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A sensitive LC-MS/MS assay for the determination of dextromethorphan and metabolites in human urine—application for drug interaction studies assessing potential CYP3A and CYP2D6 inhibition

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Abstract

The commonly used antitussive dextromethorphan can be used to simultaneously assess potential cytochrome P450 3A (CYP3A) and CYP2D6 inhibition during drug development. The metabolism of dextromethorphan to dextrorphan and subsequently to 3-hydroxymorphinan are via the 2D6 pathway, while the metabolism of dextromethorphan to 3-methoxymorphinan is via the 3A pathway. A sensitive and specific LC-MS/MS assay has been developed to determine the human urine concentrations of dextromethorphan and three metabolites (dextrorphan, 3-methoxymorphinan and 3-hydroxymorphinan) in support of drug interaction studies. Urine samples (0.5 ml), after enzymatic hydrolysis of the conjugates and containing 3-ethylmorphine as an internal standard, were extracted with chloroform under basic conditions. Following concentration and reconstitution, the samples were analyzed by LC-MS/MS. The assay was linear over the range of 5.00–500 ng/ml for dextromethorphan and 3-methoxymorphinan; and 200–3000 ng/ml for dextrorphan and 3-hydroxymorphinan using a Perkin–Elmer Sciex® triple quadrupole mass spectrometer (API 300). The intra- and inter-day relative standard deviation (RSD) across three validation runs over the entire concentration range for all analytes was less than 15%. Accuracy determined at three or four concentrations (9.00, 200, and 400 ng/ml for dextromethorphan and 3-methoxymorphinan; 250, 400, 1300 and 2500 ng/ml for dextrorphan and 3-hydroxymorphinan) ranged between 96.3 and 113.8%. The stability of analytes in urine was demonstrated for 9 months at −20 °C, 24 h under ambient conditions and for up to three freeze/thaw cycles. The method described herein is suitable for the rapid and efficient measurement of dextromethorphan and different metabolites to estimate potential CYP3A inhibition by drug candidates and for screening of extensive and poor metabolizers of CYP2D6 in the heterogeneous population. The method has subsequently been validated on a Sciex API 3000 with lower limit of quantitation; 1.00 ng/ml for dextromethorphan and 3-methoxymorphinan; 60.0 ng/ml for dextrorphan and 100 ng/ml for 3-hydroxymorphinan.

Keywords: Dextromethorphan; Quantitation; Urine; CYP450 3A; CYP450 2D6

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1. Introduction

The cytochrome P450 (CYP) superfamily plays an important role in the biotransformation of many xenobiotics. Dextromethorphan is a safe, orally administered antitussive that is universally available without prescription for which the metabolic fate in humans has been well characterized (Fig. 1). Dextromethorphan 0-demethylation to dextrorphan is primarily mediated by CYP2D6 which is polymorphically expressed in humans and is under genetic control, co-segregating with the well-described debrisoquine oxidation polymorphism [1–8] such that poor metabolizers can be distinguished from the extensive metabolizers. [9] An additional route of dextromethorphan metabolism is *N*-demethylation to 3-methoxymorphinan [10] which is primarily mediated by CYP3A4 and CYP3A5 in human liver microsomes [11] and intestinal flora. The dextrorphan and 3-methoxymorphinan are then demethylated to 3-hydroxymorphinan via 2D6. [12] It has been earlier demonstrated by Jones et al. [12,13] that biotransformation of dextromethorphan can be used as a simultaneous marker of CYP3A and CYP2D6 activity.

Various methods for the simultaneous detection of dextromethorphan and metabolites have been described such as high performance liquid chromatography (HPLC) with UV- or fluorescence detection as well as thin layer chromatography (TLC) & radioimmunoassay (RIA). [12,14–26] However, all these methods suffer from lack of sensitivity, require extensive sample clean-up as well as time consuming chromatography. In addition, for drug interaction studies, additional specificity is required to avoid potential assay interference from the co-administered drug and/or its metabolites. The liquid chromatography-mass spectrometry technique requires less extensive sample preparation and provides increased sensitivity and specificity than conventional HPLC.

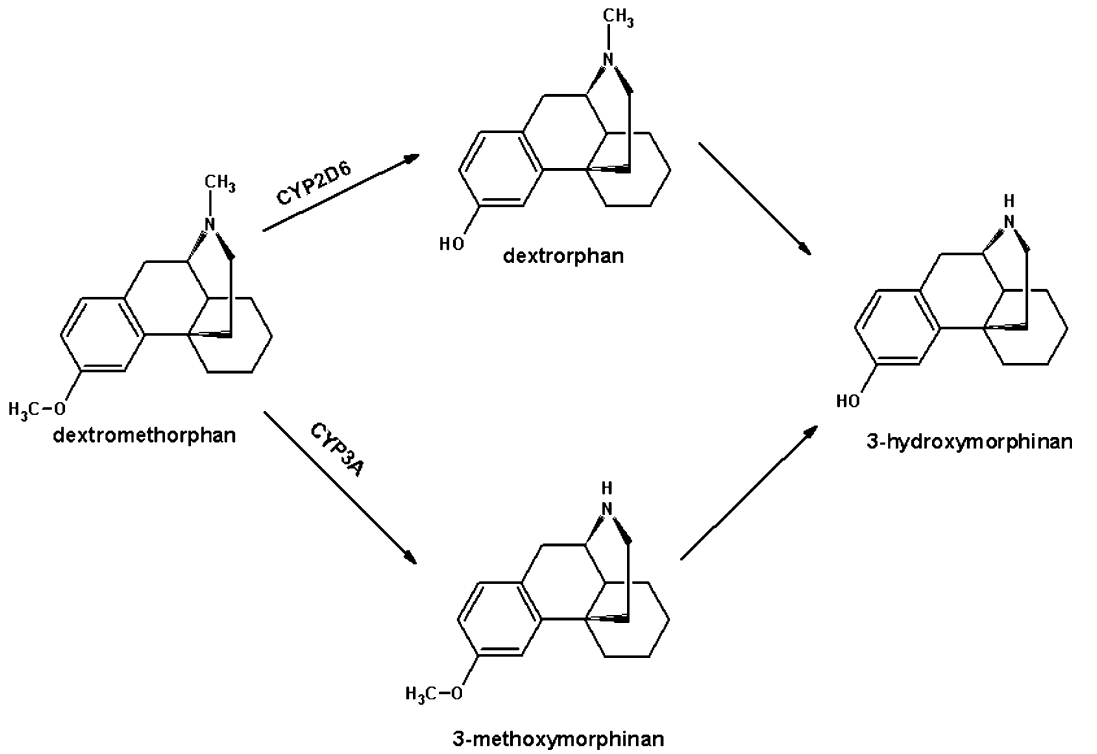

Fig. 1. Chemical structures and metabolic pathway of dextromethorphan.

The authors describe a simple and reproducible LC-MS/MS method for the simultaneous determination of urinary dextromethorphan and its metabolites. Since samples containing incurred residue are known to contain conjugates of dextrorphan and 3-hydroxymorphinan, samples are subjected to enzymatic hydrolysis prior to extraction. Analytes are removed from the samples by liquid–liquid extraction followed by concentration and LC-MS/MS analysis.

2. Experimental

2.1. Chemicals and reagents

Dextromethorphan and internal standard, 3-ethylmorphine, were purchased from Sigma-Aldrich (St. Louis, MO, USA); dextrorphan was obtained from Gentest; 3-methoxymorphinan and 3-hydroxymorphinan were purchased from Research Biochemicals Inc. (Natick, MA, USA). The stock solutions were prepared by dissolving the respective compounds in acetonitrile and were stored at −20 °C. Working solutions were prepared by dilution with control urine to concentrations of 2500 ng/ml for dextromethorphan and 3-methoxymorphinan; 10 000 ng/ml for dextrorphan and 3-hydroxymorphinan. Control human urine (void of the xenobiotic) was obtained from Biochemed (Winchester, VA, USA). Working internal standard solution was prepared in water at 25 000 ng/ml. The structures for the referenced compounds are provided in Fig. 1. β-glucuronidase, sodium carbonate, and ammonium acetate were purchased from Sigma-Aldrich. Acetonitrile, methanol and chloroform were purchased from Burdick & Jackson (Muskegon, MI, USA). Glacial acetic acid and purified water were purchased from Fisher Scientific (Pittsburgh, PA, USA). All chemicals and solvents were of analytical reagent or HPLC grade.

2.2. Preparation of calibration standards

During validation, calibration standards were freshly prepared in urine for each run covering a concentration range of 5.00–500 ng/ml for dextromethorphan and 3-methoxymorphinan, and 200–3000 ng/ml for dextrorphan and 3-hydroxymorphinan.

2.3. Preparation of quality control samples

Quality control (QC) samples were prepared from stock solutions weighed independently of the stock solutions used to prepare the calibrators. QC samples were prepared at the beginning of the validation at three or four different concentrations: 9.00, 200, and 400 ng/ml for dextromethorphan and 3-methoxymorphinan and 250, 400, 1300, and 2500 ng/ml for dextrorphan and 3-hydroxymorphinan in human urine. QC samples were stored at −20 °C to simulate the storage conditions of the study urine samples.

2.4. Sample preparation

2.4.1. Enzymatic hydrolysis

The quantity of β-glucuronidase, incubation time and the strength of ammonium acetate buffer (pH 5.0) required for complete hydrolysis of the conjugated metabolites were investigated and optimized by incubating 0.5 ml of human urine samples containing incurred residues under a variety of conditions (Table 1). As evident from Table 1, buffer strength was the most critical parameter for the activity of the enzyme. To ensure complete hydrolysis of the conjugates, a 12–18-h incubation of 0.5 ml urine sample with 5000 units of β-glucuronidase enzyme at 100 mM final buffer concentration (ammonium acetate buffer, pH 5.0) at 37 ± 2 °C was selected.

2.4.2. Liquid–liquid extraction

A 50-μl aliquot of internal standard solution (3-ethylmorphine, 25.0 μg/ml) was added to each hydrolyzed urine sample (calibration standards, QC samples, and sample with incurred residue) containing dextromethorphan and its metabolites (3-methoxymorphinan, dextrorphan, and 3-hydroxymorphinan) and vortex mixed for approximately 1 min. A 0.5 ml volume of saturated sodium carbonate was then added to each tube with vortex mixing for about 10 s followed by addition of 3 ml of chloroform to each tube. The

Table 1
Optimization of hydrolysis conditions using urine samples containing incurred residues

Sample ID	Buffer concentration (mM)	Enzyme (units)	Incubation time (h)	Analyte concentration (ng/ml)	
				Dextrorphan	3-Hydroxymorphinan
Effect of buffer concentration on hydrolysis (*ammonium acetate*, *pH 5.0*)					
001	20	5000	18	3802	1150
001	20	5000	18	3900	1214
001	100	5000	18	6316	2066
001	100	5000	18	6313	2121
Hydrolysis with variable units of enzyme activity (*β-glucuronidase*)					
002	100	5000	18	1905	820
002	100	5000	18	2119	975
002	100	10 000	18	2102	962
002	100	10 000	18	2144	940
002	100	20 000	18	2223	1011
002	100	20 000	18	1726	747
Evaluation of optimal hydrolysis time					
002	100	5000	6	1559	812
002	100	5000	6	1665	796
002	100	5000	18	1905	820
002	100	5000	18	2119	975
002	100	5000	24	2199	1015
002	100	5000	24	2279	1027

tubes were capped, mixed for 2 min on a multi-tube vortexer, and centrifuged at about 2100 rcf for 10 min. The chloroform (bottom) layer was pipetted into a clean tube leaving about 0.10 ml of chloroform in the tube behind, being careful not to remove any of the aqueous layer. The steps involving addition of chloroform were repeated twice with 2 ml of additional chloroform each time and the three chloroform extracts were combined. Solvent was evaporated with nitrogen at about 35 °C and 0.25 ml of acetonitrile was added and vortex mixed for 5 min. A 0.25 ml volume of 10 mM ammonium acetate buffer, pH 4.75, was added and vortex mixed for 5 min. The extract was transferred into an autosampler vial for LC-MS/MS analysis. The 50% organic component was necessary to assure solubility for all analytes. No untoward effects on chromatography were observed from the 50% organic reconstitution solvent interacting with the initially low organic component of the mobile phase.

2.4.3. Chromatography

The HPLC system consisted of Shimadzu (Columbia, MD, USA) LC-10AT pumps and a Shimadzu SIL-10A autosampler. Chromatographic separations were performed on a Zorbax RX-C8 column (2.1 mm × 15 cm, 5-μm) operated at ambient temperature. The injected sample volume was 100 μl for the API 300 and 10 μl for the API 3000. The mobile phases were: (A) 15 mM ammonium acetate, pH 4.75 and (B) acetonitrile/methanol (50/50; v/v) and the flow-rate was 0.50 ml/min. The gradient ranged from 1 to 99% B over 8 min. The gradient elution and run time were chosen in order to separate salts that could effect ionization and to separate dextrorphan from 3-methoxymorphinan, because they had identical precursor/product ions. The entire chromatographic effluent was passed into the mass spectrometer interface for detection. Under these conditions, the HPLC run time for each injection was approximately 11 min, including the equilibration time.

2.4.4. Mass spectrometry

The original method validation was performed on a Sciex (Concord, Ontario, Canada) Model API 300 triple quadrupole mass spectrometer interfaced to the column eluent via a Sciex Turbo Ionspray probe operating at 350 °C and 5000 V. The method has subsequently been successfully validated on a Sciex API III plus and API 3000 with a much higher sensitivity. Operating conditions for API 300 were optimized by flow injection of a mixture of all analytes at a flow-rate of 0.5 ml/min and were determined as follows: nebu-

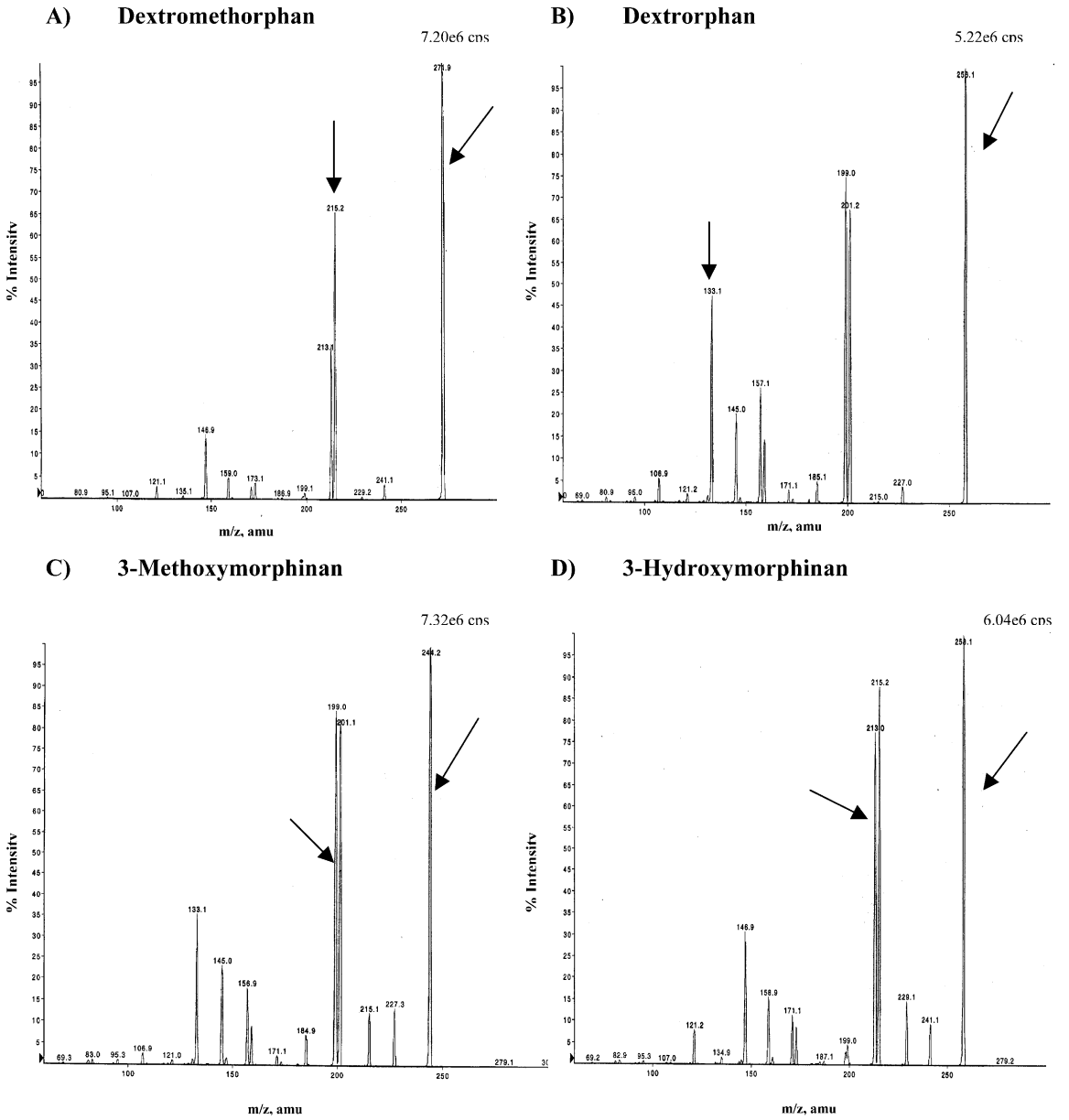

Fig. 2. Product ion mass spectra of MH^+ ions for dextromethorphan, dextrorphan, 3-methoxymorphinan, 3-hydroxymorphinan, and 3-ethylmorphine.

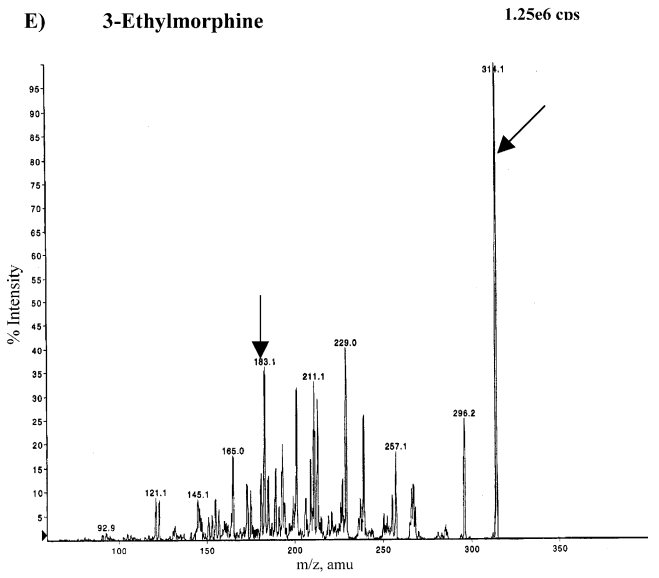

Fig. 2. (*Continued*)

lizing gas pressure, 12 psi; curtain gas, 8 psi. Multiple reaction monitoring (MRM) experiments in the positive ionization mode were performed using a dwell time of 300 ms per transition to detect ion pairs at m/z 272/215 (dextromethorphan), 258/133 (dextrorphan), 244/199 (3-hydroxymorphinan), and 258/213 (3-methoxymorphinan). These product ions were chosen based on their significance in the MS/MS spectra (Fig. 2). The MRM spectra for each analyte using LC-MS/MS method is provided in Fig. 3.

2.4.5. Quantification

To establish calibration curves, various amounts of standard working solutions were added to control human urine to yield six final concentrations at 5.00, 10.0, 50.0, 100, 250 and 500 ng/ml for dextromethorphan and 3-methoxymorphinan and 200, 300, 500, 1000, 1500 and 3000 ng/ml for dextrorphan and 3-hydroxymorphinan. Ratios of peak area of each analyte to that of 3-ethylmorphine were computed using Sciex's MACQUAN (version 1.5) software. Analyte concentrations were calculated using the internal standard method. The calibration curves were calculated from the peak area ratios of analyte/internal standard and the nominal analyte concentrations using linear regression with $1/x^2$ for dextromethorphan and 3-methoxymorphinan and $1/x$ weighting for dextrorphan and 3-hydroxymorphinan. The intra and inter-run accuracy and precision for the QC samples (control urine samples spiked with known amounts of each analyte) is presented in Table 2.

2.4.6. Lower limit of quantification (LLOQ)

Initially, the LLOQ values were 5.00 ng/ml for dextromethorphan and 3-methoxymorphinan and 200 ng/ml for dextrorphan and 3-hydroxymorphinan. Upon receipt and installation of a more sensitive LC-MS/MS API 3000 mass spectrometer, the LLOQ was subsequently lowered to 1.00 ng/ml for dextromethorphan and 3-methoxymorphinan; from 200 to 60.0 ng/ml for dextrorphan and to 100 ng/ml for 3-hydroxymorphinan. However, the extended concentration range for dextrorphan and 3-hydroxymorphinan necessitated the use of a quadratic regression model. The accuracy and precision data on the improved LLOQ is provided in Table 3 and Fig. 4.

3. Results

3.1. Specificity

HPLC conditions were optimized so that salt ions would not affect quantitation and so all analytes were satisfactorily separated. For all analytes, the tandem MS detection mode used specific precursor/product ion transitions. The product ion scan for dextromethorphan metabolites and the internal standard are shown in Fig. 2. A typical chromatogram obtained with a standard mixture of all analytes detected is shown in Fig. 3. Specificity was demonstrated by analyzing six different lots of control blank urine samples. Fig. 4A shows the MS/MS response of a control blank urine sample. Fig. 4B shows the same sample spiked at 1.00 ng/ml for dextromethorphan and 3-methoxymorphinan and 60.0 and 100 ng/ml for dextrorphan and 3-hydroxymorphinan, respectively; the new lower limit of quantification (LLOQ) for each analyte. Sample carryover effect was not observed as evidenced by the absence of MS/MS response in the regions of the peaks of

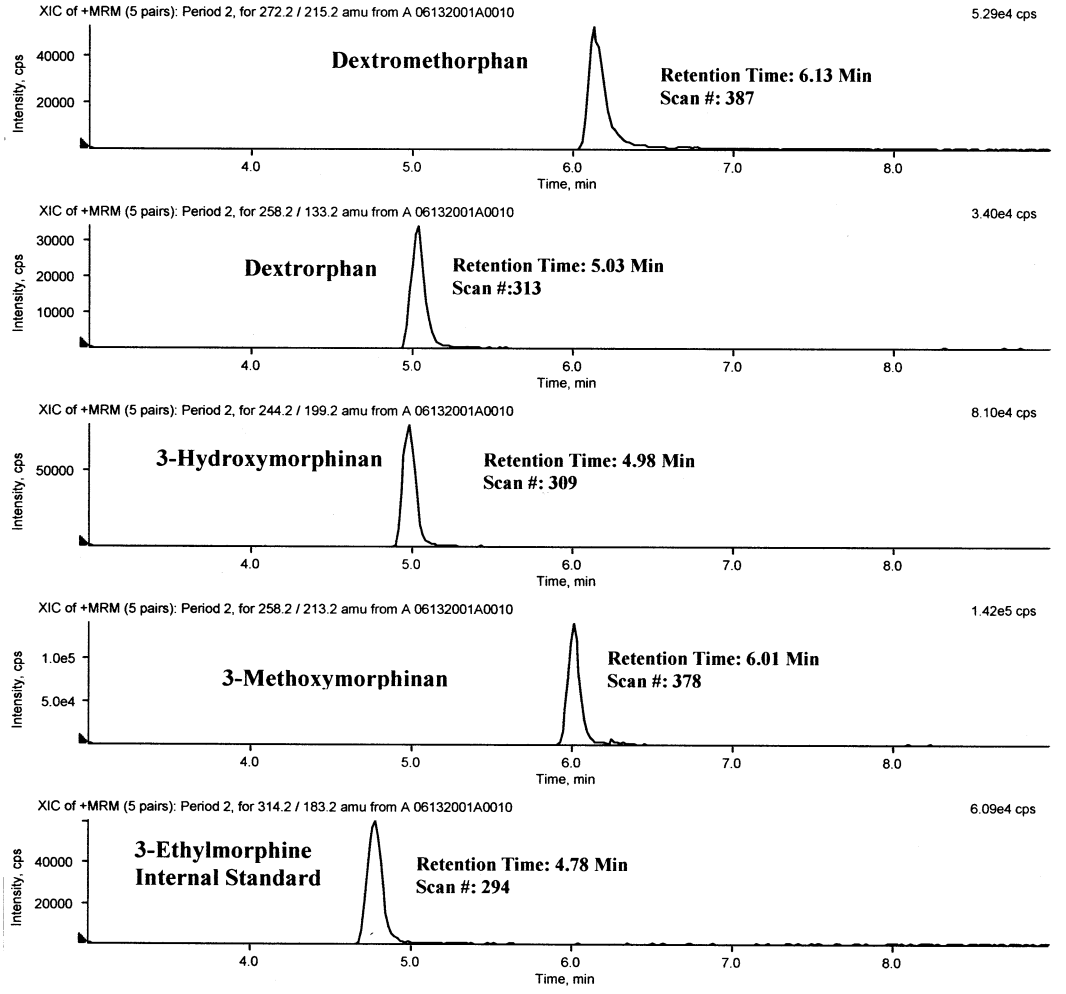

Fig. 3. Multiple reaction monitoring (MRS) of MH^+ ions for dextromethorphan, (A) dextrorphan, 3-methoxymorphinan, 3-hydroxymorphinan, and 3-ethylmorphine dextromethorphan (B) dextrorphan (C) 3-methoxymorphinan (D) 3-hydroxymorphinan (E) 3-ethylmorphine.

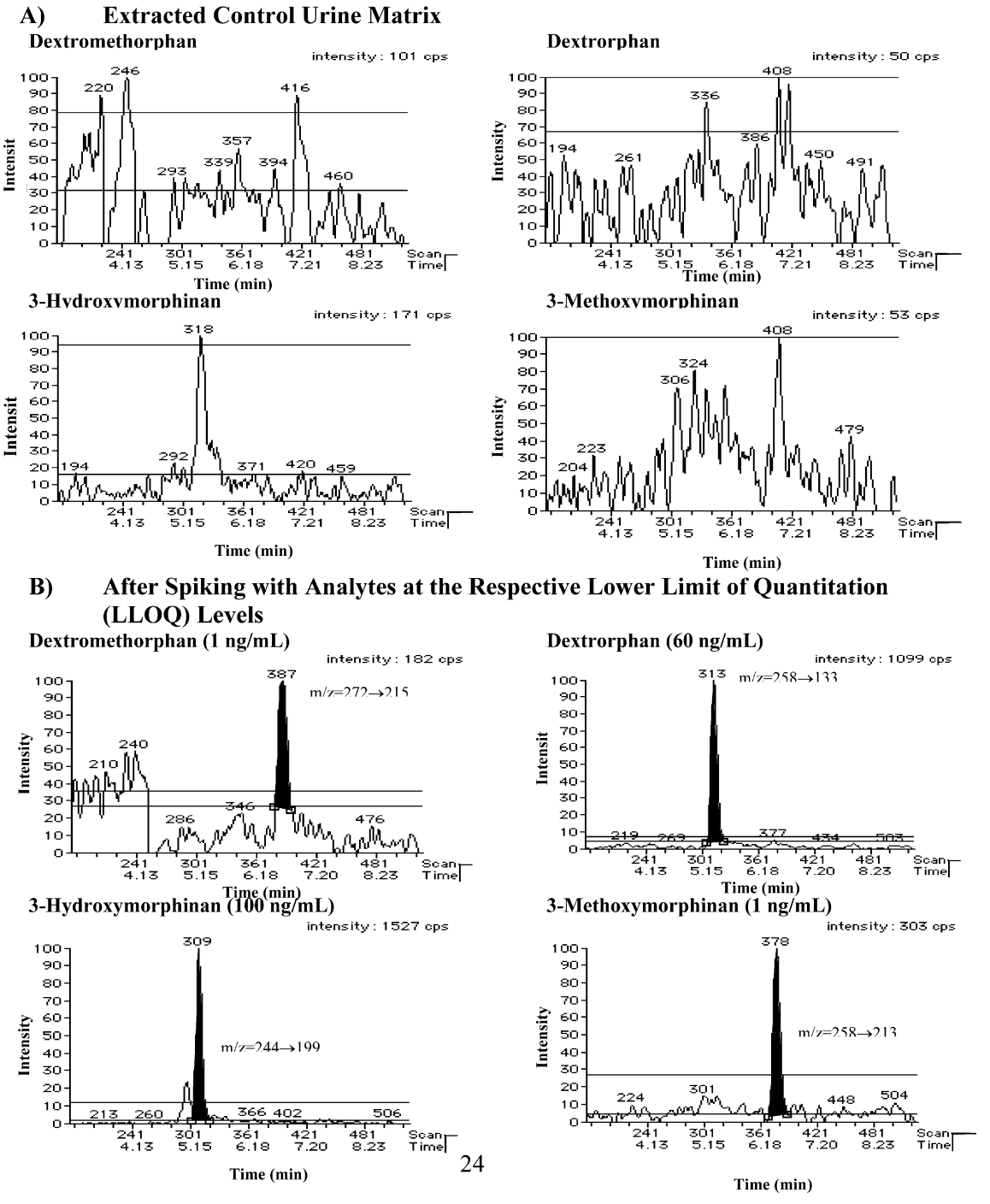

Fig. 4. MRM chromatograms of (A) an extracted control urine matrix, and (B) after spiking with analytes at the respective LLOQ concentrations.

Table 2
Accuracy and precision of dextromethorphan and its metabolites in QC samples (spiked control urine matrix)

Spiked	Concentration (ng/ml)	*n*	Mean (%) bias	% RSD
Dextromethorphan	9.00	15	3.1	7.4
	200	15	−3.7	10.2
	400	15	3.6	8.6
3-Methoxymorphinan	9.00	15	4.8	9.7
	200	15	−1.9	8.0
	400	15	13.8	5.7
Dextrorphan	250	14	3.7	13.5
	400	15	6.6	11.0
	1300	15	2.1	10.8
	2500	15	−2.0	13.5
3-Hydroxymorphinan	250	15	12.5	9.8
	400	14	11.1	6.1
	1300	15	6.3	10.3
	2500	15	1.7	13.0

Table 3
Accuracy and precision of dextromethorphan and its metabolites at LLOQ in spiked control urine matrix

Replicate	Dextromethorphan 1.00 ng/ml (% bias)	3-Methoxymorphinan 1.00 ng/ml (% bias)	Dextrorphan 60.0 ng/ml (% bias)	3-Hydroxymorphinan 100 ng/ml (% bias)
1	14.0	1.9	−3.0	−6.4
2	−10.6	−10.4	−13.1	−13.4
3	12.0	−18.4	−3.9	−9.1
4	3.0	−18.8	−4.7	−9.7
5	−16.5	−4.5	−5.7	−5.5
Mean (%) bias	0.4	−10.0	−6.1	−8.8
% RSD	13.4	9.8	4.3	3.4

interest for control blank urine extracts distributed throughout the analytical run (data not shown).

3.2. Extraction efficiency

The extraction efficiency of the analytes from the human urine was determined by comparing the peak area from the extracted sample versus those for the reconstituted blank urine extracts spiked after extraction. The extraction efficiency was determined using nine replicates each of the low and high QC samples. The mean extraction efficiency (S.D.) for dextromethorphan was 59.2% (7.4), and that of the metabolites was 69.6% (7.0), 90.8% (9.5) and 89.6% (9.9) for 3-methoxymorphinan, dextrorphan and 3-hydroxymorphinan, respectively. The mean extraction efficiency for the internal standard (3-ethylmorphine) was 68.9% (3.4).

3.3. Precision and accuracy

The accuracy and precision data for the LC-MS/MS analysis of blank human urine spiked with dextromethorphan and its metabolites at three or four concentrations (as applicable) are presented in Table 2. The accuracy across the entire range of spiked concentrations (% Bias-average of the deviations from nominal) was, in general, within 14% of the target value. The precision, as assessed by the percent relative standard deviation (RSD) for the replicate analysis, was less than 14% at all concentrations including the LLOQ (Table 3).

3.4. Stability

Stability was assessed based on the FDA Guidance for Bioanalytical Method Validation [27]. Stability was demonstrated for spiked urine samples stored at −20 °C for up to 9 months, at ambient temperatures for 24 h, or three freeze-thaw cycles. Stability of processed samples was demon-

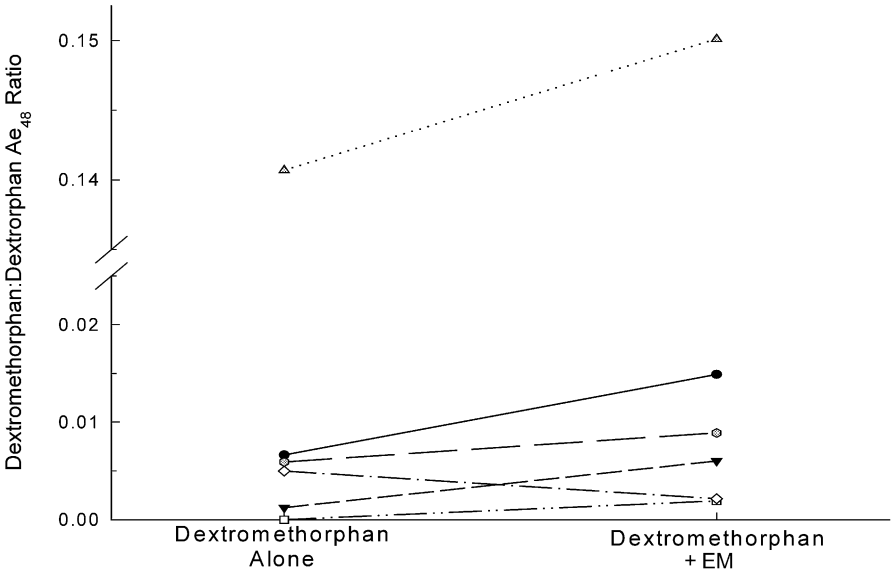

Fig. 5. Changes in individual urine dextromethorphan: 3-methoxymorphinan Ae_{48} ratios for 30 mg dextromethorphan hydrobromide given alone or in combination with an experimental drug (EM) on the 14th day of 30 mg qd EM dosing.

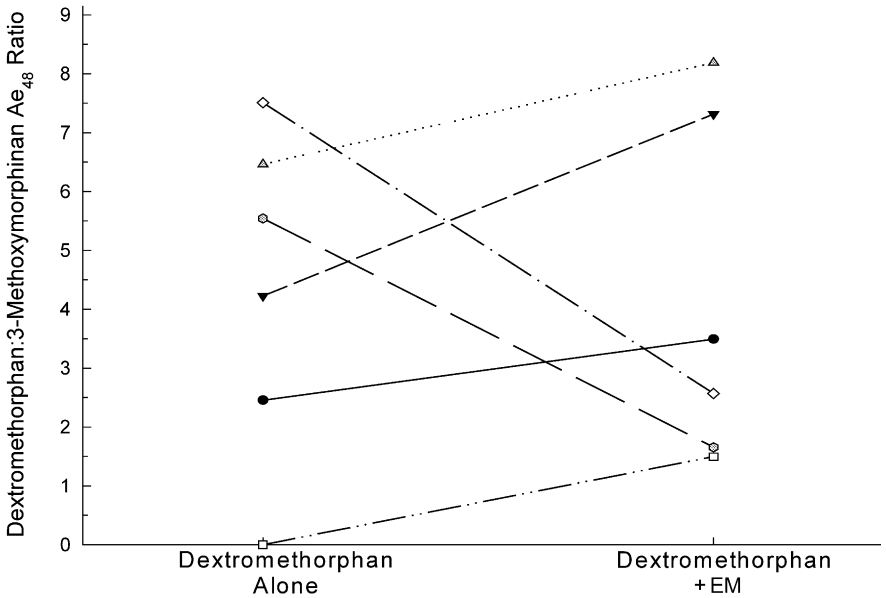

Fig. 6. Changes in individual urine dextromethorphan: 3-methoxymorphinan Ae_{48} ratios for 30 mg dextromethorphan hydrobromide given alone or in combination with an experimental drug (EM) on the 14th day of 30 mg qd EM dosing.

strated after storage for 72 h at either −20 or 4 °C and after storage in a refrigerated autosampler for 24 h. In each case, an analyte was considered adequately stable if the mean calculated concentrations were within 10% of nominal.

4. Discussion

During development of several drug candidates, the method was used to assess the potential for the drug candidate to inhibit the CYP3A or CYP2D6 isozymes. After either using the LC-MS/MS method to phenotype subjects or PCR-RFLP to genotype subjects, only fast-metabolizer subjects were enrolled in the studies. The dextromethorphan/3-methoxymorphinan and dextromethorphan/dextrorphan urinary excretion ratios through 48 h after dosing following a 30 mg dose of dextromethorphan hydrobromide were compared with or without concomitant administration of the experimental drug. For CYP3A inhibition to be significant, the ratio must exceed 10, and for CYP2D6, the ratio must exceed 0.3 to establish clear evidence of enzyme inhibition. Based on insignificant changes to either ratio (Figs. 5 and 6), it was concluded that none of the drug candidates were inhibitors of CYP3A or CYP2D6.

5. Conclusion

The results obtained during the validation of the method demonstrated that suitable sensitivity, precision, accuracy, and specificity were obtained for the determination of dextromethorphan and its metabolites (3-methoxymorphinan, dextrorphan, and 3-hydroxymorphinan) in human urine samples over the investigated concentration range of 1.00–500 ng/ml for dextromethorphan and 3-methoxymorphinan, 60.0–3000 ng/ml for dextrorphan and 100–3000 ng/ml 3-hydroxymorphinan using an LC-MS/MS assay. This method has been successfully applied to several clinical studies in which dextromethorphan was the substrate for both CYP3A and CYP2D6 when co-administered with drug candidates under development.

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