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Simultaneous determination of dextromethorphan and dextrophan in rat plasma by LC-MS/MS and its application to a pharmacokinetic study

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A highly selective and sensitive liquid chromatography-tandem mass spectrometric (LC-MS/MS) method was developed and validated for quantitating dextromethorphan (DXM) and its metabolite dextrophan (DXO) in rat plasma using pirfenidone as an internal standard. Protein precipitation with acetonitrile was employed for the sample preparation. Chromatographic separation was achieved on a SB-C_{18} column at 25 °C, with a gradient elution programme of which acetonitrile-0.1% formic acid in water as mobile phase. The flow rate was 0.4 mL/min. Detection is carried out by multiple reaction monitoring (MRM) on a ion-trap LC-MS/MS system with an electrospray ionization interface. The assay is linear over the range 1–500 ng/mL for DXM and 1–250 ng/mL for DXO, with a lower limit of quantitation of 1 ng/mL for both. Intra- and inter-day precision of the assay were less than 9.80% and the accuracy were in the range 96.35–106.39%. The developed method was successfully applied to analyze the drug in samples of rat plasma for pharmacokinetic study.

1. Introduction

Dextromethorphan (DXM, Fig. 1), (+)-3-methoxy-17-methyl-(9α,13α,14α)-morphinan, is a dextrorotatory morphinan with a chemical structure related to the levorotatory morphinans of levorphanol, codenine and morphine (Chen et al. 2007). DXM, which is widely used as an over-the-counter antitussive drug for the treatment of pain and cough, is normally used as a kind of ideal probe drug for phenotyping study because of its safety and the wide availability (Chladek et al. 1999; Schmid et al. 1985). As shown in Fig. 1, it has been reported that DXM is mainly transformed to dextrorphan (DXO, Fig. 1) via *O*-demethylation by the polymorphic cytochrome P450 2D6 (CYP2D6), of which polymorphism is highly expressed in humans who can be sorted as poor, intermediate, and extensive metabolizer (Schmid et al. 1985). DXM and DXO can undergo *N*-demethylation to 3-methoxymorphinan (3-MEM) and 3-hydroxymorphinan (3-HM), respectively, primarily mediated by CYP3A4 (Gorski et al. 1994; Jacqz-Aigrain et al. 1993). As for the *O*-demethylation of 3-MEM to 3-HM CYP2D6 is involved. Finally, DXO and 3-HM are glucuronidated to their *O*-glucuronides which are mainly excreted into urine (Koppel et al. 1987; Lutz et al. 2008). Therefore, the simultaneous analysis of DXM and DXO would be practically beneficial for assessing CYP2D6 activity.

In fact, it is widely believed that the therapeutic effect of DXM is caused by both the drug and DXO. Because of the low systemic levels of DXM and DXO in most individuals, highly sensitive methods are required for determinating these analytes in plasma samples. A variety of methods for the simultaneous detection of DXM and its metabolites have been described such as capillary electrophoresis (CE) (Aumatell and Wells 1993; Kristensen 1998), high performance liquid chromatography (HPLC) (Afshar et al. 2004; Bendriss et al. 2001; Hendrickson et al. 2003; Kim et al. 2006b; Lin et al. 2007), gas chromatography-mass spectrometry (GC-MS) (Bagheri et al. 2005; Kim et al. 2006a; Rodrigues et al. 2008; Spanakis et al. 2009), thin layer chromatography (TLC) (Guttendorf et al. 1988) and radioimmunoassay (RIA) (Dixon et al. 1978). However, all these methods suffer from lack of sensitivity, require extensive sample clean-up and time consuming chromatography. Furthermore, additional specificity is necessary to avoid potential assay interference from the co-administered drug and/or its metabolites.

The liquid chromatography-mass spectrometry (LC-MS) technique requires less extensive sample preparation and provides better sensitivity and specificity than the conventional methods mentioned above. So far, some LC-MS methods for the simultaneous determination of DXM and its metabolites in various matrices such as plasma, saliva and urine have been reported (Constanzer et al. 2005; Kikura-Hanajiri et al. 2011; Liang et al. 2009; Lutz et al. 2004; Vengurlekar et al. 2002). In this study, we describe a sensitive, simple and reproducible LC-MS-MS method for the simultaneous determination of DXM and DXO, and its application to a pharmacokinetic study in rat plasma.

2. Investigations and results

2.1. LC Method

To avoid interference from exogenous compounds co-eluted with the target compound, MS/MS detection, offering unique selectivity against matrix background and requiring very limited sample preparation was performed. And in order to select

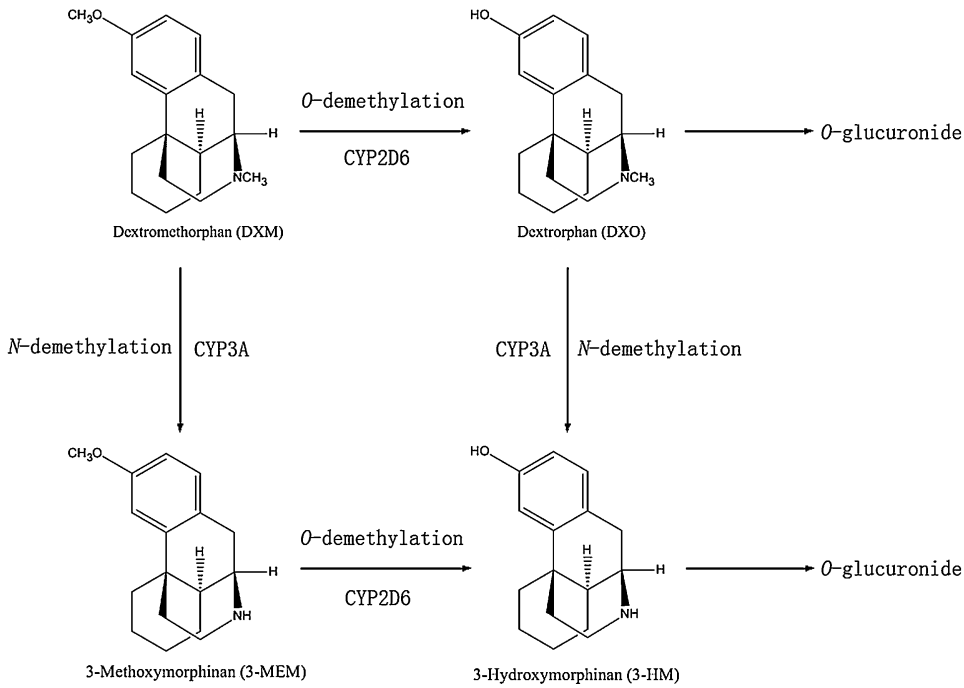

Fig. 1: Chemical structure and typical metabolic pathway of dextromethorphan in humans

an appropriate ionization mode in LC-MS analysis, the mass spectra were compared in ESI and APCI, both positive and negative mode. As a result, ionization of analytes was chosen using the ESI technique with positive polarity and multiple reaction monitoring (MRM) mode. MRM was performed at unit resolution using the mass reansition ion-pairs *m/z* 271.8→214.6 for DXM, *m/z* 257.9→200.6 for DXO and *m/z* 185.9→157.9 for pirfenidone (IS), shown in Fig. 2.

The separation of DXM, DXO and the IS were influenced by composition of the mobile phase. Water-acetonitrile/methanol, water containing formic acid-acetonitrile/methanol were evaluated for optimizing the condition of mobile phase. It was found that acetonitrile had to higher sensitivity and lower background noise than methanol. In addition, 0.1% formic acid made the peak shape shaper. Above all, gradient elution improved separation and the running time. Therefore, a gradient elution programme was employed for the chromatographic separation with mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile) as follows: 0–1.5 min (10–85% B), 1.5–6.0 min (85–85% B), 6.0–7.0 min (85–10% B), 7.0–10.0 min (10–10% B). Under such conditions, DXM and DXO were perfectly separated based on their chromatographic and extraction behaviors as well as IS.

A critical process for the determination of drugs in biological samples is sample preparation. Ethyl acetate, diethyl ether, chloroform were taken as solvent to extract analytes by liquid-liquid extraction and methanol, acetonitrile, 10% perchloric acid were tested for protein precipitation. Acetonitrile precipitation was simpler, faster, and had higher recovery than other precipitants, so that sample pretreatment with acetonitrile was used. Above all, the sample processing time was shorter than in other reported methods (Hendrickson et al. 2003; Liang et al. 2009).

2.2. Specificity and matrix effects

Typical chromatograms of blank plasma and plasma spiked with DXM, DXO and IS are shown as well as samples from rats are shown in Fig. 3. Under the conditions described in the experimental part, the assay was highly specific, and no interfering endogenous plasma materials were observed during the analysis of plasma samples. The peak shapes of DXM, DXO and IS were symmetrical and the retention times were 3.6, 3.2 and 3.5 min, respectively. Interestingly, the method also shorted the retention time compared with other published LC-MS/MS methods which were more time-consuming (Arellano et al. 2005; Lutz et al. 2004; Vengurlekar et al. 2002).

The matrix effect of the assay for DXM (2, 20, 400 ng/mL) and DXO (2, 20, 200 ng/mL) were evaluated. The percent nominal concentrations determined were 102.7 ± 9.3, 108.5 ± 5.8, and $109.4 \pm 6.6\%$ for DXM, 103.8 ± 2.7, 108.9 ± 7.2, and $106.7 \pm 5.6\%$ for DXO, respectively, at each concentration level ($n = 3$). The evaluation was also performed for IS and the percent concentration was $97.6 \pm 7.6\%$ ($n = 3$). As a result, the ME from plasma was negligible in this analytical method.

2.3. Linearity and lower limit of quantification

The standard calibration curves showed good linearity within the range using leasts quares regression analysis. The linearity for DXM and DXO were investigated by linear regression of peak area ratios against concentrations. The regression equation for the calibration plot was $Y = 0.0189C + 0.0197$, $r^2 = 0.9996$ for DXM and $Y = 0.011C - 0.1155$, $r^2 = 0.9994$ for DXO (Y is the peak ratio of analyte to IS, and C is the concentration of analyte in plasma), for concentrations in the range 1–500 ng/mL for DXM and 1–250 ng/mL for DXO, respectively. The detection limit, defined as a signal-noise ratio of 3, was 0.2 ng/mL for DXM and 0.3 ng/mL for DXO in plasma. The lower limit of quantitation (LLOQ), defined as the concentration giving a signal-noise ratio of 10, was 1.0 ng/mL for both DXM and DXO in plasma, which are sufficient for pharmacokinetics and clinical studies.

2.4. Precision and accuracy

Accuracy and precision of the method were determined with QC samples as described above. The relative standard deviation (RSD) and relative error (RE) were calculated. As shown in the Table, this method allowed good precision and accuracy. Intra- and inter-day precision (RSD) were 4.63–8.82 and

Table 1: Precision and accuracy of method for the determination of DXM and DXO in rat plasma (n = 5)

	Concentration added (ng/mL)	Concentration measured (ng/mL) Mean ± SD	RSD (%)	RE (%)
DXM				
Intra-day	2	2.04 ± 0.18	8.82	2.05
	20	19.27 ± 1.62	8.41	−3.65
	400	416.74 ± 19.30	4.63	4.18
Inter-day	2	1.94 ± 0.19	9.80	−3.00
	20	21.39 ± 1.77	8.27	6.39
	400	420.75 ± 21.50	5.11	5.19
DXO				
Intra-day	2	1.97 ± 0.19	9.64	−1.52
	20	20.46 ± 0.97	4.74	2.31
	200	208.57 ± 15.35	7.36	4.29
Inter-day	2	2.12 ± 0.15	7.08	6.10
	20	19.44 ± 1.40	7.20	−2.83
	200	211.23 ± 17.63	8.35	5.62

5.11–9.80% for DXM, 4.74–9.64 and 7.08–8.35% for DXO. Accuracy were 96.35–106.39% for DXM and 97.17–106.10% for DXO, respectively.

2.5. Recovery

The mean recovery of DXM from plasma at concentrations of 2, 20 and 400 ng/mL were found to be 85.2, 87.3 and 89.2% (n = 5), respectively. And the mean recovery of DXO at concentrations of 2, 20 and 200 ng/mL were 81.3, 84.0 and 85.8% (n = 5). The recovery of the IS was 86.6% (n = 5).

2.6. Stability

All the stability studies of DXM and DXO in plasma were conducted at three concentration levels (2, 20, and 400 ng/mL for DXM, and 2, 20, and 200 ng/mL for DXO) with five determinations for each under different storage conditions. The RSDs of the mean test responses was within 10% in all stability tests of DXM and DXO in plasma.

No effect on the quantitation was observed for plasma samples kept at room temperature for 6 h. Samples were also stable for at least 12 h in an autosampler. There was also no significant degradation when samples of DXM and DXO in plasma were taken through three freeze (−20 °C)-thaw (room temperature) cycles. DXM and DXO in plasma was stable at −20 °C for 3 weeks.

2.7. Assay application

The validated method has been successfully applied to a pharmacokinetic study in six rats. The rats received a single oral dose of 15 mg/kg of DXM and pharmacokinetic parameters of DXM and DXO were determined. The representative DXM and its metabolite DXO concentration versus time profiles were presented in Fig. 4.

The data of DXM and DXO plasma concentrations-time were fitted using DAS ver. 2.0 and the values of AIC (Akaike's Information Criterion). As the results showing that the value of AIC is minimum of all under the two-compartment model (weight coefficient = $1/c^2$), we take the two-compartment model to analyze the data. The main pharmacokinetic parameters of DXM and DXO in rats were as follow: $T_{1/2}$ were 10.03 ± 9.01 h and 10.96 ± 5.24 h; T_{max} were 0.98 ± 0.31 h and 0.75 ± 0.20 h; C_{max} were 388.59 ± 19.76 ng/mL and 290.87 ± 16.30 ng/mL; $AUC_{0\rightarrow t}$ were 3848.34 ± 331.22 ng/mL•h and 1089.76 ± 306.37 ng/mL•h; $AUC_{0\rightarrow\infty}$ were 4927.16 ± 1831.89 ng/mL•h and 1249.39 ± 291.82 ng/mL•h, respectively.

2.8. Conclusions

A sensitive LC-MS/MS method has been developed and validated for the simultaneous determination of DXM and its metabolite DXO in rat plasma. The precision and accuracy for calibration and QC samples were well within the acceptable limits. A simplified plasma extraction procedure and reproducibility of the assay make it suitable for pharmacokinetics studies of DXM and this method is successfully employed in a pharmacokinetic study of DXM in rats.

3. Experimental

3.1. Chemicals and reagents

DXM and DXO (both > 98.0% purity) was purchased from Sigma-Aldrich Company (St. Louis, USA), and pirfenidone (IS, purity > 98.0%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China). LC-grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). All other chemicals were analytical grade and used without further purification. Ultrapure water (resistance > 18 mΩ) prepared by a Millipore Milli-Q purification system (Bedford, USA) was used to make mobile phase and all other solutions.

3.2. Apparatus and chromatographic conditions

All analysis was performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment, and a Bruker Esquire HCT mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software.

Chromatographic separation was achieved on an Agilent Zorbax SB-C18 (2.1 mm × 50 mm, 3.5 μm) column at 25 °C. A gradient elution programme was conducted for chromatographic separation with mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile) as follows: 0–1.5 min (10–85% B), 1.5–6.0 min (85–85% B), 6.0–7.0 min (85–10% B), 7.0–10.0 min (10–10% B). The flow rate was 0.4 mL/min.

The determination of DXM and DXO was performed in MRM mode (*m/z* 271.8→214.6 for DXM, *m/z* 257.9→200.6 for DXO and *m/z* 185.9→157.9 for pirfenidone (IS)) and positive ion electrospray ionization interface. Drying gas flow was set to 7 L min^{-1} and temperature to 350 °C. Nebuliser pressure and capillary voltage of the system were adjusted to 25 psi and 3,500 V, respectively.

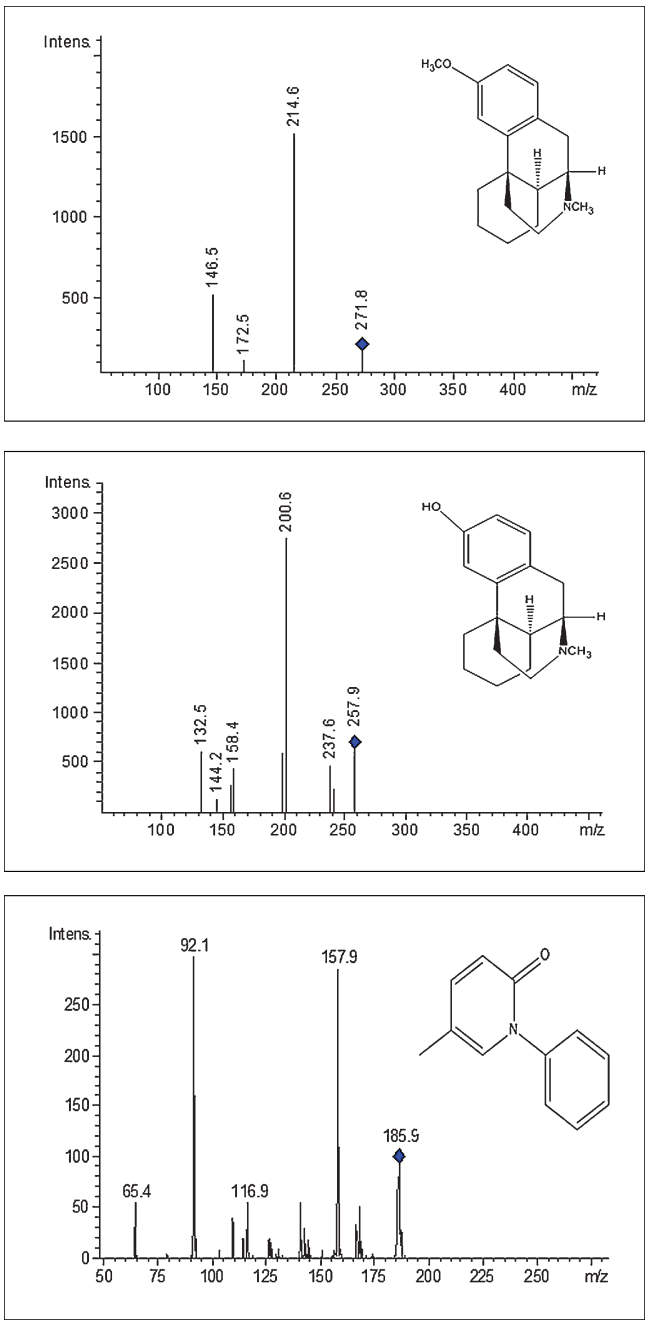

Fig. 2: MS spectrum of the peak corresponding to DXM, DXO and pirfenidone (IS)

3.3. *Sample preparation*

In a 1.5 mL centrifuge tube, an aliquot of 10 µL of the internal standard working solution (200 ng/mL) was added to 0.1 mL of collected plasma sample followed by the addition of 0.2 mL of acetonitrile. After the tube was vortex-mixed for 1.0 min, the sample was centrifuged at 15,000 rmp for 10 min. The supernatant (10 µL) was injected into the LC-MS system for analysis. The standards were prepared in the same way.

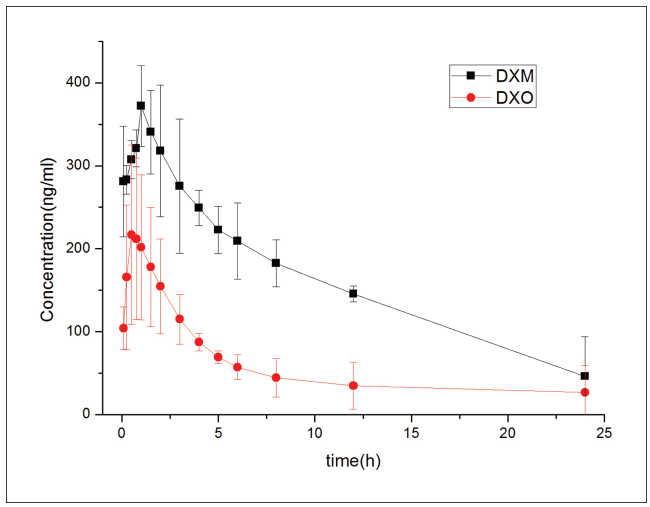

Fig. 4: Mean plasma concentration-time profile of DXM and DXO following a single oral dose of 15 ng/ml to rats (n= 6)

3.4. *Preparation of calibration standards and quality-control (QC) samples*

Individual stock solutions of DXM (1.0 mg/mL), DXO (1.0 mg/mL) and pirfenidone (IS) (1.0 mg/mL) were separately prepared in methanol and stored at 4 °C. Working standard solutions for calibration and controls were prepared by serial dilution of the stock solution with methanol. The working standard solution of IS (200 ng/mL) was prepared by diluting its stock solution with methanol. Calibration standards were prepared by spiking blank rat plasma with appropriate amounts of the working solutions. Calibration plots were constructed at concentrations of 1, 2, 5, 10, 50, 100, 200, 500 ng/mL for DXM and 1, 2, 5, 10, 25, 50, 100, 250 ng/mL for DXO in rat plasma. Low, medium, and high quality control (QC) samples at 2, 20, 400 ng/mL for DXM and 2, 20, 200 ng/mL for DXO were prepared by the same way as the calibration standards.

3.5. *Method validation*

To evaluate the linearity, the calibration curves were generated using the analyte to IS peak area ratios by weighted ($1/x^2$) least-squares linear regression on three consecutive days. The lower limit of quantitation (LLOQ) was determined at the concentration with a signal-to-noise ratio of 2.

Specificity was determined by analysis of blank plasma from five different rats, without addition of DXM, DXO and the IS to determine possible interference with these compounds.

To evaluate the matrix effect (ME), blank rat plasma were precipitated and then spiked with the analyte at 2, 20, 400 ng/mL for DXM and 2, 20, 200 ng/mL for DXO. The corresponding peak areas were then compared with those of neat standard solutions at equivalent concentrations, and this peak area ratio is defined as the matrix effect. The ME of IS was evaluated at the concentration (20 ng/mL) in the same manner.

The intra-day precision and accuracy were evaluated by analyzing QC samples with five replicates for each concentration. The inter-day precision and accuracy were tested by analyzing QC samples with five replicates for each concentration over five consecutive days. The assay precision for each QC level was determined as the relative standard deviation (RSD) of the mea-

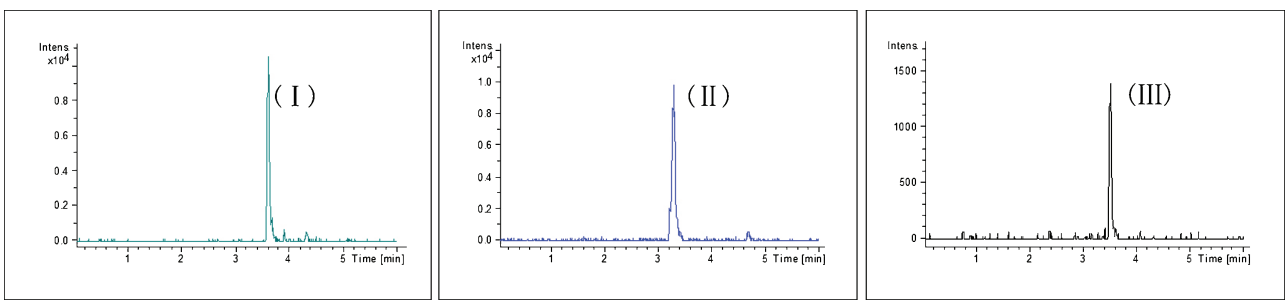

Fig. 3: Representative LC-MS/MS chromatograms for DXM (I), DXO (II) and pirfenidone (IS, III) in a rat plasma sample 30 min after oral administration of single dosage 15 mg/kg DXM

sured concentrations. The assay accuracy was calculated as relative error (RE). The intra- and inter-day precisions were required to be below 15%, and the accuracy to be within ± 15%.

Extraction recovery experiments of DXM and DXO were evaluated by comparing the peak areas obtained from extracted QC samples (2, 20, 400 ng/mL for DXM and 2, 20, 200 ng/mL for DXO) with unextracted standard working solutions at three concentrations in the same solvent. Recovery of IS from plasma was determined at a concentration of 20 ng/mL.

Sample stability was tested by analyzing QC samples after shorterm (6 h) storage at room temperature, 12 h storage in an autosampler and on storage at −20 °C for 3 weeks. The effect of three freeze (−20 °C)-thaw (room temperature) cycles was also investigated.

3.6. Pharmacokinetic study

Six Wistar male rats (220 ± 10 g) raised from Wenzhou Medical College Laboratory Animal Center (Wenzhou, China), were used to study the pharmacokinetics of DXM. All six animals were housed at the Wenzhou Medical College Laboratory Animal Research Center. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the Guide for the Care and Use of Laboratory Animals. After overnight fast (12 h), rats were given intragastrically with DXM at a dose of 15 mg/kg. Water was freely accessible after the rat had been given the drug for 3 h, but no food was allowed until the rats had been given the drug for six hours. Blood samples (0.4 mL) were directly collected into the heparinized tube through the tail vein at 0 (prior to dosing), 0.08, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24 h after the administration. The samples were immediately centrifuged at 5,000 rpm for 10 min, 100 μL plasma were transferred to another tube and stored at −20 °C until analysis. Plasma DXM and DXO concentration versus time data for each rat was analyzed by DAS software (Version 2.0, Medical College of Wenzhou, China).

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