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Metabolism of mequindox and its metabolites identification in chickens using LC–LTQ-Orbitrap mass spectrometry

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ABSTRACT

Mequindox (MEQ), 3-methyl-2-quinoxalinacetyl-1,4-dioxide, is widely used in Chinese veterinary medicine as an antimicrobial and feed additive. Its toxicities have been reported to be closely related to its metabolism. To understand more clearly the metabolic pathways of MEQ, its metabolism in chickens was studied using liquid chromatography coupled with electrospray ionization hybrid linear trap quadrupole orbitrap (LC–LTQ-Orbitrap) mass spectrometry. The structures of the MEQ metabolites and their product ions were easily and reliably characterized based on the accurate MS-squared spectra and known structure of MEQ. Twenty-four metabolites were detected in chicken plasma, bile, faeces, and tissues, of which 12 were detected *in vivo* for the first time. The major metabolic pathways reported previously for *in vitro* metabolism of MEQ in chicken microsomes were confirmed in this study, including N → O group reduction, carbonyl reduction, and methyl mono-hydroxylation. In addition, deacetylation and acetyl-hydroxylation of MEQ were shown to be important metabolic pathways. Collectively, these data contribute to our understanding of the *in vivo* metabolism of MEQ.
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1. Introduction

Mequindox (MEQ), 3-methyl-2-quinoxalinacetyl-1,4-dioxide is a synthetic quinoxaline veterinary drug developed by Lanzhou Institute of Animal Husbandry and Veterinary Drugs, Chinese Academy of Agricultural Sciences (Lanzhou, China). MEQ can inhibit bacterial DNA synthesis and has broad-spectrum antimicrobial activity against many Gram-negative bacteria, including *E. coli*, *Pasteurella*, *Salmonella* spp. and *Bacillus proteus*, and against several Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus* spp. [1]. In particular, MEQ has excellent activity against *Treponema hyodysenteriae* [2]. As a result of its broad antimicrobial properties, MEQ has been widely used as both feed additive and parenteral solution to prevent and treat infectious diseases in livestock in China [3]. However, several recent studies *in vivo* and *in vitro* have revealed potential adrenal [4], endocrine, reproductive [5], and kidney toxicities [6], as well as adrenal mutagenicity [4], which have been shown to be closely related to oxidative free radicals produced during MEQ biotransformation [4]. Therefore, it is important to thoroughly investigate the *in vivo* biotransformation of MEQ in order to evaluate its safety.

Recently, an *in vitro* study with chicken microsomes identified 14 metabolites of MEQ using high-performance liquid chromatography combined with ion trap/time-of-flight mass spectrometry (HPLC/ITTOF-MS) [7]. In another study, 5 metabolites were identified in chicken blood using the same MS technique [8]. However, there has been no comprehensive study to date of the *in vivo* metabolic pathways of MEQ.

In recent years, liquid chromatography coupled with electrospray ionization hybrid linear trap quadrupole orbitrap (LC–LTQ-Orbitrap) mass spectrometry has proven to be a powerful and reliable analytical tool for identifying *in vivo*- and *in vitro*-generated drug metabolites [9–11]. Firstly, the Orbitrap is capable of providing high mass accuracy of <2 ppm using internal standards and <5 ppm with external calibration, and high mass resolution of up to 100,000 fwhm [12], thereby allowing a greater number of metabolites of similar accurate mass to be identified with a high level of confidence. Secondly, the combination of 2 mass analysers enables both scan types to be acquired simultaneously and consecutively, allowing the metabolite structures to be confirmed in one analytical run by the simultaneous acquisition of exact mass precursor and fragment ion data. Furthermore, the mass resolution of the Orbitrap is correlated to acquisition time with longer acquisition times providing higher mass resolution. An acquisition time of 0.4 s provides a mass resolution of 30,000, which is 1.5–6-fold greater than that observed for high resolution TOF instruments [13].

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Thus, the combination of the high mass resolution and mass accuracy over a wider dynamic range together with a limited trade-off between sensitivity and resolving power suggests that the LTQ-Orbitrap is an ideal analytical tool for structural elucidation of drug metabolites in biological samples [11].

In this study, we identified *in vivo* metabolites of MEQ in chicken plasma, bile, faeces, and tissues using LC–LTQ-Orbitrap mass spectrometry. Twenty-four phase I metabolites were detected and their structures were identified. Finally, we propose the pathways of MEQ metabolism in chickens on the basis of the identified metabolites.

2. Experimental

2.1. Chemicals and reagents

The MEQ reference standard (99.8%) was purchased from the China Institute of Veterinary Drug Control (Beijing, China). MEQ (99.5%) was provided by Jiangsu Tiancheng Health Care Products Co., Ltd. (Haian, Jiangsu, China). 1-Desoxymequindox (98.7%), bisdesoxymequindox (99.3%), 2-isoethanol 1-desoxymequindox (99.1%) and 2-isoethanol mequindox (98.8%) were synthesized in our laboratory. HPLC-grade methanol was obtained from Merck and Co., Inc. (West Point, PA, USA). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). HPLC-grade formic acid was supplied by CNW Technologies GmbH (Düsseldorf, Germany). Cellulose sodium carboxymethyl (CMC-Na) was purchased from Guoyao Chemical Co. (Shanghai, China). All other chemicals and reagents were of the highest analytical grade.

2.2. Animals

Four male and 4 female Kebao-500 chickens weighing 1.4–1.7 kg were purchased from Chia Tai Kang in (Dongguan) Co., Ltd (Dongguan, Guangdong, China), and were kept under conditions of controlled temperature (25 °C), humidity (65%), and light (12 h light–dark cycle). Birds were acclimatized in metabolic cages for at least 1 week before the experiment with free access to standard food and water. Animals were fasted for 12 h prior to the experiment and had free access to water. Six chickens (3 male, 3 female) were administered a single dose of MEQ (30 mg/kg, adding 0.5% CMC-Na) by oral gavage. The 2 remaining chickens served as controls and were administered the same volume of 0.5% CMC-Na solution. All studies on animals were performed with the approval of the Institutional Authority for Laboratory Animal Care.

2.3. Samples

Faecal samples were collected during the periods 0–2 h, 2–12 h, and 12–24 h post-dose. To reduce contamination, the metabolic cages were cleaned after each sampling. Whole blood was collected from the armpit vein into heparinized tubes at 2 h, 12 h and 24 h post-dosing. Bile and tissues (heart, liver, spleen, lung, kidney, small intestine, large intestine, glandular stomach, muscle, fat, and skin) were obtained after exsanguination of animals 24 h after dosing. Blood samples were placed in an ice bath for at least 30 min before plasma was isolated by centrifuging at 4 °C for 10 min at 1726 × *g*. Tissues were cut into small pieces and homogenized at 4000 × *g* for 1 min. All samples were assayed immediately or frozen at −20 °C until analysis.

2.4. Sample pretreatment

2.4.1. Bile and plasma

Aliquots of bile (0.5 mL) or plasma (1 mL) were mixed with 3 mL of 5% (w/v) trichloroacetic acid and then centrifuged at 6903 × *g* for 10 min. The supernatant was loaded onto a Strata-X cartridge (60 mg/3 mL), the cartridge was washed with 3 mL 5% methanol/water, the aqueous phase was removed using an air stream, and the analytes were eluted from the cartridge with 2 mL methanol. The eluate was evaporated to dryness under nitrogen at 40 °C and the residue was finally reconstituted in either 0.5 mL or 1 mL of 30% methanol for bile and plasma, respectively.

2.4.2. Faeces and tissues

Three mL of 5% trichloroacetic acid was added to an aliquot of 1.0 g of faecal or tissue sample. The mixture was ultrasonicated for 10 min, shaken for 20 min, centrifuged at 6903 × *g* for 10 min, and the supernatant then transferred to a glass tube. These extraction steps were repeated once more. The residue was then mixed with 3 mL methylene chloride/n-hexane (1/1, v/v) and ultrasonicated, shaken and centrifuged as before. The organic phase was evaporated to dryness in a 40 °C water bath under a gentle stream of nitrogen and reconstituted with 0.5 mL of 30% methanol. The methanol sample was combined with the supernatants from the first 2 extractions and loaded onto a Strata-X cartridge. The cartridge was washed and processed as described for bile and plasma, above.

2.5. LC–LTQ-Orbitrap conditions

The LC system consisted of a Finnigan Surveyor LC system with a built-in degasser and autosampler. The separation was performed on an Agilent XDB (100 mm × 2.1 mm, i.d., 3.5 μm) C18 column and the column temperature was set at 30 °C. The mobile phases were 0.1% formic acid in water (A) and methanol (B). Gradient elution was linearly programmed as follows: 0.00 min 10% B, 15.00 min 90% B, 18.00 min 90% B, 18.01 min 10% B, 25.00 min 10% B, at a constant flow rate of 0.2 mL/min. The injection volume was 10 μL.

Mass spectra were analyzed on a Finnigan LTQ-Orbitrap XL instrument with an ESI source (Thermo Electron, Bremen, Germany). Nitrogen was used as sheath gas (30 arbitrary units) and auxiliary gas (5 arbitrary unit), helium served as the collision gas. Values of tube lens and capillary voltages were set to 70 V and 200 V, respectively. The scan event cycle used a full scan mass spectrum at a resolution of 30,000 and 3 corresponding data-dependent MS-squared events acquired at a resolving power of 7500. Data-dependent MS-squared analyses were triggered by the 3 most abundant ions from the precursor list of predicted metabolites. The most abundant ion was selected to trigger data-dependent scans if there was no match with the precursor list. Collision-induced dissociation (CID) was conducted with an isolation width of 2 Da, and the activation time was set at 30 ms. An external calibration for mass accuracy was performed on the day of analysis using a mixture of caffeine, MRFA peptide, and Ultramark 1600. The mass spectrometric data was collected from *m/z* 100–400 in positive ion mode.

2.6. Data processing

MetWorks 1.2 software was set to automatically identify metabolites by comparing the sample with the control. Twenty possible metabolic pathways of MEQ were defined in the modification manager. The mass tolerance and baseline window were set to 5 ppm and 40 s, respectively. An intensity ratio threshold of 5× and match threshold of 0.8 were set as limits to perform the component-detected subtraction.

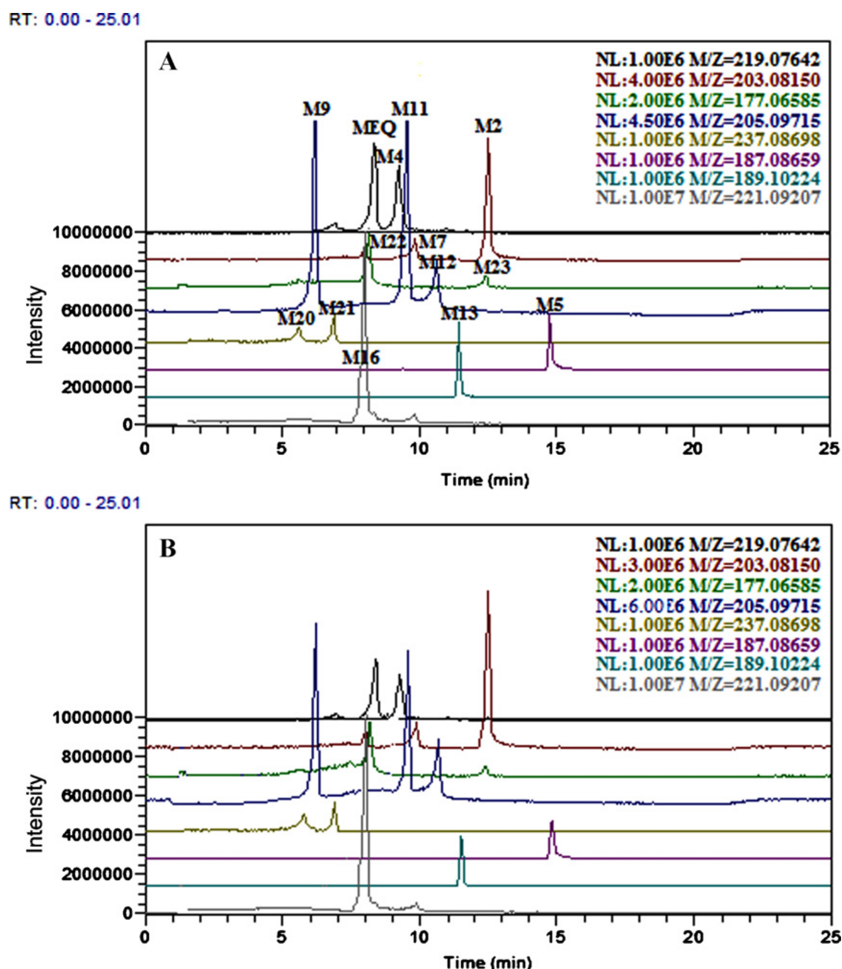

Fig. 1. The accurate extracted mass chromatograms (EIC) of MEQ metabolites in female (A) and male (B) chicken plasma at 2 h after a 30 mg/kg oral dose.

3. Results and discussion

3.1. Metabolite profiles of MEQ in plasma

The accurate extracted ion chromatograms (EIC) of female and male chicken plasma at 2 h after oral administration of MEQ are shown in Fig. 1. The parent and 14 metabolites were detected, indicating that MEQ was rapidly absorbed from the gastrointestinal tract of chickens and metabolized extensively. No additional metabolites were detected in the female and male chicken plasma at 12 h after oral administration of MEQ. However, 3 additional metabolites, M14, M15, and M24 were found in the female and male chicken plasma at 24 h post-dose. The results also revealed that there was no qualitative difference in the metabolite profiles of MEQ in plasma of female and male chickens.

3.2. Metabolite profiles of MEQ in faeces

The accurate EIC for female and male chicken faeces during the first 2 h after MEQ administration revealed 21 and 20 metabolites in female and male faeces, respectively (Fig. 2). With the exception of M8, the parent drug and 17 metabolites that were present in female and male plasma at 2 h were also detected in the female and male faeces during the first 2 h post-dose. Eight additional metabolites, M1, M3, M18 (detected in 0–2 h male, but not female faeces), M14, M15, M17, M19 and M24, were also observed in the 0–2 h female and male faeces samples. In samples collected between 2 and 12 h post-dose, 2 further metabolites, M6 and M8, were detected in both female and male chicken faeces. The retention times, protonated molecules, and metabolite appearance in faeces are summarized in Table 1.

3.3. Metabolite profiles of MEQ in bile and tissues

Table 2 lists the metabolites detected in chicken bile and tissues at 24 h after dosing with MEQ. Most metabolites were widely distributed, including M2, M5, M7–M9, M11, M13–M16, M18, and M22. However, M1 and M20 were detected only in the male liver and in female and male bile. Both M1 and M20 were also found in faecal samples, suggesting they were excreted into the faeces after secretion in bile. A large number of metabolites were found in the bile, liver and kidney, suggesting the liver and kidney were the main organs of MEQ metabolism. In addition, many of the metabolites were found in the glandular stomach, indicating that metabolism could occur at this site. Small amounts of the parent drug were detected in the bile and 8 tissues (heart, liver, spleen, lung, kidney, small intestine, large intestine, and skin) of males, while parent drug was found only in bile, kidney, and skin of females, indicating that the metabolism of MEQ in females occurs more rapidly than in male chickens.

3.4. Identification of metabolites of MEQ

Parent drug and 24 phase I metabolites were detected in chicken plasma, bile, faeces and tissues with the help of MetWorks 1.2. No phase II metabolites were detected. The predicted elemental

Table 1
The retention times (RT), protonated molecules ([M+H]$^+$), and summary of mequindox (MEQ) metabolites detected in female and male chicken faeces.

Compound	RT	[M+H]$^+$	Female chicken			Male chicken		
			0–2 h	2–12 h	12–24 h	0–2 h	2–12 h	12–24 h
MEQ	8.35	219	√	√	ND	√	√	ND
M1	11.32	203	√	√	√	√	ND	ND
M2	12.55	203	√	√	√	√	√	√
M3	10.97	219	√	ND	ND	√	ND	ND
M4	9.24	219	√	√	√	√	√	√
M5	14.85	187	√	√	√	√	√	√
M6	8.79	203	ND	√	√	ND	√	√
M7	9.87	203	√	√	√	√	√	√
M8	5.99	205	ND	√	ND	ND	√	ND
M9	9.58	205	√	√	√	√	√	√
M11	10.68	189	√	√	√	√	√	√
M12	11.27	205	√	ND	√	√	ND	√
M13	11.52	189	√	√	√	√	√	√
M14	8.81	205	√	√	√	√	√	√
M15	6.89	221	√	√	√	√	√	√
M16	8.05	221	√	√	√	√	√	√
M17	10.03	221	√	√	√	√	√	√
M18	8.79	221	√	√	√	ND	√	√
M19	7.05	235	√	√	ND	√	√	√
M20	5.62	237	√	√	√	√	√	√
M21	6.91	237	√	√	√	√	√	√
M22	8.23	177	√	√	√	√	√	√
M23	12.51	177	√	√	√	√	√	√
M24	7.17	193	√	√	√	√	√	ND

M followed by number, metabolite; √, detected; ND, not detected.

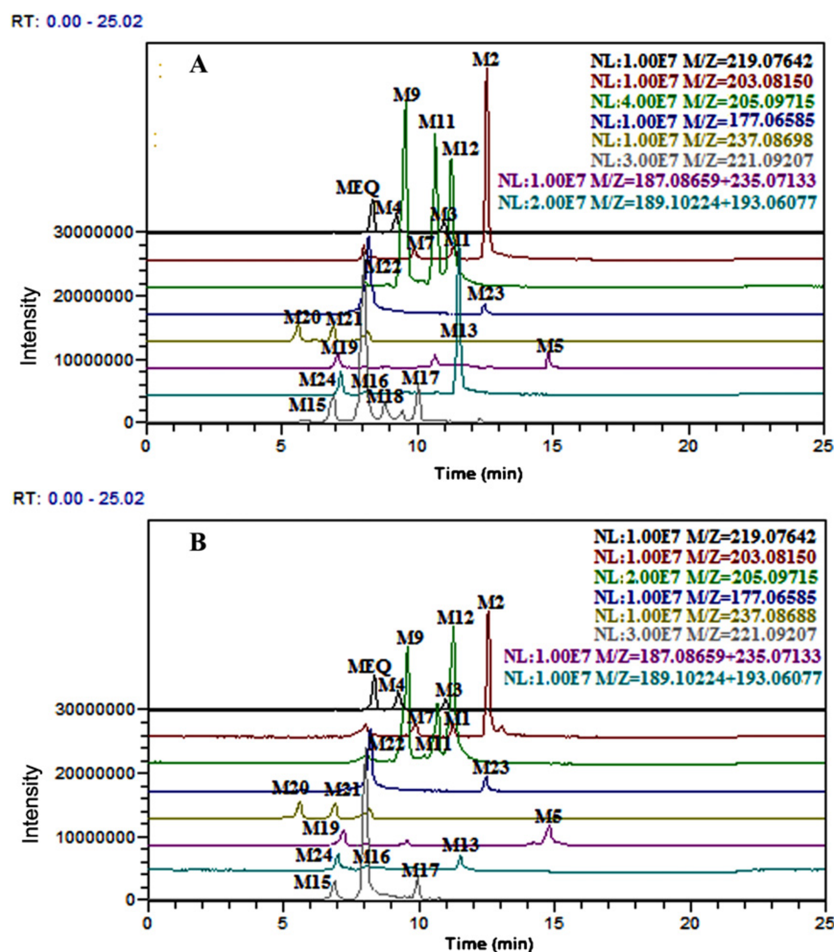

Fig. 2. The accurate extracted mass chromatograms (EIC) of MEQ metabolites in female (A) and male (B) chicken faeces collected between 0 and 2 h post-dose.

Table 2
Summary of MEQ metabolites detected in chicken bile and tissues.

Compound	Female chicken											
	Bi	He	Li	Sp	Lu	Ki	Mu	Fa	Sk	Si	Li	Gs
MEQ	√	ND	ND	ND	ND	√	ND	ND	√	ND	ND	ND
M1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
M2	√	√	√	√	√	√	√	ND	√	√	√	√
M4	√	√	ND	√	√	√	√	ND	ND	ND	ND	ND
M5	√	√	√	√	√	√	√	ND	ND	√	√	√
M7	√	√	ND	√	√	ND	√	ND	√	√	√	√
M8	√	√	√	√	√	√	√	√	√	√	√	√
M9	√	√	ND	ND	√	√	√	√	√	√	√	√
M10	√	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
M11	√	√	ND	ND	√	√	√	√	√	√	√	√
M12	√	ND	ND	ND	ND	ND	ND	ND	ND	ND	√	ND
M13	√	√	√	√	√	√	√	√	√	√	√	√
M14	√	√	ND	ND	√	√	√	√	√	√	√	√
M15	√	√	√	√	√	√	√	ND	√	√	√	√
M16	√	√	√	√	√	√	√	ND	ND	√	√	√
M17	√	ND	ND	√	ND	√	ND	ND	ND	ND	√	ND
M18	√	√	√	ND	√	√	√	ND	ND	√	√	√
M19	ND	ND	√	ND	ND	√	ND	ND	ND	ND	ND	ND
M20	√	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
M21	√	√	ND	√	√	ND	√	ND	ND	ND	ND	√
M22	√	√	√	ND	√	√	√	ND	√	√	√	√
M24	ND	√	ND	ND	ND	ND	√	ND	ND	ND	ND	ND

Compound	Male chicken											
	Bi	He	Li	Sp	Lu	Ki	Mu	Fa	Sk	Si	Li	Gs
MEQ	√	√	√	√	√	√	ND	ND	√	√	√	ND
M1	ND	ND	√	ND	ND	ND	ND	ND	ND	ND	ND	ND
M2	√	√	√	√	√	√	√	√	√	√	√	√
M4	ND	√	ND	ND	ND	√	√	ND	ND	ND	√	ND
M5	√	√	√	√	√	√	√	ND	ND	√	√	√
M7	√	√	ND	√	√	ND	√	√	√	√	√	√
M8	ND	√	√	√	√	√	√	√	√	√	√	√
M9	√	√	ND	ND	√	√	√	√	√	√	√	√
M10	√	ND	ND	ND	ND	ND	ND	ND	ND	ND	√	ND
M11	√	√	ND	ND	√	√	√	√	√	√	√	√
M12	√	ND	ND	ND	ND	ND	ND	ND	ND	ND	√	ND
M13	√	√	√	√	√	√	√	√	√	√	√	√
M14	√	√	ND	ND	√	√	√	√	√	√	√	√
M15	√	√	√	√	√	√	√	√	√	√	√	√
M16	√	√	√	√	√	√	√	√	ND	√	√	√
M17	√	ND	ND	√	ND	√	ND	√	ND	ND	√	ND
M18	√	√	√	ND	√	√	√	ND	ND	√	√	√
M19	ND	ND	√	ND	ND	√	ND	ND	ND	ND	ND	ND
M20	√	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
M21	√	√	ND	√	√	ND	√	ND	ND	ND	ND	√
M22	√	√	√	ND	√	√	√	√	√	√	√	√
M24	ND	√	ND	ND	ND	ND	√	ND	ND	ND	ND	ND

M followed by number, metabolite; Bi, bile; He, heart; Li, liver; Sp, spleen; Lu, lung; Ki, kidney; Mu, muscle; Fa, fat; Sk, skin; Si, small intestine; li, large intestine; Gs, glandular stomach; √, detected; ND, not detected.

compositions, observed and calculated masses, mass errors, and characteristic fragment ions for the proposed metabolites are presented in Table 3. The observed and calculated masses agree to within less than 4 ppm, providing a high level of confidence in the proposed elemental composition of the metabolites.

MEQ was eluted at a retention time of 8.35 min and showed a protonated molecule at *m/z* 219. The product ions of protonated MEQ are shown in Fig. 3. These product ions were used as references to interpret the product ions of the metabolites, as well as to examine the high resolution and mass accuracy of the instrument. Table 4 lists the formulae, the observed and calculated masses, and the mass errors of protonated MEQ and its fragmentations. The mass errors between the observed and calculated masses were less than 3 ppm, which demonstrated the high mass accuracy of the instrument.

As shown in Table 4, protonated MEQ lost an OH radical to form the product ion radical at *m/z* 202, the latter lost an OH radical to form the product ion at *m/z* 185 following loss of C_2H_2O to form *m/z* 143. The loss of C_2H_2O from the protonated MEQ formed the fragment ion at *m/z* 177. The fragment ion at *m/z* 160 was formed by the loss of an OH radical from *m/z* 177. The fragment ion at *m/z* 160 retained one oxide at position 4, as the methyl on the side chain of MEQ would inhibit loss of oxygen from its adjacent N → O group. Subsequently, the fragment ion at *m/z* 131 was formed by the loss of COH from *m/z* 160, and the fragment ion at *m/z* 143 could be formed by the loss of an OH radical from *m/z* 160. According to the results obtained, the fragmentation pathways of MEQ are proposed in Fig. 4.

3.4.1. Metabolites M1 and M2

The accurate MS-squared spectra for MEQ metabolites are shown in Fig. 5 (M1–M12) and Fig. 6 (M13–M24). Metabolites M1 and M2 were eluted at retention times of 11.32 and 12.55 min, respectively. Both metabolites showed a protonated molecule at *m/z* 203 and a fragment ion at *m/z* 186 which were 16 Da lower than that of the protonated molecule of MEQ at *m/z* 219 and its

Table 3
The predicted elemental compositions, observed masses and calculated masses, retention times (RT), characteristic fragment ions, and description of MEQ metabolites in chicken plasma, bile, faeces, and tissues.

Metabolite	Elemental compositions $[M+H]^+$	RT (min)	Observed $[M+H]^+$ (m/z)	Calculated $[M+H]^+$ (m/z)	Error (ppm)	Fragment ions formula	Metabolite description
M1	$C_{11}H_{11}N_2O_2{}^+$	11.32	203.08211	203.08150	2.983	185, 175, 161, 157, 144	4-Desoxymequindox
M2	$C_{11}H_{11}N_2O_2{}^+$	12.55	203.08200	203.08150	2.442	186, 177, 166, 157, 143	1-Desoxymequindox
M3	$C_{11}H_{11}N_2O_3{}^+$	10.97	219.07693	219.07642	2.334	202, 177, 173, 159, 143	3-Hydroxymethyl 1-desoxymequindox
M4	$C_{11}H_{11}N_2O_3{}^+$	9.24	219.07693	219.07642	2.334	201, 184, 177, 160, 143	2-Hydroxyacetyl 1-desoxymequindox.
M5	$C_{11}H_{11}N_2O^+$	14.85	187.08713	187.08659	2.889	187, 159, 145	Bisdesoxymequindox
M6	$C_{11}H_{11}N_2O_2{}^+$	8.79	203.08212	203.08150	3.033	186, 174, 157	2-Hydroxyacetyl bisdesoxymequindox
M7	$C_{11}H_{11}N_2O_2{}^+$	9.87	203.08212	203.08150	3.033	186, 175, 157, 147	3-Hydroxymethyl bisdesoxymequindox
M8	$C_{11}H_{13}N_2O_2{}^+$	5.99	205.09601	205.09715	-1.191	188, 163, 149	2-Isoethanol 4-desoxymequindox
M9	$C_{11}H_{13}N_2O_2{}^+$	9.58	205.09755	205.09715	1.930	188, 170, 157, 145	2-Isoethanol 1-desoxymequindox
M10	$C_{11}H_{13}N_2O_2{}^+$	10.09	205.09698	205.09715	-0.849	187, 175, 153	3-Hydroxymethyl-2-isoethanol bisdesoxymequindox
M11	$C_{11}H_{13}N_2O_2{}^+$	10.68	205.09756	205.09715	1.979	188, 173, 161, 145	3-Methyl-2-quinoxalinethylene glycol
M12	$C_{11}H_{13}N_2O_2{}^+$	11.27	205.09764	205.09715	2.369	188, 173, 161, 145	3-Methyl-2-quinoxalinethylene glycol
M13	$C_{11}H_{13}N_2O^+$	11.52	189.10263	189.10224	2.065	171, 159	2-Isoethanol bisdesoxymequindox.
M14	$C_{11}H_{13}N_2O_2{}^+$	8.81	205.09689	205.09715	-1.288	187, 175, 159, 145	Hydroxyl-2-isoethanol bisdesoxymequindox
M15	$C_{11}H_{13}N_2O_3{}^+$	6.89	221.09259	221.09207	2.357	203, 186, 158	3-Methyl-2-quinoxalinethylene glycol 4-oxide
M16	$C_{11}H_{13}N_2O_3{}^+$	8.05	221.09261	221.09207	2.448	203, 187, 177, 144	2-Isoethanol mequindox
M17	$C_{11}H_{13}N_2O_3{}^+$	10.03	221.09261	221.09207	2.448	204, 203, 177, 175, 161	3-Hydroxymethyl-2-quinoxalinethylene glycol 4-oxide
M18	$C_{11}H_{13}N_2O_3{}^+$	8.79	221.09264	221.09207	2.584	204, 203, 185, 177, 157	3-Hydroxymethyl-2-isoethanol 1-desoxymequindox
M19	$C_{11}H_{11}N_2O_4{}^+$	7.05	235.07185	235.07133	2.198	218, 201, 183, 159	3-Hydroxymethyl-2-hydroxyacetyl 1-desoxymequindox
M20	$C_{11}H_{13}N_2O_4{}^+$	5.62	237.08771	237.08698	3.065	219, 203, 177, 161, 144	3-Hydroxymethyl-2-quinoxalinethylene glycol 4-oxide
M21	$C_{11}H_{13}N_2O_4{}^+$	6.91	237.08759	237.08698	2.559	219, 201, 185, 166, 145	3-Hydroxymethyl-2-isoethanol mequindox
M22	$C_9H_9N_2O_2{}^+$	8.23	177.06639	177.06585	3.027	160, 159, 144, 135, 131	3-Hydroxymethyl quinoxaline 4-oxide
M23	$C_9H_9N_2O_2{}^+$	12.51	177.06615	177.06585	1.671	160, 144, 135, 131	Deacetylation mequindox.
M24	$C_9H_9N_2O_3{}^+$	7.17	193.06131	193.06077	2.804	176, 159, 147, 136	3-Hydroxymethyl quinoxaline-1,4-dioxide

fragment ion at m/z 202, suggesting that they were N → O group reduction metabolites of MEQ. We observed the intensity of M2 was much higher than that of M1. N → O group reduction at position 1 is easier when the electronic effect is considered, as the methyl on the side chain of MEQ would inhibit loss of an OH radical from its adjacent N → O group [14,15]. M2 showed the same retention time and characteristic fragment ions as the authentic 1-desoxymequindox standard. Therefore, M1 and M2 were identified as 4-desoxymequindox and 1-desoxymequindox, respectively.

3.4.2. Metabolites M3 and M4

Metabolites M3 and M4 were eluted at retention times of 10.97 and 9.24 min, respectively, and showed a protonated molecule at m/z 219, which was 16 Da higher than that of m/z 203, suggesting that they were hydroxylation metabolites of M1 or M2. In the MS-squared spectra of M3, fragment ions at m/z 202, 173, and 159 were 16 Da greater than fragment ions at m/z 186, 157, and 143 from 1-desoxymequindox (M2), respectively. In the MS-squared spectra of M3, the presence of the fragment ion at m/z 143, identical to M2, indicating that hydroxylation had not occurred on the phenyl ring. In addition, the fragment ion at m/z 177 was formed by the loss of the acetyl side chain (C_2H_2O, observed 42.0102 Da, calculated 42.0100 Da) from the protonated M3, indicating that the hydroxylation had occurred on the methyl group rather than the acetyl group. Based on these observations, M3 was identified as 3-hydroxymethyl 1-desoxymequindox. The MS-squared spectra of M4 showed a fragment ion at m/z 173, which was 16 Da greater than the fragment ion at m/z 157 of 1-desoxymequindox (M2). M4 contained a fragment ion at m/z 201, which was formed by loss of H_2O (18.0108 Da, calculated 18.0100 Da) from m/z 219, indicating that there was a hydroxyl group adjacent to the acetyl. The fragment ions at m/z 184 and 173 were formed by the loss of OH (observed 17.0028 Da, calculated 17.0021 Da) and CO (observed 27.9947 Da, calculated 27.9943 Da) from m/z 201, respectively. Moreover, the fragment ions at m/z 143 were identical to those from M2, indicating that hydroxylation had not occurred on the quinoxaline ring. According to these findings, M4 was identified as 2′-hydroxyacetyl 1-desoxymequindox.

3.4.3. Metabolite M5

Metabolite M5 had a retention time of 14.85 min on HPLC and showed a protonated molecule at m/z 187; both are identical to those of synthesized bisdesoxymequindox. The MS-squared spectra of M5 showed fragment ions at m/z 169, 159, and 145, consistent with those of synthesized bisdesoxymequindox. Therefore, M5 was identified as bisdesoxymequindox.

3.4.4. Metabolites M6 and M7

Metabolites M6 and M7 were eluted at retention times of 8.79 and 9.87 min, respectively. Both metabolites showed a protonated molecule at m/z 203, which was 16 Da higher than that of bisdesoxymequindox (M5), suggesting that they were hydroxylation metabolites of M5. Moreover, both metabolites M6 and M7 contained a fragment ion at m/z 157, indicating that the hydroxylation position was not on the quinoxaline ring. Hydroxylation on the acetyl group enables the metabolite to have a relatively higher polarity than when on the methyl group, as the carbonyl in the acetyl group has a strong electron-absorbing effect on the adjacent hydroxyl group. M6 had an earlier retention time than that of M7. Therefore, M6 and M7 were identified as 2′-hydroxyacetyl bisdesoxymequindox and 3-hydroxymethyl bisdesoxymequindox, respectively.

3.4.5. Metabolites M8, M9, M10, M11, and M12

Metabolites M8, M9, M10, M11, and M12 were eluted at retention times of 5.99, 9.58, 10.09, 10.68, and 11.27 min, respectively.

Table 4
Formula, observed and calculated masses, ring and double-bond equivalents (RBE), and mass errors of protonated MEQ and its fragmentations.

Formula (M+H$^+$)	Observed mass (Da)	Calculated mass (Da)	RBE	Error (mDa)	Error (ppm)
$C_{11}H_{10}N_2O_3 + H^+$	219.07704	219.07642	7.5	0.62	2.836
$C_{11}H_9N_2O_2 + H^+$	202.07394	202.07368	8.5	0.26	1.291
$C_{11}H_8N_2O + H^+$	185.07117	185.07094	8.5	0.23	1.246
$C_9H_8N_2O_2 + H^+$	177.06602	177.06585	6.5	0.17	0.937
$C_9H_7N_2O + H^+$	160.06328	160.06311	7.0	0.17	1.034
$C_9H_6N_2 + H^+$	143.06053	143.06037	7.5	0.16	1.085
$C_8H_6N_2 + H^+$	131.06056	131.06037	6.5	0.19	1.413

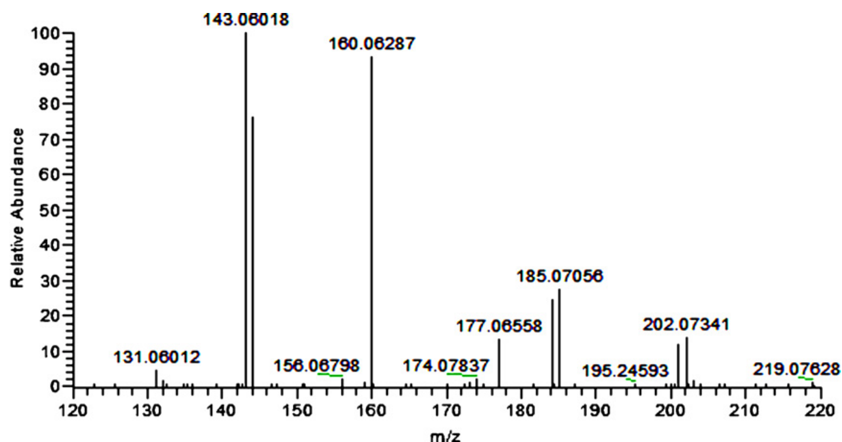

Fig. 3. Accurate MS-squared spectra of MEQ.

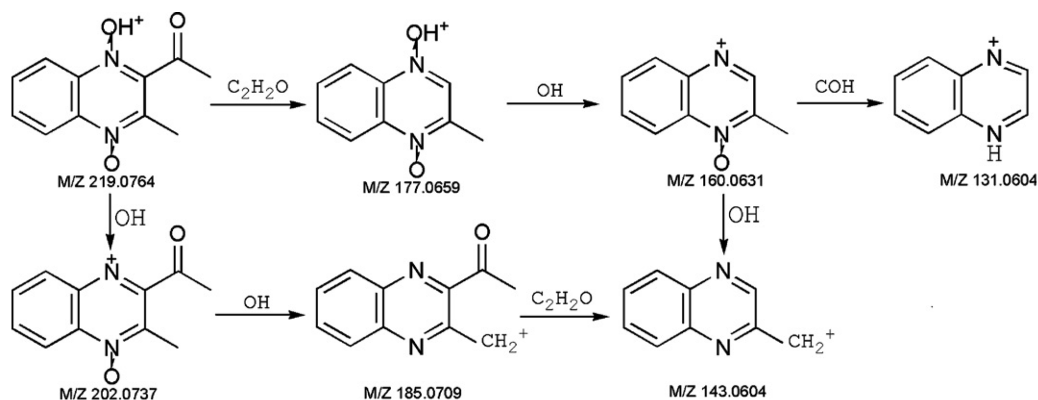

Fig. 4. Fragmentation pathways of MEQ.

All showed a protonated molecule at *m/z* 205, 2 Da higher than that of *m/z* 203, suggesting hydrogenation had occurred. The MS-squared spectrum of M8 showed fragment ions at *m/z* 188, 187, and 163, which were 2 Da higher than fragment ions at *m/z* 186, 185, and 161 of 4-desoxymequindox (M1), respectively, suggesting hydrogenation had occurred on M1. Therefore, M8 was identified as 2-isoethanol 4-desoxymequindox. The molecular ion at *m/z* 205 of M9 and its fragment ions at *m/z* 187 and 145 were all 2 Da higher than that of 1-desoxymequindox (M2), for which the molecular ion was at *m/z* 203 and the fragment ions were at *m/z* 186 and 143, respectively. Moreover, M9 was eluted at the same retention time and showed the same fragment ions in the MS-squared spectrum as the synthesized 2-isoethanol 1-desoxymequindox standard. Therefore, M9 was identified as 2-isoethanol 1-desoxymequindox. The MS-squared spectrum of M10 showed fragment ions at *m/z* 187 and 175, which were 2 Da higher than the fragment ions at *m/z* 185 and 173 of 3-hydroxymethyl bisdesoxymequindox (M7), respectively, indicating that hydrogenation had occurred on M7. Therefore, M10 was identified as 3-hydroxymethyl-2-isoethanol bisdesoxymequindox. The molecular ion at *m/z* 205 of M11 and its fragment ions at *m/z* 188 were all 2 Da higher than that of 2′-hydroxyacetyl bisdesoxymequindox (M6), for which the molecular ion was at *m/z* 203 and the fragment ions were at *m/z* 186, respectively, indicating that hydrogenation had occurred on M6. M11 and M12 had similar MS-squared spectra, indicating they were cis/trans-isomers. Therefore, M11 and M12 were both identified as 3-methyl-2-quinoxalinethylene glycol.

3.4.6. *Metabolite M13*

Metabolite M13 was eluted at a retention time of 11.52 min, and showed a protonated molecule at *m/z* 189, which was 2 Da higher than that of the protonated molecule of bisdesoxymequindox (M5), indicating that hydrogenation had occurred on M5. The fragment ion at *m/z* 171 was formed by loss of a molecule of H_2O (observed 18.0106 Da) from the protonated molecule M13. Therefore, M13 was identified as 2-isoethanol bisdesoxymequindox.

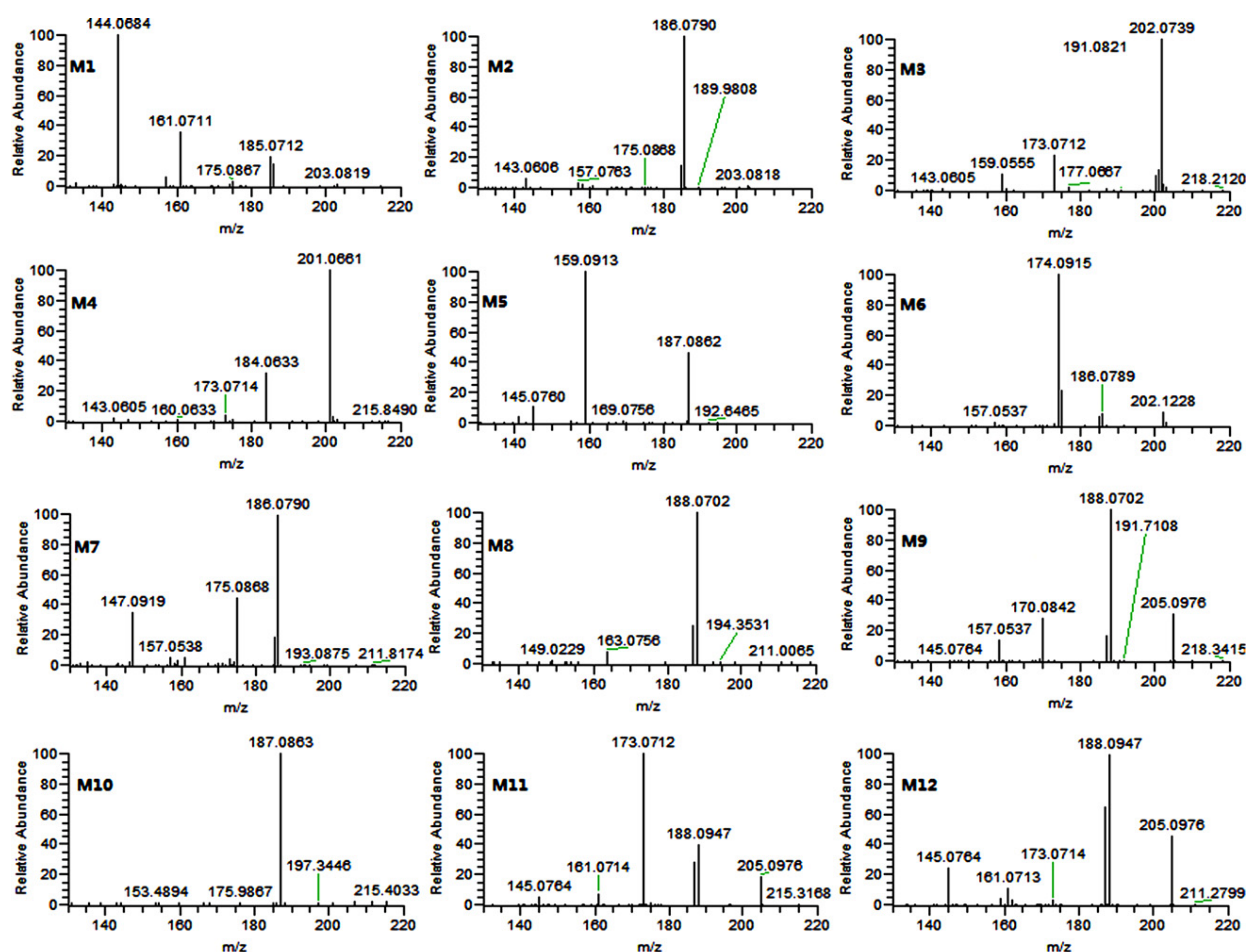

Fig. 5. Accurate MS-squared spectra of MEQ metabolites (M1–M12).

3.4.7. Metabolite M14

Metabolite M14 had a retention time of 8.81 min and showed a protonated molecule at *m/z* 205. The molecular ion and its fragment ions at *m/z* 187 were all 16 Da higher than that of 2-isoethanol bisdesoxymequindox (M13), for which the molecular ion was at *m/z* 189 and the fragment ions were at *m/z* 171, respectively, suggesting hydroxylation had occurred on M13. The metabolite M14 contained a fragment ion at *m/z* 159, and no fragment ion at *m/z* 143 was observed as for M2, suggesting the hydroxylation had occurred on the quinoxaline ring. The exact position of hydroxylation on the phenyl ring could not be characterized. Therefore, M14 was identified as a hydroxylation metabolite of 2-isoethanol bisdesoxymequindox at one of the positions 5, 6, 7, or 8 on the quinoxaline ring.

3.4.8. Metabolites M15, M16 and M17

Metabolites M15, M16 and M17 were eluted at retention times of 6.89, 8.05 and 10.03 min, respectively, and all showed a protonated molecule at *m/z* 221, 2 Da higher than that of *m/z* 219, suggesting hydrogenation had occurred. The molecular ion at *m/z* 221 of M15 and its fragment ions at *m/z* 203 and 186 were all 2 Da higher than that of 2′-hydroxyacetyl 1-desoxymequindox (M4), for which the molecular ion was at *m/z* 219 and the fragment ions were at *m/z* 201 and 184, respectively, suggesting hydrogenation had occurred on M4. Therefore, M15 was identified as 3-methyl-2-quinoxalinethylene glycol 4-oxide. The MS-squared spectrum of M16 showed fragment ions at *m/z* 204 and 187, which were 2 Da higher than fragment ions at *m/z* 202 and 185 of MEQ, respectively. Moreover, M16 showed the same retention time and characteristic fragment ions as the authentic 2-isoethanol mequindox standard. Therefore, M16 was identified as 2-isoethanol mequindox. The molecular ion at *m/z* 221 of M17 and its fragment ions at *m/z* 204 and 161 were all 2 Da higher than that of 3-hydroxymethyl 1-desoxymequindox (M3), for which the molecular ion was at *m/z* 219 and the fragment ions were at *m/z* 202 and 159, respectively, suggesting hydrogenation had occurred on M3. Therefore, M17 was identified as 3-hydroxymethyl-2-isoethanol 1-desoxymequindox.

3.4.9. Metabolite M18

Metabolite M18 had a retention time of 8.79 min and showed a protonated molecule at *m/z* 221. The molecular ion at *m/z* 221 of M18 and its fragment ions at *m/z* 204 and 203 were all 16 Da higher than that of 2-isoethanol 4-desoxymequindox (M8), for which the molecular ion was at *m/z* 205 and the fragment ions were at *m/z* 188 and 187, respectively, suggesting hydroxylation had occurred on M8. Moreover, the metabolite M18 contained a fragment ion at *m/z* 157, suggesting the hydroxylation position was not on the quinoxaline ring. Furthermore, the fragment ion at *m/z* 177 was formed by the loss of the isoethanol side chain (C_2H_4O, observed 44.0265 Da, calculated 44.0257 Da) from the molecular ion M18. Based on these observations, the hydroxylation position was likely on the methyl group. Therefore, M18 was identified as 3-hydroxymethyl-2-isoethanol 4-desoxymequindox.

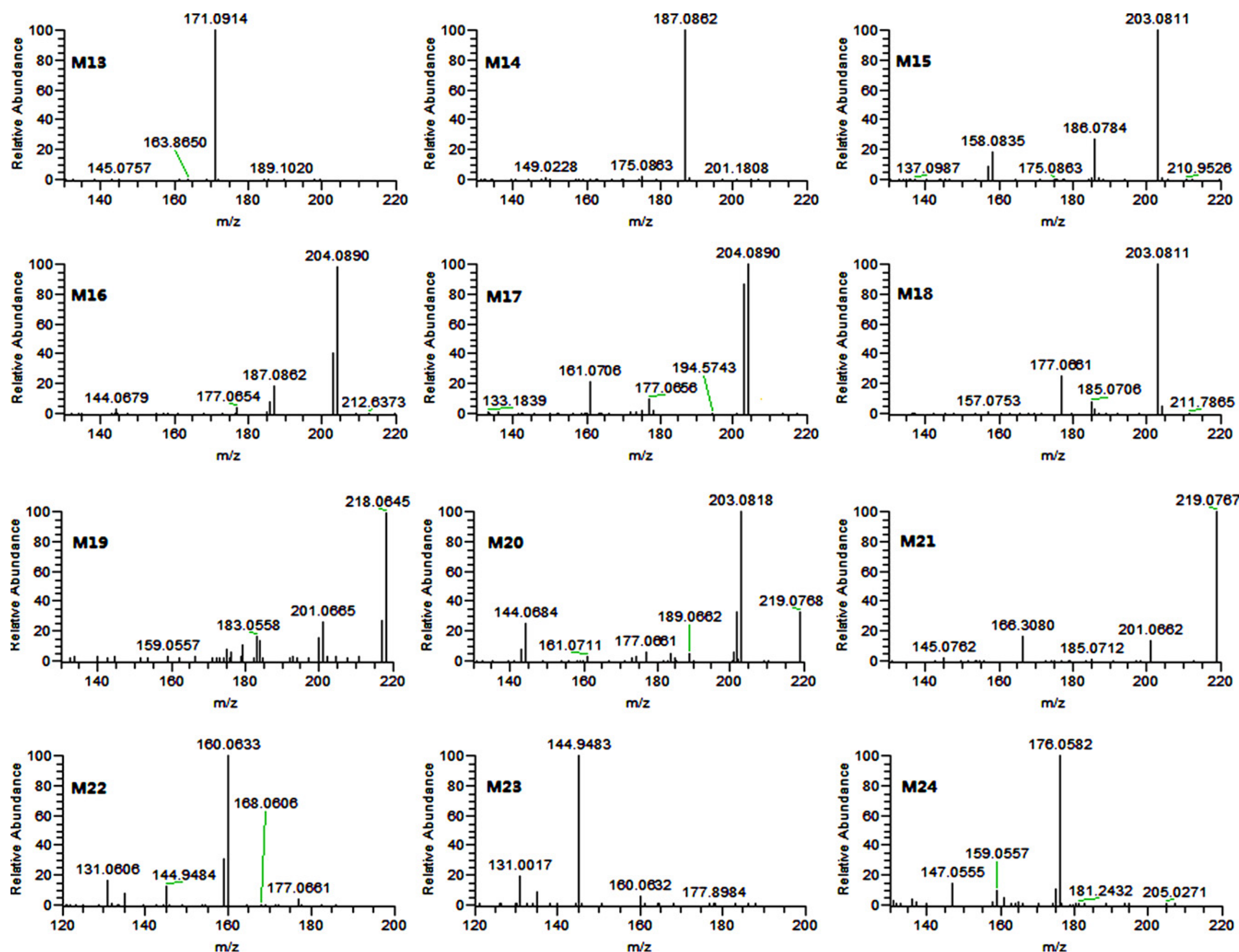

Fig. 6. Accurate MS-squared spectra of MEQ metabolites (M13–M24).

3.4.10. Metabolite M19

Metabolite M19 had a retention time of 7.05 min and showed a protonated molecule at *m/z* 235. In the MS-squared spectra of M19, fragment ions at *m/z* 218, 201 and 159 were all 16 Da higher than the fragment ions at *m/z* 202, 185 and 143 of MEQ, respectively, suggesting hydroxylation had occurred. In addition, protonated M19 lost 2 OH radical (observed 34.0053 Da, calculated 34.0049 Da) successively to generate the product ion at *m/z* 201. The latter lost a molecule of H_2O (observed 18.0107 Da) to form the product ion at *m/z* 183. The fragment ion at *m/z* 159 was formed by loss of the side chain (C_2H_2O, observed 42.0108 Da) from *m/z* 201, indicating that there was likely a hydroxyl group on the methyl group at position 3 of the mequindox. According to the above, M19 was identified as 3-hydroxymethyl mequindox.

3.4.11. Metabolite M20

Metabolite M20 was eluted at a retention time of 5.62 min, and showed a protonated molecule at *m/z* 237. The molecular ion at *m/z* 237 of M20 and its fragment ions at *m/z* 219 and 202 were all 16 Da greater than that of 3-methyl-2-quinoxalinethylene glycol 4-oxide (M15), for which the molecular ion was at *m/z* 221 and the fragment ions were at *m/z* 203 and 186, respectively, suggesting hydroxylation had occurred on M15. In addition, the presence of the fragment ion at *m/z* 144 indicated hydroxylation position was not on the quinoxaline ring. Therefore, M20 was identified as 3-hydroxymethyl-2-quinoxalinethylene glycol 4-oxide.

3.4.12. Metabolite M21

Metabolite M21 had a retention time of 6.91 min, and showed a protonated molecule at *m/z* 237. The molecular ion M21 at *m/z* 237 and its product ions at *m/z* 219, and 185 were all 2 Da higher than that of 3-hydroxymethyl mequindox (M19), for which the molecular ion was at *m/z* 235 and the fragment ions were at *m/z* 217 and 183, respectively, suggesting hydrogenation had occurred. Therefore, M21 was identified as 3-hydroxymethyl-2-isoethanol mequindox.

3.4.13. Metabolites M22 and M23

Metabolites M22 and M23 were eluted at retention times of 8.23 and 12.51 min, respectively. Both metabolites showed a protonated molecule at *m/z* 177, 42 Da lower than that of *m/z* 219, suggesting deacetylation had occurred. The MS-squared spectrum of M22 showed fragment ions at *m/z* 160, 135 and 131, which were 42 Da lower than the fragment ions at *m/z* 202, 177 and 173 of 3-hydroxymethyl 1-desoxymequindox (M3), respectively, suggesting deacetylation had occurred on M3. Moreover, M22 contained a fragment ion at *m/z* 159, which was formed by the loss of H_2O (observed 18.0104 Da) from *m/z* 177, suggesting there was a hydroxyl group on the side chain. Therefore, M22 was identified as 3-hydroxymethyl quinoxaline 4-oxide. The molecular ion M23 at *m/z* 177 and its product ions at *m/z* 160 and 135 were all 42 Da lower than that of parent drug, for which the molecular ion was at *m/z* 219 and the fragment ions were at *m/z* 202 and 177,

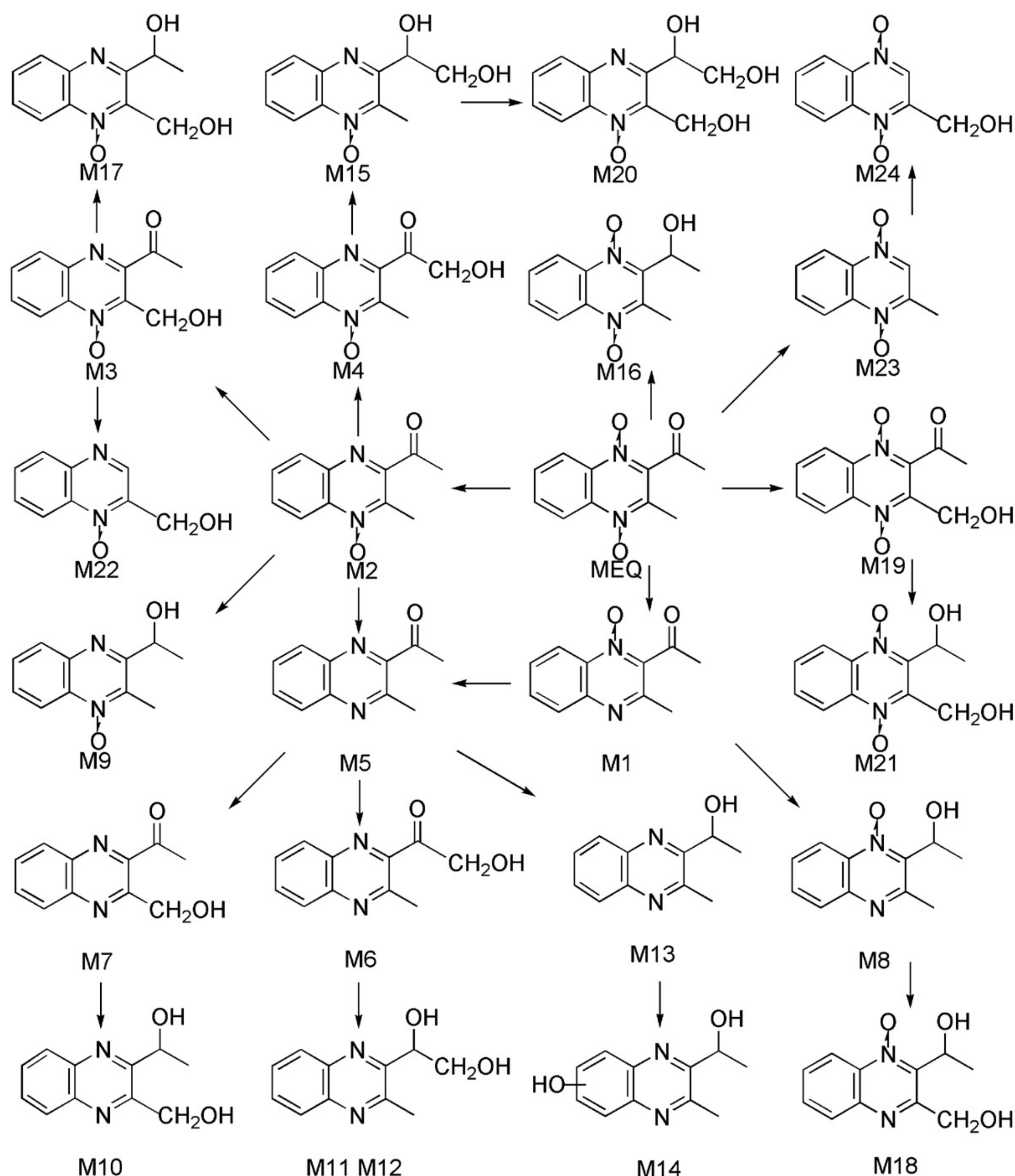

Fig. 7. Proposed *in vivo* metabolism of MEQ in chickens.

respectively. No fragment ion at *m/z* 159 was found as for M22, indicating M23 was the deacetylation metabolite of MEQ. Therefore, M23 was identified as deacetylated MEQ.

3.4.14. Metabolite M24

Metabolite M24 had a retention time of 7.17 and showed a protonated molecule at *m/z* 193. The molecular ion M24 at *m/z* 193 and its product ions at *m/z* 176, 175, and 147 were all 16 Da higher than that of 3-hydroxymethyl quinoxaline 4-oxide (M22), for which the molecular ion was at *m/z* 177 and the fragment ions were at *m/z* 160, 159 and 131, respectively. In addition, the metabolite M24 contained a fragment ion at *m/z* 159, which was formed by loss of 2 OH radical (observed 34.0056 Da) from *m/z* 193, indicating that both of the 2N → O groups were retained. Therefore, M24 was identified as 3-hydroxymethyl quinoxaline-1, 4-dioxide.

3.5. The metabolism of MEQ in chickens

In an earlier *in vitro* metabolism study, 14 metabolites of MEQ were identified in chicken microsomes using LC-ITTOF-MS. Based on those results, the proposed metabolic pathways of MEQ consisted of a series of N → O group reduction, carbonyl reduction, and methyl mono-hydroxylation [7]. In this study, the *in vivo* metabolism of MEQ in chickens was investigated using LC–LTQ-Orbitrap, which has higher mass resolution and accuracy. Twelve (M1–M9, M13, M15 and M16) of the 14 metabolites found in the

in vitro study were also found in the present *in vivo* study; the exceptions were hydroxyl-1-desoxymequindox and the cis/trans-isomers of M9. This suggests that the *in vitro* metabolism of MEQ correlated well with its *in vivo* metabolism. In addition, 12 new metabolites formed *in vivo* were detected and characterized. Based on these results, we propose the possible metabolic pathways of MEQ in chickens (Fig. 7).

The present results showed that N → O group reduction at position 1, carbonyl reduction, and the hydroxylation reaction occurring at the methyl group are the main metabolic pathways of MEQ in chickens. In addition, deacetylation was found to be a new metabolic pathway, as 3 metabolites (M22–M24) were attributed to deacetylation of the parent drug. In addition, the formation of 6 metabolites (M4, M11, M12, M15, M19, and M20) provided further support for the hydroxylation at the acetyl group. No significant qualitative differences were observed in the metabolite profiles of MEQ between female and male chickens. However, the metabolism of MEQ in female chickens appears to be more rapid than in males. After oral administration of MEQ, the drug was quickly absorbed and metabolized. Large quantities of metabolites were found in the plasma at 2 h and in the faeces between 0 and 2 h post-dose. However, MEQ was not detected in the plasma at 24 h or in the faeces during the 12–24 h post-dose period. These results are in good agreement with previous results on the pharmacokinetics of MEQ in goats [3]. Finally, during the process of data analysis, we observed that the LC–LTQ-Orbitrap produced more fragments than LC–ITTOF-MS for the same compound, making the structural elucidation of metabolites more direct and accurate.

4. Conclusions

The metabolism of MEQ in male and female chickens was studied extensively using liquid chromatography coupled with electrospray ionization hybrid linear trap quadrupole orbitrap (LC–LTQ-Orbitrap). Twenty-four metabolites were detected with the help of MetWorks 1.2 software, of which 12 were detected in an earlier *in vitro* study and the remaining 12 were found for the first time *in vivo*. The metabolite structures were elucidated based on the retention times on the LC system, the accurate molecular mass and characteristic fragment ions. The results show that in addition to N → O group reduction, carbonyl reduction and the hydroxylation reaction occurring at the methyl group, hydroxylation at the acetyl group and deacetylation are also possible metabolic pathways of MEQ in chickens. These results have also demonstrated that LC–LTQ-Orbitrap is a rapid, sensitive, and reliable tool for the characterization of unknown metabolites in biological samples.

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