on a high-performance liquid chromatography (HPLC) column and full-scan MS and product ion scan MS/MS data generated on-line. By producing the MS^n ions associated with these basic structural features as a substructural template based on the parent drug, the structures of the metabolites may be rapidly characterized by comparing their product ions with those of the parent drug, even without standards.[6] Moreover, multiple reaction monitoring (MRM) as a highly sensitive acquisition method in triple quadrupole mass spectrometry can provide good signal intensity by parallel monitoring of several product ions from the same precursor ion and has been used as a method of detecting drug residues in animal-borne food.[7]

On the basis of *in vitro* metabolism research, we have been able to develop new drugs from possible metabolites, in some cases the metabolite exhibits the same pharmacological potential as the parent drug and is less toxic than the parent;

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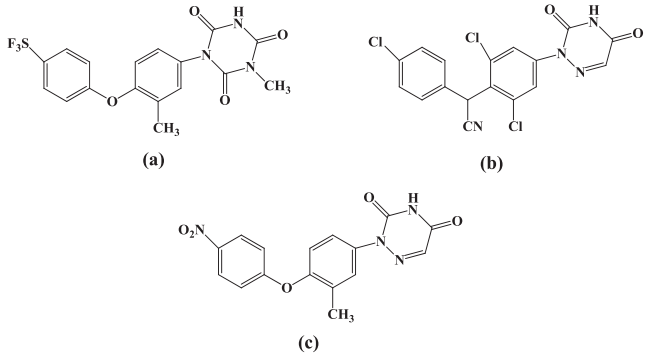


Scheme 1. Chemical structure of toltrazuril (a), diclazuril (b), and nitromezuril (c).

besides, we have also conducted *in vivo* metabolism, as possible metabolic pathways could provide a hint for appropriate radioactive labeled spots which guarantee the success of radioactive label research of pharmacokinetics. In addition, the liver constitutes a crucial step before the distribution of oral drugs, so hepatocytes are widely used in *in vitro* studies of toxicity and the metabolism of xenobiotics.[8–10]

The comprehensive metabolism of nitromezuril *in vitro* and *in vivo* has not been investigated till now. The study firstly assumed possible metabolites of nitromezuril according to common metabolic paths and made use of LC/MS/MS to obtain the fragmentation behavior of nitromezuril and predicted the product ions of possible metabolites. Then we obtained the molecular weights of possible metabolites by recording chromatograms from their peaks in total ion chromatograms (TICs) and detected low levels of possible metabolites by extracted ion chromatograms (EICs) on the basis of assumed metabolites. By comparing product ion scan spectra of possible metabolites of blank and incubation samples with available authentic standards we were able to identify them. Finally, the MRM method was used to detect blank and incubation samples to compensate for the lower sensitivity of the results obtained from product ion scans. The objective of this study was to offer scientific grounds for research of pharmacodynamics and food safety evaluations.

EXPERIMENTAL

Reagents and chemicals

Nitromezuril (purity 99.8%), M1 (purity 99.0%) and M2 (purity 98.9%) were synthesized by the Shanghai Veterinary Research Institute of the Chinese Academy of Agricultural Science and characterized by LC-UV, LC-MS and NMR methods (data not shown). HepG2 cells were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) for culturing and fetal bovine serum (FBS) were obtained from Life Technologies (Carlsbad, CA, USA). Glucose 6-phosphate dehydrogenase, glucose-6-phosphate, β-nicotinamide adenine dinucleotide phosphate sodium salt (NADP) and arochlor-induced rat liver S9 homogenate (S9 mix) were purchased from Roche Chemical Co. (Beijing, China). Acetonitrile (LC grade) was obtained from Fisher Chemicals (Fair Lawn, NJ, USA). Collagenase IV, rat tail collagen and formic acid were purchased from Sigma (St. Louis, MO, USA). Ethyl acetate and other analytical reagents were of analytical grade and purchased from Shanghai Experimental Reagent Co., Ltd (Shanghai, China). Ultra-pure water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). All other chemicals and reagents were of the highest analytical grade available.

Preparation of cells

The frozen tube of HepG2 cells was taken out of the liquid nitrogen tank, put into a water bath at 37°C and shaken until totally thawed. The cell suspension was transferred to a 15 mL centrifuge tube, 4 mL of complete medium was added and the tube was centrifuged at 1000 rpm for 5 min. The sediment was resuspended in pre-heated 37°C culture medium, the cell suspension was implanted to 24-well plates at a density of 5.0×10^5 cm^{-2} (area 1.9 cm^2), and the cells were cultivated in a humidified incubator at 37°C and atmosphere containing 5% CO_2.

Preparation of rat primary hepatocytes was performed using the two-step perfusion method.[11] Isolated hepatocytes were resuspended in DMEM. Then 180 μL of cell suspension was mixed with 20 μL 0.4% Trypan blue solution, and the the viable (white) and dead (blue) cells were counted in a hemocytometer under a light microscope; four fields were counted per chamber. The viability and concentration of the cells were calculated and reached 95% and 9×10^7cells/mL, respectively. Primary hepatocytes began to adhere after 4 h stabilization.

Stability of nitromezuril solution

Nitromezuril was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/mL. The stock solution was mixed with complete medium (DMEM with 10% FBS) at a proportion of 1% (v/v), then the mixed solution was placed in 37°C humidified incubator containing 5% CO_2 for 48 h. The culture medium was extracted with ethyl acetate then centrifuged at 12000 rpm for 20 min at 4°C. The organic supernatant was evaporated under nitrogen atmosphere at 40°C and the residue was dissolved in 200 μL acetonitrile. The aliquot were filtered by a 0.22 μm membrane then analyzed.

Preparation of sample with HepG2 cells

HepG2 cells were allowed to adhere for 12 h. Then, the culture medium was replaced with DMEM culture medium with nitromezuril (20 μg/mL). After 48 h incubation, the medium was collected and extracted with ethyl acetate then centrifuged at 12000 rpm for 20 min at 4°C. The organic supernatant was evaporated under nitrogen atmosphere at 40°C, the residue was dissolved in 200 μL acetonitrile. The aliquot were filtered using a 0.22 μm membrane to identify unchanged nitromezuril and its metabolites. The blank was conducted in the absence of nitromezuril.

Preparation of sample with rat liver S9

To investigate rat liver S9 mix metabolism of nitromezuril, 2 mg protein/mL of S9 mix and an NADPH-generating system consisting of 1 mmol/L NADP, 10 mmol/L glucose-6-phosphate, 0.8 U glucose-6-phosphate dehydrogenase, and 7 mmol/L $MgCl_2$ were mixed. The mixture was preincubated

at 37°C for 5 min. To initiate the reaction, 50 µL nitromezuril was added, and the mixture was maintained at 37°C for another 2.5 h. Control incubations were performed in the absence of nitromezuril and added 50 µL 0.1 mol/L phosphate buffer (pH 7.4). The reaction was terminated by the addition of twice the volume of ethyl acetate, and the mixture was vortexed and centrifuged at 5000 rpm for 15 min at 4°C.[12] The organic supernatant was evaporated under nitrogen atmosphere at 40°C, the residue was dissolved in 200 µL acetonitrile. The aliquot were filtered using a 0.22 µm membrane to identify metabolites by LC/MS/MS.

Preparation of samples with rat primary hepatocytes

Rat primary liver cells were seeded on collagen-coated six-well plates (area 9.4cm^2) at a density of 5 × 10^5 cells cm^{-2} and allowed to adhere in 37°C humidified incubator containing 5% CO_2 for 4 h, then the culture medium was replaced with fresh warm complete medium and cells allowed to recover for 12 h before incubation with nitromezuril (20 µg/mL) for 48 h.[13] The medium was collected and treated as for the preparation of sample with HepG2 cells.

LC/MS/MS analysis of nitromezuril and its metabolites

All experiments were performed with a Waters Alliance 2695 LC system (Waters) and a quattro micro API quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) source.

LC analysis was carried out on an XTerra C_{18} HPLC column (2.1 mm × 100 mm, 3.5 µm; Waters, Milford, MA, USA) equipped with a guard column (XTerra C_{18} 2.1 mm × 100 mm, 3.5 µm; Waters, Milford, MA, USA). The mobile phase consisted of A (water containing 0.1% formic acid, v/v) and B (acetonitrile) with a gradient elution: 0–2 min, 95% A; 2–10 min, 95%–50% A; 10.01 min, 95% A. The flow rate during the complete runs was 0.2 mL/min. Following the gradient elution, the initial conditions were used and stayed under the initial conditions for 2 min for re-equilibration and to get ready for the next injection. The injection of the pretreated sample was 10 µL and the column oven temperature was set at 30°C.

The mass spectrometer was used for analysis operating in negative ion mode. The main parameters were optimized by infusing 1 µg/mL of nitromezuril standard dissolved in

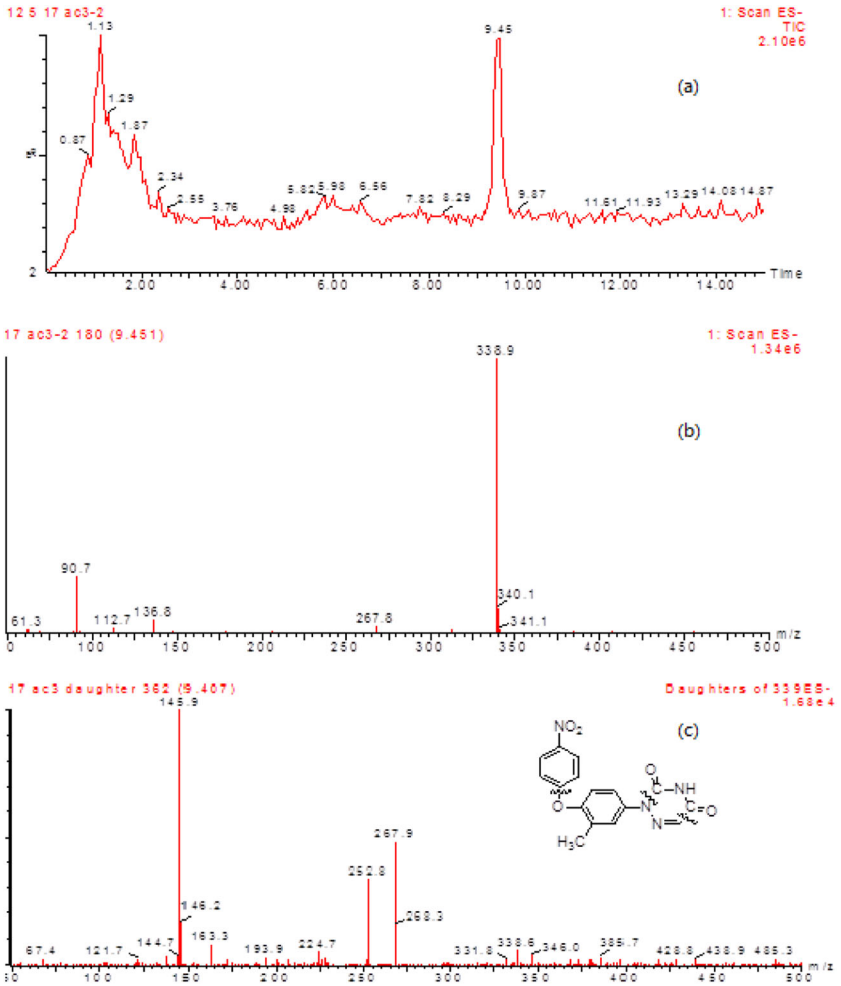


Figure 1. Total ion chromatogram (TIC) (a), full scan mass spectrum (b), and low-energy collision-induced dissociation product ion spectrum (c) behavior of authentic standard nitromezuril. The deprotonated molecule of nitromezuril was *m/z* 339 and it was eluted at 9.4 min (a, b). MS/MS analysis of *m/z* 339 revealed prominent product ions at *m/z* 268 formed by the loss of CONHCO (71 Da). The fragment ion at *m/z* 268 lost $C_6H_4NO_2$ (122 Da) to form *m/z* 146.

acetonitrile at 10 μL/min using a syringe needle (Hamilton, Switzerland). Nitrogen was used as desolvation gas (400 L/h) and nebulizing gas (50 L/h). The source temperature was held at 100°C. The capillary and cone voltages were 4.5 kV and 30 V, respectively. Argon was used as collision gas at a pressure of 3.3×10^{-4} mbar, desolvation temperature 300°C and collision energy of 25 eV. The spectra were recorded in the range *m/z* 50–600 for full scan MS analysis.

Identification of metabolites was based on comparing their retention times, full scan, product ion scan to available authentic standards. Multiple reaction monitoring (MRM) was also used to confirm the conclusion. System control and data acquisition were completed by using the software Masslynx 4.0 (Waters, Milford, MA, USA).

RESULTS AND DISCUSSION

Stability of nitromezuril solution

Stock solutions of nitromezuril was stable for at least 2 months when stored at 4°C (data not shown). On this basis the stability of nitromezuril in DMEM was investigated, and results show that the DMEM samples were stable for 48 h at 37°C in a 5% CO_2 incubator at 95% humidity (data not shown). We assumed nitromezuril was stable under incubation conditions for 48 h and that the metabolism of nitromezuril could be investigated *in vitro*.

Sample preparation

In this study, protein precipitation with acetonitrile and liquid-liquid extraction (LLE) methods were used in order to extract metabolic substances from samples. It turned out that direct protein precipitation was less effective because of its inferior ability to remove endogenous substances. Dichloromethane and ethyl acetate were tried to compare the extraction effects: ethyl acetate demonstrated good elimination of endogenous substances and good extraction efficiency. Therefore, ethyl acetate was selected to prepare incubation samples. All the extracts were dried under nitrogen atmosphere at 40°C and then dissolved; pure acetonitrile and 50% acetonitrile both were tried as solvents. Pure acetonitrile indicated better extraction specificity of metabolites. The injection volume was 10 μL for LC/MS analysis.

LC/MS and LC/MS/MS analyses of nitromezuril

In the present study, both positive and negative ion modes were tried and it turned out that the negative ion mode provided a dramatically stronger signal for nitromezuril. The characterization of nitromezuril was conducted with the conditions mentioned above. Full scan analysis indicated that the deprotonated molecule of nitromezuril was *m/z* 339 and it was eluted at 9.4 min (Figs. 1(a) and 1(b)). MS/MS analysis of *m/z* 339 revealed prominent product ions at *m/z* 268 formed by the loss of CONHCO (71 Da). The fragment ion at *m/z* 268 lost $C_6H_4NO_2$ (122 Da) to form *m/z* 146. The fragmentation of *m/z* 339 resulted in the cleavage of the triazine ring and the ether bond (Fig. 1(c)).

The characteristic product ions and cleavage patterns reported above provided us with scientific grounds for the identification of possible metabolites of incubation samples of nitromezuril with S9, HepG2 cells and primary hepatocytes.

LC/MS and LC/MS/MS for the analysis of the HepG2 sample

In recent years, scientists have taken advantage of quantitative polymer chain reaction (PCR) to measure the expression levels of CYPs in both HepG2 cells and human primary hepatocytes. The result showed that main CYPs, such as CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4, were expressed in HepG2 cells.[14] Besides, HepG2 cells have the ability to proliferate and are easy to obtain, which indicates a good model for metabolism research. In this article, we focused on the *in vitro* metabolism of

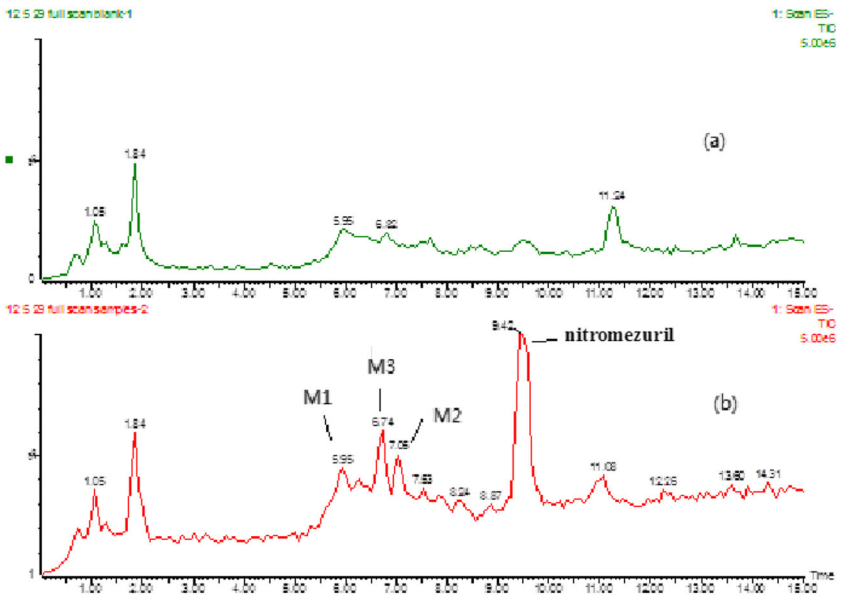


Figure 2. Total ion chromatograms (TICs) of the extracts of both blank (a) and incubation (b) samples in the HepG2 cells system.

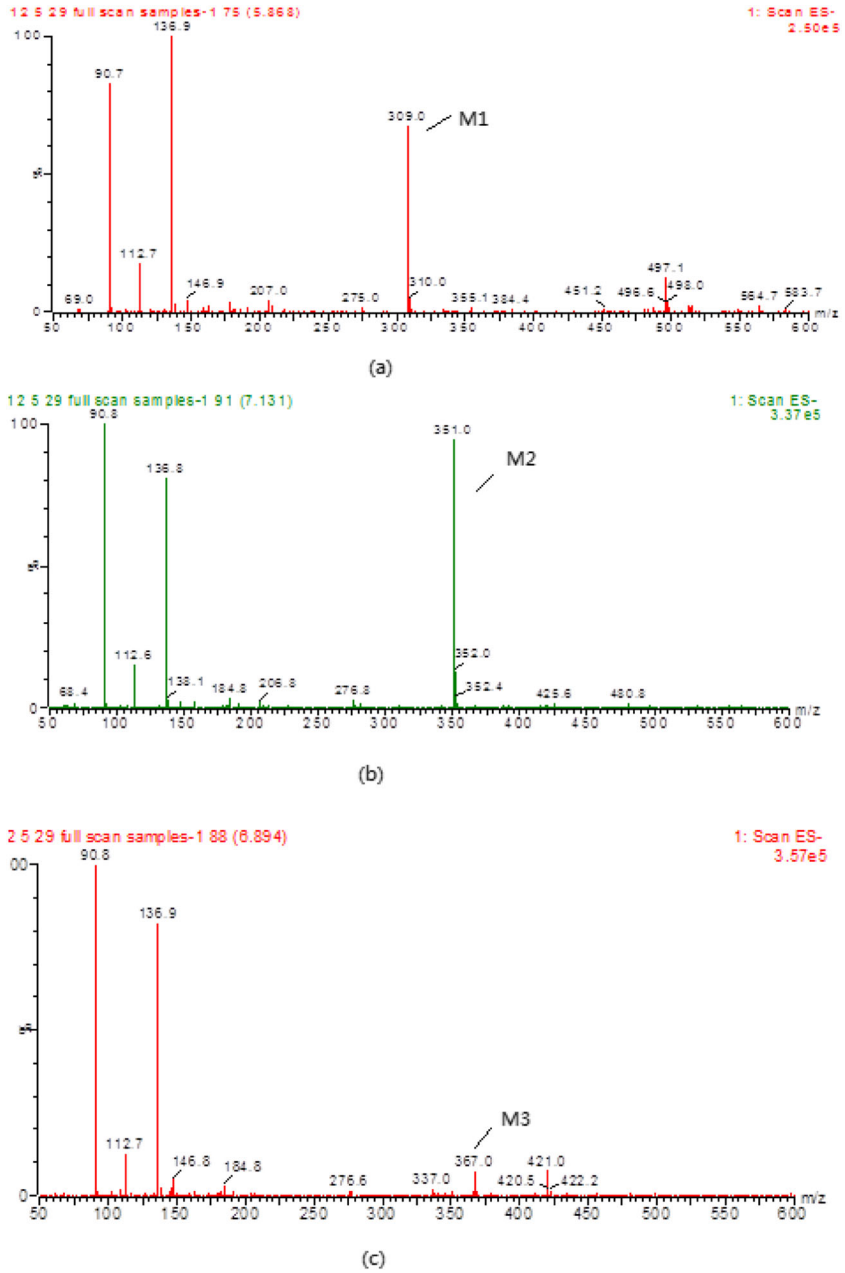


Figure 3. Deprotonated molecular ions of metabolites by taking spectra at the top of the peaks in the TIC of the incubation group. The deprotonated molecules of these metabolites were *m/z* 309 (M1), *m/z* 351 (M2) and *m/z* 367 (M3), respectively. The response of *m/z* 367 is far lower than that of the other metabolites.

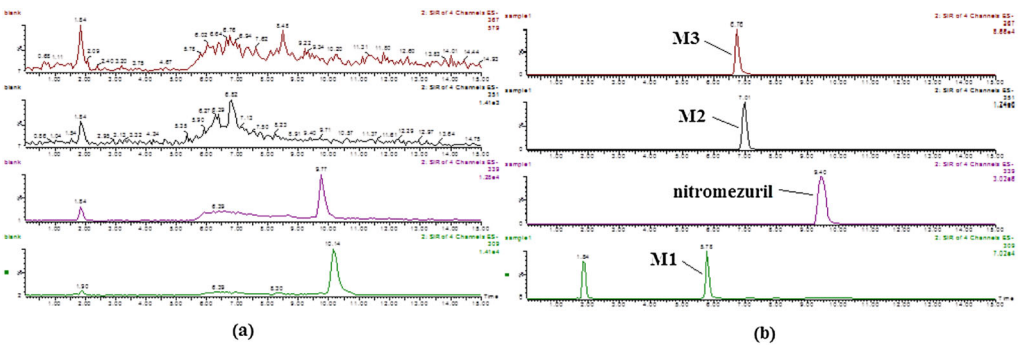
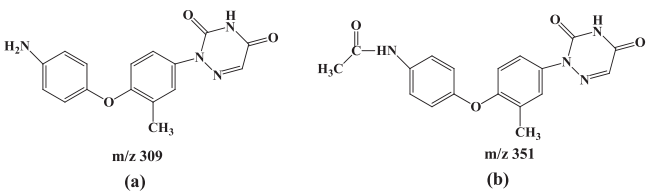


Figure 4. Extracted ion chromatograms of both blank (a) and incubation (b) samples with HepG2 cells.

nitromezuril in HepG2 cell and rat primary hepatocyte systems and aimed to find out possible metabolites, which could provide scientific grounds for further research.

We firstly speculated possible metabolites according to the most common biotransformation pathways, the structure of the parent drug and similarly structured drugs, and eight possible metabolites were assumed. Then, the full scan mass spectra and extracted ion chromatograms (EICs) of the extraction of incubation sample were compared with those obtained from the extraction of blank culture medium to find out possible metabolites.

According to comparing both TICs (Fig. 2) and EICs of pretreated samples, taking the chromatogram at the top of the peaks in the TICs produced the deprotonated molecules of nitromezuril and its metabolites. The deprotonated molecules of these metabolites were *m/z* 309 (M1), *m/z* 351 (M2) and *m/z* 367 (M3), respectively. The response of *m/z* 367 is far lower than that of the other metabolites (Fig. 3). Detecting low levels of possible metabolites according to EICs, metabolites had the same signal intensities as those found in the TICs. The retention times of M1, M2 and M3 were 5.9, 7.0 and 6.8 min, respectively (Fig. 4).



Scheme 2. Chemical structures of M1 (a) and M2 (b) standards.

Combining the results reported above with common metabolic pathways, we tentatively identified the two main metabolites as 2-(4-(4-aminophenoxy)-3-methylphenyl)-1,2,4-triazine-3,5-(2*H*,4*H*)-dione and *N*-(4-(4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2-(3*H*)-yl)-2-methylphenoxy)phenyl) acetamide (M1 and M2 standards) (Scheme 2). Then the two main possible metabolites were synthesized.

The characterization of the two possible metabolites standards was studied in both positive and negative ion mode. The signal response intensity of the M2 standard was the same as nitromezuril. The signal of the M1 standard was a little stronger in the positive ion mode than that in the negative ion mode because of the influence of its amino moiety. Finally, we chose the negative ion mode to analyze the pretreated samples. Their retention times were 5.9 and 7.0 min, respectively, which were the same as those of the metabolites. MS/MS analysis of M1 and M2 standards showed that prominent product ions of *m/z* 309 (M1 standard) were *m/z* 238 formed by the loss of CONHCO (71 Da) and *m/z* 146 formed by the consecutive loss of $C_6H_4NH_2$ (92 Da), which revealed the cleavage was the same with nitromezuril as well as *m/z* 351 (M2 standard), fragment ions of which were at *m/z* 280 and *m/z* 146. The MS/MS spectra of M1 and M2 are shown in Fig. 5.

Studies on metabolism have shown that nearly 90% unchanged diclazuril excretes through faeces, which was the main metabolic pathway. Only four minor metabolites of diclazuril were detected and each of them was less than 1%.[15]

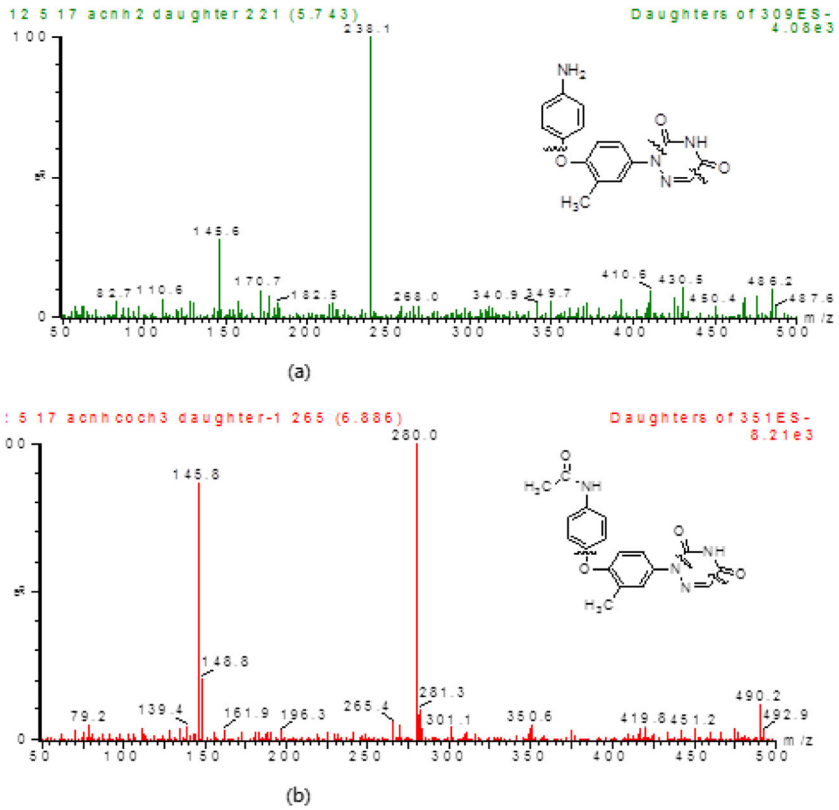
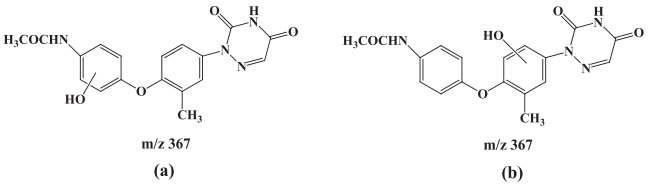


Figure 5. LC/MS/MS product ion spectra of the authentic standards of M1 (a) and M2 (b) standards.



Scheme 3. Possible chemical structures of M3.

Moreover, unchanged toltrazuril was the main component, four metabolites were identified, one major metabolite, toltrazuril sulfone, represented 4.6–16%.[16] Thus we can infer that the metabolism of an anticoccidial drug of the triazine family is relatively stable. Diclazuril possesses the same trizaine ring as nitromezuril, the only fragment of which is formed by the loss of CONHCO (71 Da).[16] So we can infer that the 1,2,4-triazine ring is inclined to cleave by the loss of CONHCO (71 Da).

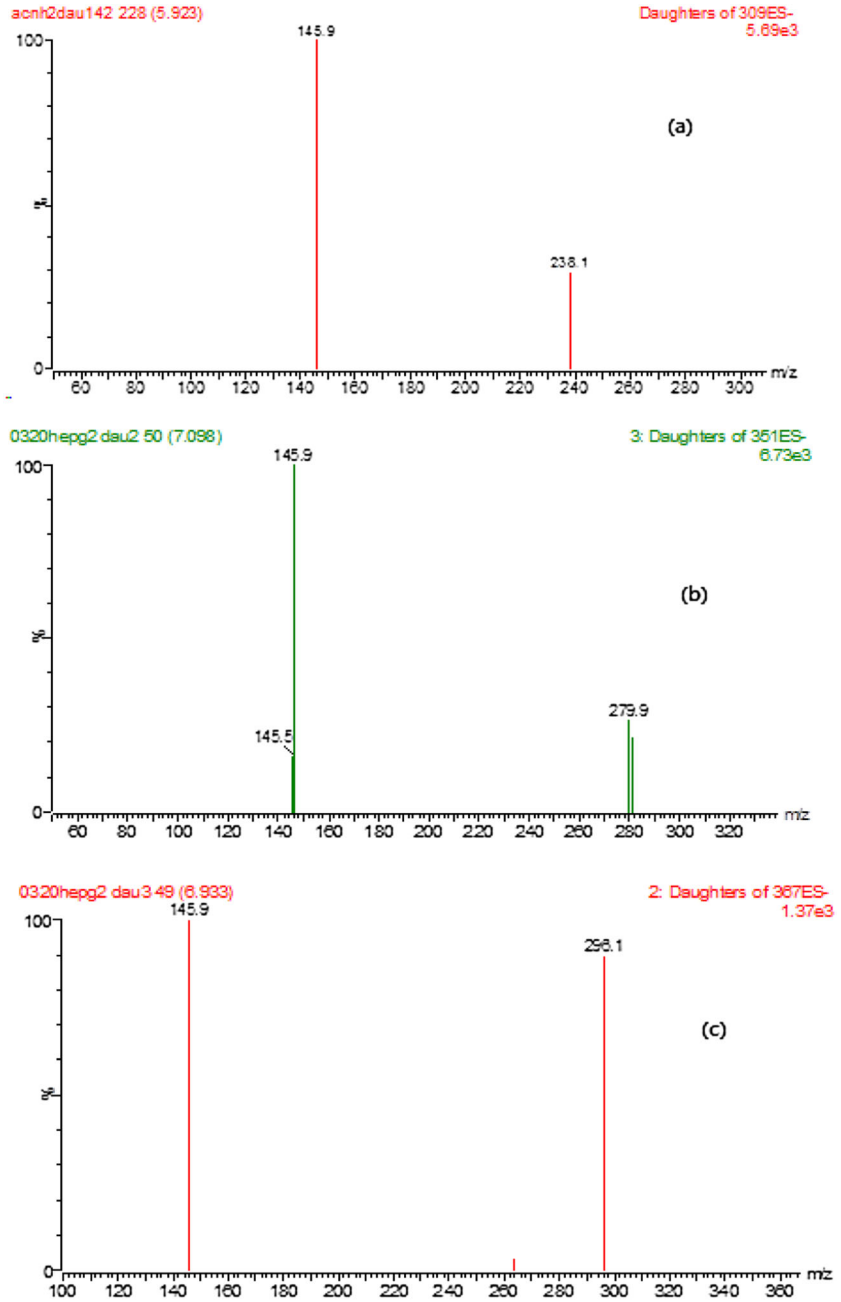


Figure 6. LC/MS/MS product ion scan spectra of metabolites in incubation sample with HepG2 cells: (a) (M1 were *m/z* 238 formed by the loss of CONHCO (71 Da) and *m/z* 146 formed by the loss of $C_6H_4NH_2$ (92 Da)); (b) (M2 were *m/z* 280 formed by the loss of CONHCO (71 Da) and *m/z* 146 formed by the loss of C_8H_8NO (134 Da);and (c) (M3 was the product of phenyl hydroxylation of M2).

The molecular weight of M3 was 16 Da more than that of M2, so we inferred that M3 was the product of phenyl hydroxylation of M2. However, we were unsure about the position of the hydroxyl moiety. According to the cleavage pattern of the parent drug and other metabolites, we speculated possible ion pairs of M3, which were *m/z* 367→296, *m/z* 367→162 and (or) *m/z* 367→146 (Scheme 3).

Product ion scans were conducted in both the blank and incubation groups to obtain the product ions of the possible metabolites and compare them with the authentic standards. The product ions of the possible metabolites were the same as those of the M1 and M2 standards (Figs. 6(a) and 6(b)), and the main product ions of M3 were *m/z* 296 and 146 (Fig. 6(c)), so we tentatively identified M3 as structure **a** shown in Scheme 3.

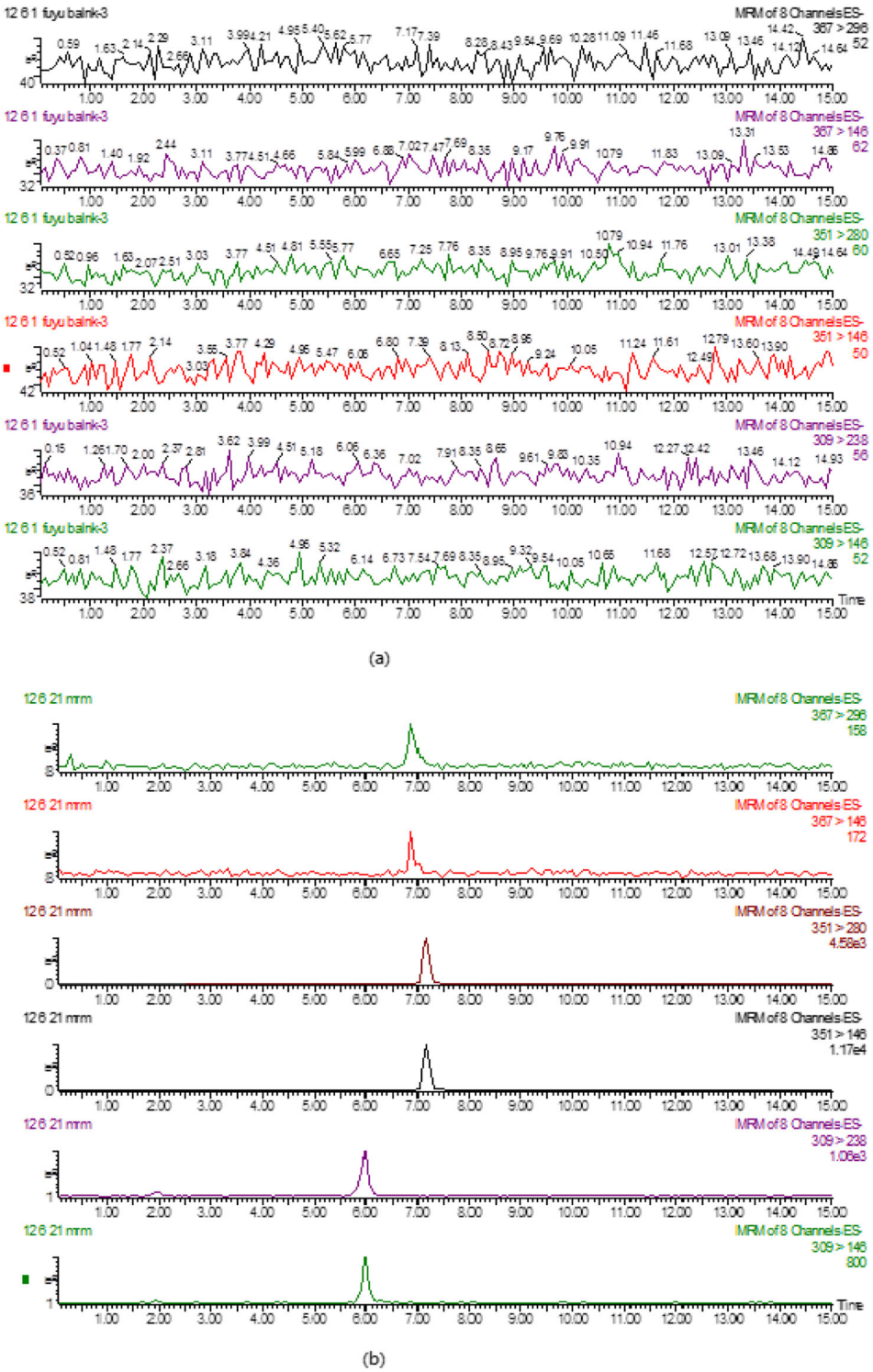


Figure 7. Qualitative detection of M1, M2 and M3 in both blank (a) and incubation (b) samples by the multiple reaction monitoring (MRM) method in the HepG2 cell sytem.

Neutral loss scan analysis was also performed and this facilitated the detection of the glucuronide and sulfate conjugates by monitoring losses of 176 Da and 80 Da, respectively. However, no sulfate or glucuronide conjugates of nitromezuril, M1, M2 or M3 were found.

Multiple reaction monitoring (MRM) was applied to compensate for for the lower sensitivity of the product ion scan and enhance the reliability of the metabolism results. Six transitions were used as follows: *m/z* 367→296, *m/z* 367→146, *m/z* 351→280, *m/z* 351→ 146, *m/z* 309→238, and *m/z* 309→146. No signal was shown in the blank sample whereas ion pairs could be detected in the incubation sample (Fig. 7).

Drug metabolism often converts lipophilic chemical compounds into more readily excreted hydrophilic products. If the metabolites are sufficiently polar, they may be readily

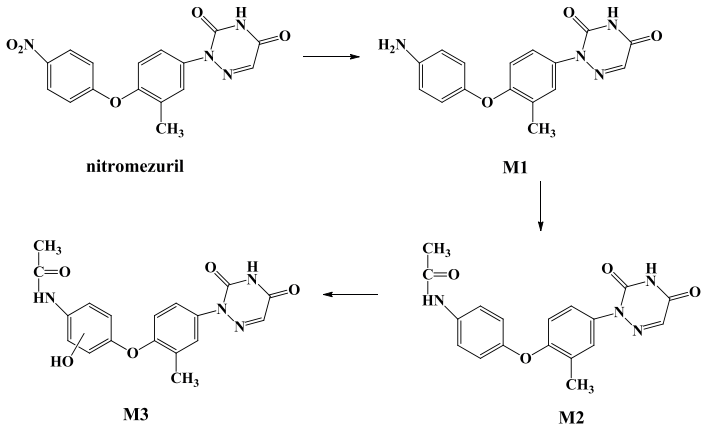


Figure 8. Proposed metabolic pathways of nitromezuril incubated with HepG2 cells *in vitro*.

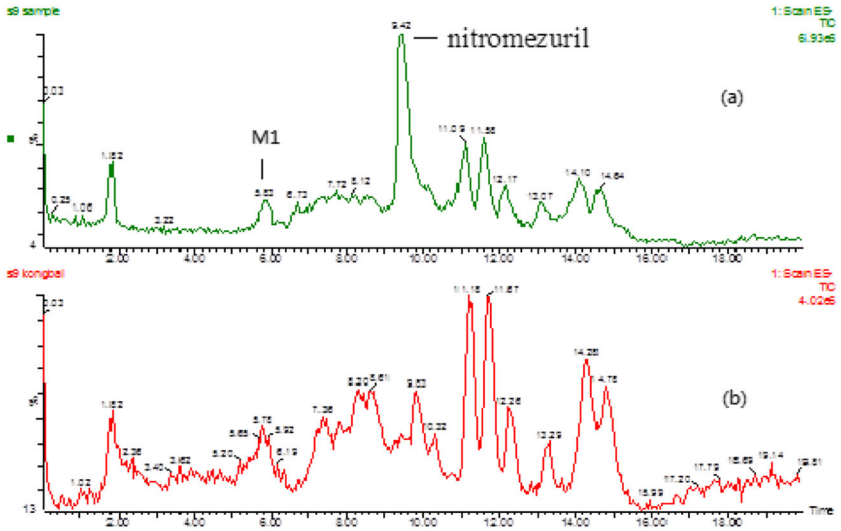


Figure 9. Total ion chromatograms (TICs) of the extracts of both blank (a) and incubation (b) samples in the S9 mix.

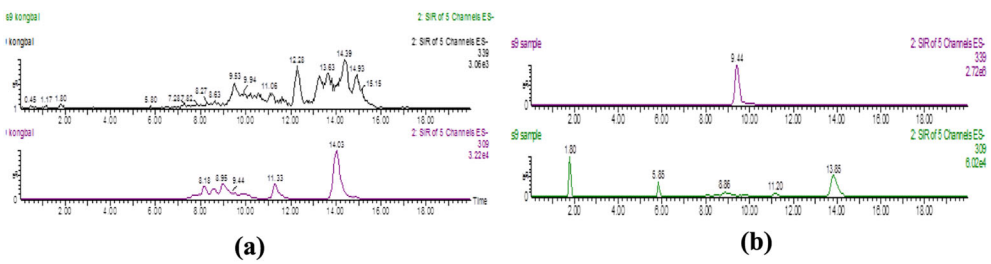


Figure 10. Extracted ion chromatograms (EICs) of both blank and incubation samples with S9. No more metabolites were detected in the EICs of the potential metabolites of nitromezuril.

excreted. Based on these results, the major metabolic pathways of nitromezuril metabolism in HepG2 cells are completed and summarized in Fig. 8. Nitromezuril was metabolized by reduction of nitro to amino followed by acetylation of amino to amide and followed by hydroxylation of the benzene ring of the amide.

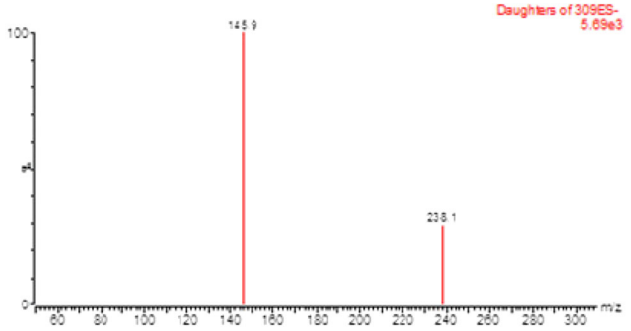


Figure 11. LC/MS/MS product ion scan spectra of metabolite in incubation sample with S9.

LC/MS and LC/MS/MS for the analysis of the S9 sample

S9 is a crude cell preparation and contains both cytosolic enzymes (e.g. sulfotranferase, aldehyde oxidase) and membrane-bound enzymes (e.g. CYP450, FMO). The S9 mix is useful for studying both phase I and phase II metabolism. Thus, incubation of potential drug candidates with the S9 fraction can help understand the metabolic fate of compounds.[17]

We firstly speculated possible metabolites according to the most common biotransformation pathways, the structure of the parent drug and similarly structured drugs, and eight possible metabolites were assumed. Then, the full scan mass spectra and extracted ion chromatograms (EICs) of the extraction of incubation sample were compared with the extraction of a blank culture medium to discover possible metabolites. Detecting possible metabolites in rat S9, only one metabolite was found and identified as M1.

The total ion chromatograms (TICs) are shown in Fig. 9; by taking the chromatogram at the top of the peaks, a possible metabolite eluting at 5.9 min was detected, which could be M1. No more metabolites were detected according to the EICs (Fig. 10).

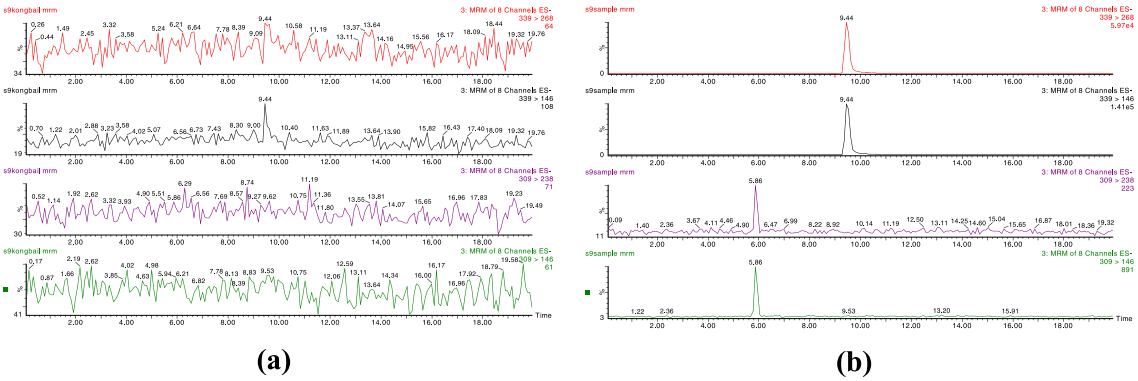


Figure 12. Qualitative detection of M1 in both blank (a) and incubation (b) samples by the multiple reaction monitoring (MRM) method in the S9 mix.

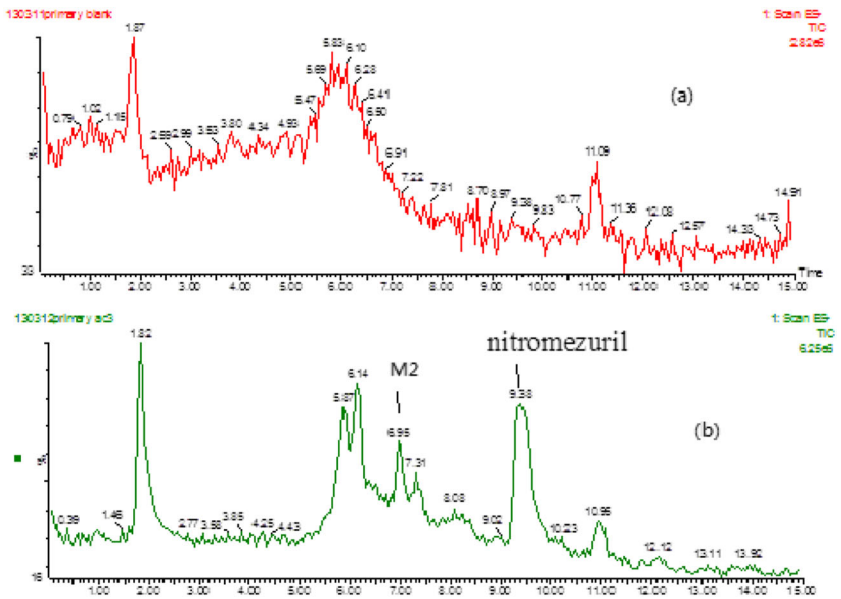


Figure 13. Total ion chromatograms (TICs) of the extracts of both blank (a) and incubation (b) samples in the rat primary hepatocytes system.

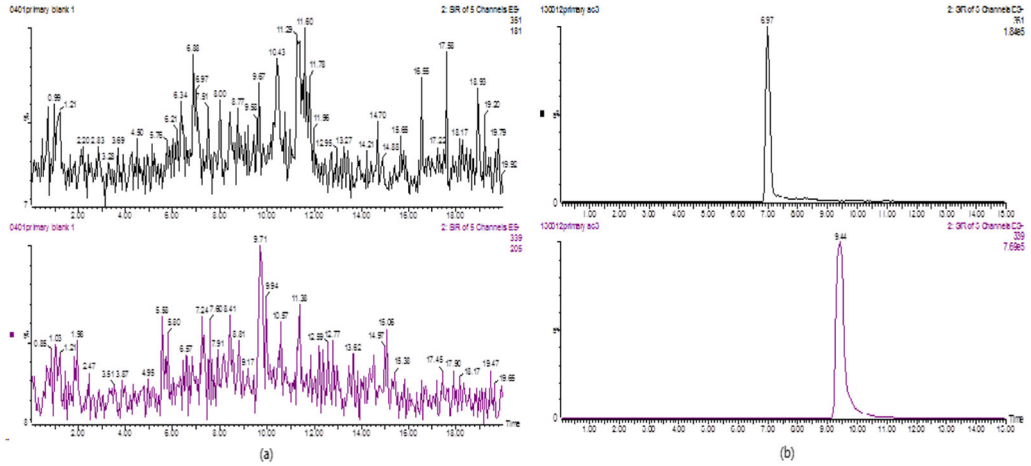


Figure 14. Extracted ion chromatograms (EICs) of both blank and incubation samples with primary hepatocytes. No more metabolites were detected in the EICs of the potential metabolites of nitromezuril.

Product ion scans were conducted in both the blank and incubation samples to obtain the product ions of possible metabolites and compare them with the authentic standards. MS/MS analysis of metabolite M1 and authentic standard M1 showed that prominent product ions of *m/z* 309 both were *m/z* 238 formed by the loss of CONHCO (71 Da) and *m/z* 146 formed by the consecutive loss of $C_6H_4NH_2$ (92 Da), which revealed the product ions of metabolite were the same as those of the M1 standard (Figs. 5 and 11). The MRM comparison between blank and incubation samples is shown in Fig. 12: *m/z* 309→238, *m/z* 309→146, *m/z* 339→268, *m/z* 339→146 were set as the detecting ion pairs.

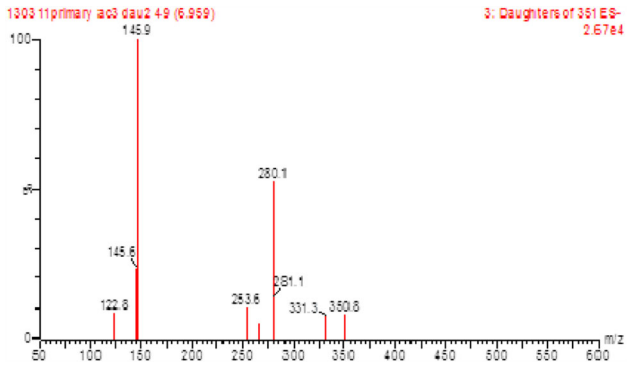


Figure 15. LC/MS/MS product ion scan spectra of metabolite in incubation sample with primary hepatocytes.

LC/MS and LC/MS/MS for the analysis of primary hepatocytes sample

The same procedure of identification was used to detect possible metabolites in rat primary hepatocytes. Only one metabolite was found and identified as M2.

The TICs are shown in Fig. 13; by taking the chromatogram at the top of the peaks, a possible metabolite that eluted at 7.0 min was detected, which could be M2. No more metabolites were detected according to the EICs (Fig. 14).

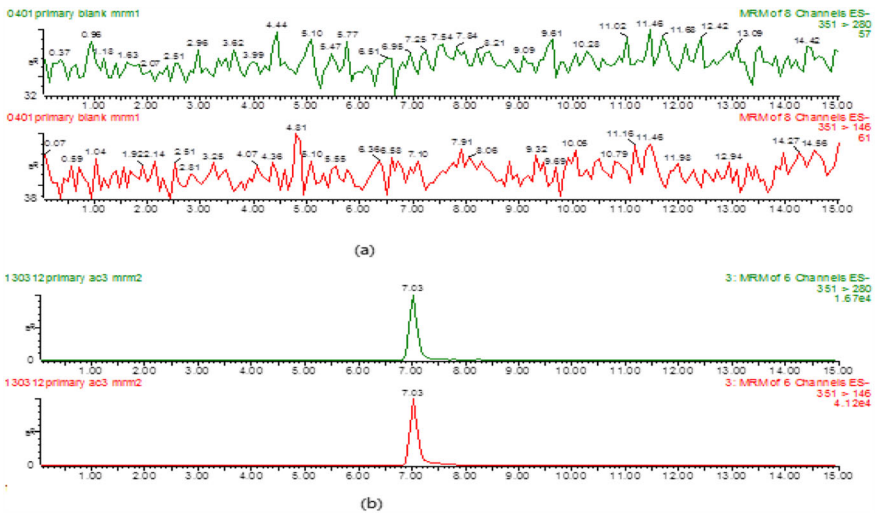


Figure 16. Qualitative detection of M2 in both blank (a) and incubation (b) samples by the multiple reaction monitoring (MRM) method in the primary hepatocytes system.

Product ion scans were conducted in both the blank and incubation samples to obtain the product ions of possible metabolites and compare them with the authentic standards. The product ions of the metabolite were the same as those of the M2 standard (Fig. 15). An MRM comparison between the blank and incubation samples is shown in Fig. 16: *m/z* 351→280 and *m/z* 351→146 were set as detecting ion pairs.

Product M1 which, according to chemical transformation formula, was the premise for producing M2 was not detected in the system. We considered the reason responsible for this result: the acetylase catalyzing this reaction was more active in rat primary hepatocytes than that in HepG2 cells, M2 was the main metabolic component in the rat primary hepatocytes system.[18]

A neutral loss scan was also conducted by monitoring the loss of 176 and 80 to detect common conjugates, no sulfate or glucuronide conjugates of nitromezuril, M1, M2 or M3 were found.

CONCLUSIONS

The metabolism of the new anticoccidial drug nitromezuril was studied for the first time. HepG2 cells, rat liver S9 and primary hepatocytes incubation systems were used to study the metabolism. Apart from its convenience and easy availability, HepG2 cells contained the main CYPs that anticipated drug metabolism, S9 and primary hepatocytes were regarded as gold standards for metabolism research. Finally, a total of three metabolites (M1–M3) were confirmed or tentatively identified in HepG2 cells and one metabolite was confirmed respectively as M1 in rat S9 and M2 in the rat hepatocytes system. Overall, nitromezuril demonstrated no obvious species difference in metabolism. M1 and M2 could be an important objective in pharmacodynamics and residue research. This study supports further research with scientific data and hints.

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