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Determination of dextromethorphan and its metabolites in rat serum by liquid–liquid extraction and liquid chromatography with fluorescence detection

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Abstract

Dextromethorphan is an effective and safe antitussive, but has liabilities with respect to its abuse potential at doses above the therapeutic dose. At these higher doses, people report phencyclidine-like effects from the drug. A number of animal models have suggested that dextrorphan, an active metabolite of dextromethorphan, is responsible for the abuse liability of the parent compound when dextromethorphan is taken at high doses. Full pharmacokinetic profiles in single animals have not been demonstrated in these studies due to a lack of analytical sensitivity and/or selectivity for dextromethorphan and its metabolites. We have developed a low-cost liquid chromatographic method capable of characterizing the concentration–time profile for dextromethorphan and dextrorphan for 8 h in rats following an 18 mg/kg i.p. dose of dextromethorphan. Limits of quantitation ($S/N = 10$) in 100 μL of serum were 0.25, 0.19, 0.27, and 0.22 nmol/mL for 3-hydroxymorphinan, dextrorphan, 3-methoxymorphinan, and dextromethorphan, respectively. Inter-day precision was better than 11% across the dynamic range of the method.

Keywords: Dextromethorphan; 3-Hydroxymorphinan; Dextrorphan; 3-Methoxymorphinan

1. Introduction

Dextromethorphan is an antitussive agent used in many nonprescription cough and cold medications. At recommended therapeutic doses (20–30 mg), dextromethorphan has an excellent safety profile; however, at doses associated with abuse (>100 mg), commonly observed side effects include euphoria, disorientation, hallucinations, visual disturbances, and psychosis [1,2]. In recent years, dextromethorphan abuse has increased markedly, earning it a reputation as an inexpensive alternative to the illegal street drug methylenedioxymethamphetamine (ecstasy) [3].

In humans, dextromethorphan is metabolized extensively to dextrorphan and to a lesser extent to 3-methoxymorphinan and 3-hydroxymorphinan (Fig. 1). Dextrorphan is of particular interest since it binds

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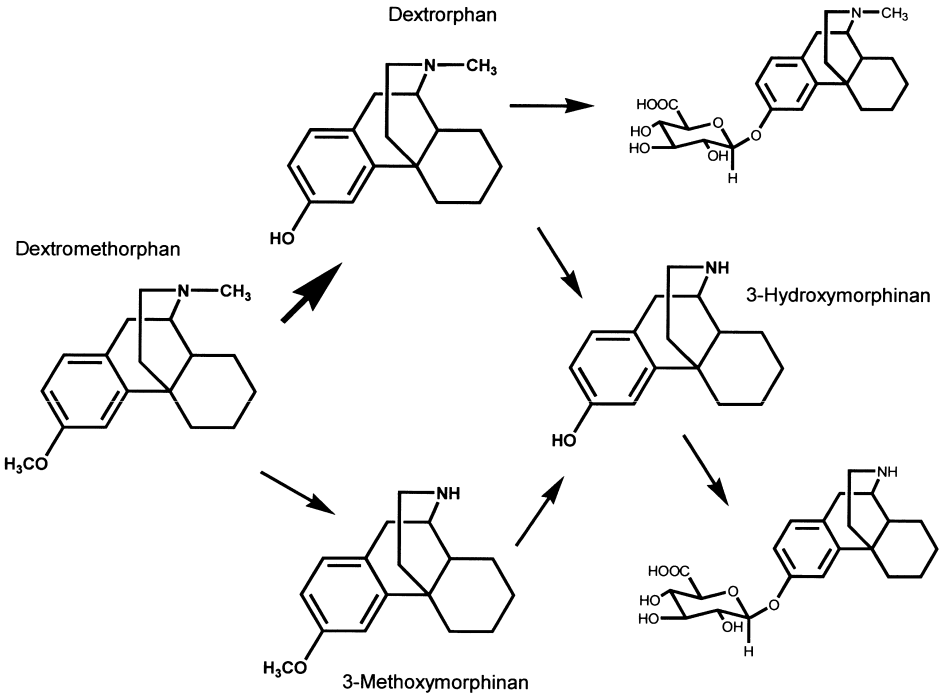

Fig. 1. Dextromethorphan metabolic pathways.

with relatively high affinity to the NMDA receptor at the same site implicated in mediating the effects of phencyclidine [4–6]. It is unclear whether dextromethorphan, dextrorphan, or both are responsible for the effects that contribute to its abuse potential.

A number of investigators have employed animal models to investigate the pharmacological mechanisms of dextromethorphan abuse [7–9]. These studies have investigated both behavioral and pharmacokinetic aspects of dextromethorphan administration. Others have utilized pharmacokinetic–pharmacodynamic (PK–PD) modeling to determine the relative contribution of active metabolites to dextromethorphan's pharmacological response [5]. One impediment to dextromethorphan PK–PD assessment in rodent models is the lack of sensitive analytical methodologies for determining the parent compound and metabolites in biological matrices. Investigators are currently forced to employ a single animal for each time point when generating dextromethorphan pharmacokinetic concentration–time profiles. We have interests in using the rat as a model for the pharmacological effects of dextromethorphan at doses reflective of abused doses in humans. Since it is possible that the pharmacokinetics are relevant to the pharmacodynamic effects, we have developed a cost-effective analytical method for the determination of dextromethorphan and its three metabolites in rat serum.

Many analytical methods have been described for the separation of dextromethorphan and its metabolites in biological matrices [10–12], the most versatile being liquid chromatography (LC). Despite a wide variety of LC detection methods (ultraviolet absorption [10], fluorescence [12], and electrochemical detection [13]), large sample volumes ($>$2 mL) of blood or whole organs are required to attain adequate sensitivity. Large sample volumes, in turn, necessitate the use of multiple animals. Liquid chromatography with mass spectrometric detection [14–16] is the preferred technique in terms of sensitivity but not necessarily in terms of capital expenditures. In fact, Bolden et al. demonstrated excellent sensitivity with only 200 μL of whole blood in human subject samples [16].

We describe a liquid chromatographic methodology for the determination of dextromethorphan and its three principal metabolites in microliter serum sam-

ples using an external standard approach. Utilizing liquid–liquid extraction and fluorescence detection, we were able to characterize concentration–time profiles for dextromethorphan and free dextrorphan following an 18 mg/kg i.p. dose in rats.

2. Experimental procedures

2.1. Reagents

Dextromethorphan hydrobromide monohydrate and 3-hydroxymorphinan hydrochloride were purchased from Sigma–RBI (St. Louis, MO, USA). 3-Methoxymorphinan hydrobromide was obtained from the National Institute on Drug Abuse (Bethesda, MD, USA). Dextrorphan tartrate monohydrate was synthesized in-house from dextromethorphan.

Briefly, 30 g (81 mmol) of the dextromethorphan salt was dissolved in 150 mL of 48% HBr (1.3 mol) (Aldrich, Milwaukee, WI, USA) and stirred under reflux for 1.5 h. The solution was cooled in an ice bath and the pH adjusted to 9 with ammonium hydroxide (Fisher Scientific, Houston, TX, USA). The resulting white precipitate was extracted with chloroform (3×300 mL) and the combined organics were washed with 150 mL of water. The chloroform solution was dried over sodium sulfate and evaporated to a white foam. This was dissolved in 75 mL of water under reflux and 12.9 g of L(+)-tartaric acid (Sigma, St. Louis, MO, USA) was added. The resulting crystalline dextrorphan salt was filtered and washed with cold water, 2-propanol, and ether. The white crystals (25 g) were then dried overnight in a vacuum oven (77% yield). M_p = 185–188 °C (lit. 183–185 °C); % C/H/N, 58.27:7.23:3.25 (theory 59.28:7.34:3.29).

Sodium heptane sulfonic acid, HPLC grade methanol and acetonitrile were purchased from Fisher. Tetrahydrofuran, diethyl either, chloroform, and 2-propanol were HPLC grade and purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). The tetrahydrofuran was stored under nitrogen. Water was filtered through a Milli-Q^{50} Ultra Pure Water System (Millipore, Bedford, MA, USA) prior to use. The mobile phase was filtered through 0.45-μm Durapore Nylon membrane filters (Millipore) and subsequently degassed using an in-line degassing system (Shimadzu, Columbia, MD, USA).

2.2. Liquid chromatography

A Shimadzu liquid chromatography system consisting of a 10AD pump coupled to a 10A autosampler and an RF-10xL fluorescence detector was used. Dextromethorphan and its metabolites were separated on a 15×0.46 cm (I.D.) Microsorb CV 5 μm phenyl column (Varian, Palo Alto, CA, USA) and a 0.75×0.46 cm Alltima 5 μm phenyl guard column (Alltech, Deerfield, IL, USA) maintained at 30 °C with a Shimadzu Model 10A vp column oven. The mobile phase consisted of 0.05 *M* potassium phosphate, pH 3.8, 1 m*M* heptane sulfonic acid, 20% (v/v) acetonitrile, 2.5% (v/v) methanol, and 1% (v/v) tetrahydrofuran. The flow-rate was 1.0 mL/min and a 500-μL injection loop was used. The fluorescence detector was set to an excitation wavelength of 230 nm and an emission wavelength of 330 nm. These wavelengths were optimized in preliminary experiments. The fluorescence for all analytes was superior at 280 and 306 nm but because of a peak which coeluted with dextrorphan the fluorescence was monitored at 330 nm. This peak was detected in all blank serum and in the injection solvent. The intensity of the peak was about eight times the noise. Chromatographic data were collected using a Shimadzu Class Vp 5.01 data acquisition interface and software.

2.3. Sample preparation

Standards containing 1.2, 2.4, 6.0, 8.4, 12, and 30 nmol/mL were prepared in water and stored at −20 °C until used. Fresh samples were prepared every 4 months. The stability was not compromised by repeated freeze–thaw cycles. Standard curves were generated by adding 10 μL of a thawed standard to 100 μL of rat serum or water in a 15-mL conical borosilicate tube. Serum samples were then treated with 200 μL of saturated sodium carbonate (166 g in 500 mL of water) and briefly vortexed. A 1-mL volume of diethyl ether–chloroform–2-propranol (40:18:2) was added and the sample was vortex-mixed for 12 min and centrifuged at 1500 *g* for 5 min. The organic layer was transferred to a

separate tube containing 100 μL of 0.05 *M* phosphoric acid and the analytes were back-extracted into the aqueous layer by vortexing for 12 min. Samples were vortex-mixed on an S|P Multitube Vortexer (Baxter Scientific). The organic layer was removed by aspiration and 50 μL of the aqueous layer injected onto the column for LC analysis.

2.4. Assay validation

2.4.1. Calibration

The linearity of the response for each analyte was established by plotting the peak area of the compound versus the serum concentration. A serum concentration range of 0.12–3.0 nmol/mL was used to generate calibration curves. Slopes, *y*-intercepts and standard deviations for these values were determined using Prism 3.0 (GraphPad Software, San Diego, CA, USA). Curves were fit to the data using Prism's linear regression analysis routine.

2.4.2. Precision and bias

The precision of the method was determined by replicate analyses ($N=4$) of quality control samples (0.12, 0.84, and 1.2 nmol/mL) on three different days. The bias of the method was expressed as

$$\%\ \text{bias} = \frac{(\text{observed} - \text{actual})}{\text{actual}} \cdot 100\%$$

where ''observed'' is the mean concentration calculated from the standard curve in serum. The assay recoveries for dextromethorphan and its metabolites were assessed at all six standard concentrations. Recoveries were determined from the peak area observed in the spiked serum sample and the concentration extrapolated or interpolated from the calibration curves generated in water. The limit of quantitation (LOQ) and limit of detection (LOD) were calculated at a signal-to-noise ratio of 10 and 2, respectively. The noise was evaluated by averaging the peak-to-peak noise across a 30-s time window of 10 blank serum injections.

2.5. Dextromethorphan administration and serum sampling

Female Sprague–Dawley rats (Harlan, Madison, WI, USA) weighing 250–290 g were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (20 mg/kg) administered intramuscularly. When an appropriate level of anesthesia was achieved (monitored by lack of response to a toe pinch), a midline incision over the left exterior jugular vein was made and an intravenous catheter (PE 50, Becton-Dickson, Franklin Lakes, NJ, USA) was placed into the vein and secured with suture. The distal end of the catheter was routed subcutaneously to an exit point in the mid-scapular region. When not in use the catheters were filled with lock solution and plugged with monofilament. The animals were then allowed to recover until their presurgical body weight was attained (generally 2 to 3 days). The catheters were flushed daily with lock solution that consisted of 40 μL of 0.9% normal saline containing 1.25 IU/mL sodium heparin and 0.4 mg/mL gentamicin (Abbott Laboratories, North Chicago, IL, USA). On the day of the experiment, rats were administered 18 mg/kg dextromethorphan i.p. in a volume of 1 mL/kg. The high dose was chosen to appropriately scale to a likely human abuse dose. To characterize the pharmacokinetics of dextromethorphan and its metabolites, blood samples (100 to 400 μL) were collected at 5, 10, 15, 45, 200, and 400 min following the injection. On a second occasion (2 to 4 days following the first dose), 18 mg/kg of dextromethorphan hydrobromide was administered again to the same rat and blood samples (200 to 1500 μL) were collected at 100, 200, and 500 min. The final assayed serum volumes were approximately half the blood volume. Larger blood samples were taken at later time points to ensure an adequate amount of analyte in the final extracted samples. The total amount of blood removed from the animal each day was less that 10% of the calculated total blood volume. In preliminary experiments, it was determined that this sampling regimen decreased the hematocrit by less that 10%. Samples were allowed to coagulate for 1 h at room temperature (protected from light) and then centrifuged at room temperature for 15 min at 15 800 *g*. The final serum volume assayed was 40 to 600 μL. Serum was aspirated from the samples and subsequently stored at −20 °C until analyzed. When the serum volume analyzed was equal to or exceeded 400 μL, the sodium carbonate volume was increased to 400 μL. These experimental protocols were approved by the

University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee.

2.6. Pharmacokinetic analysis

Dextromethorphan and dextrorphan serum concentration–time profiles were fit to select polyexponential equations using the nonlinear least-squares routine in Prism 3.0 (GraphPad). Pharmacokinetic parameters were calculated from computer-generated coefficients via compartmental analysis.

3. Results and discussion

3.1. Analytical method validation

Fig. 2 shows chromatograms of an extracted serum blank (Fig. 2A), and an extracted serum sample containing dextromethorphan and its three metabolites (Fig. 2B). Retention times for 3-hydroxymorphinan, dextrorphan, 3-methoxymorphinan and dextromethorphan were 5.95, 6.61, 22.25, and 24.5 min, respectively. The actual concentrations for each compound present in the chromatogram shown in Fig. 2B were 0.12, 0.09, 0.14 and 0.11 nmol/ml for 3-hydroxymorphinan, dextrorphan, 3-methoxymorphinan, and dextromethorphan, respectively. The S/N for dextromethorphan at this level was 2 and, clearly, the peak for dextromethorphan is distinguishable from the noise. In Fig. 2C a representative chromatogram from a 70 μL extracted serum sample obtained 15 min after an i.p. injection of 18 mg/kg of dextromethorphan is shown.

3.1.1. Calibration

The precision in the calibration curves is particularly relevant to the quantitative determination of these compounds in rat serum since calibration curve slopes were used to calculate concentrations in pharmacokinetic samples. Specifically, linear least squares equations for 3-hydroxymorphinan, dextrorphan, 3-methoxymorphinan, and dextromethorphan were $y = 0.011x + 0.005$, $y = 0.014x + 0.003$, $y = 0.021x + 0.004$, and $y = 0.023x + 0.004$, respectively. The inter-day variability in the slopes was less than 5%.

Since the volume of serum in the pharmacokinetic

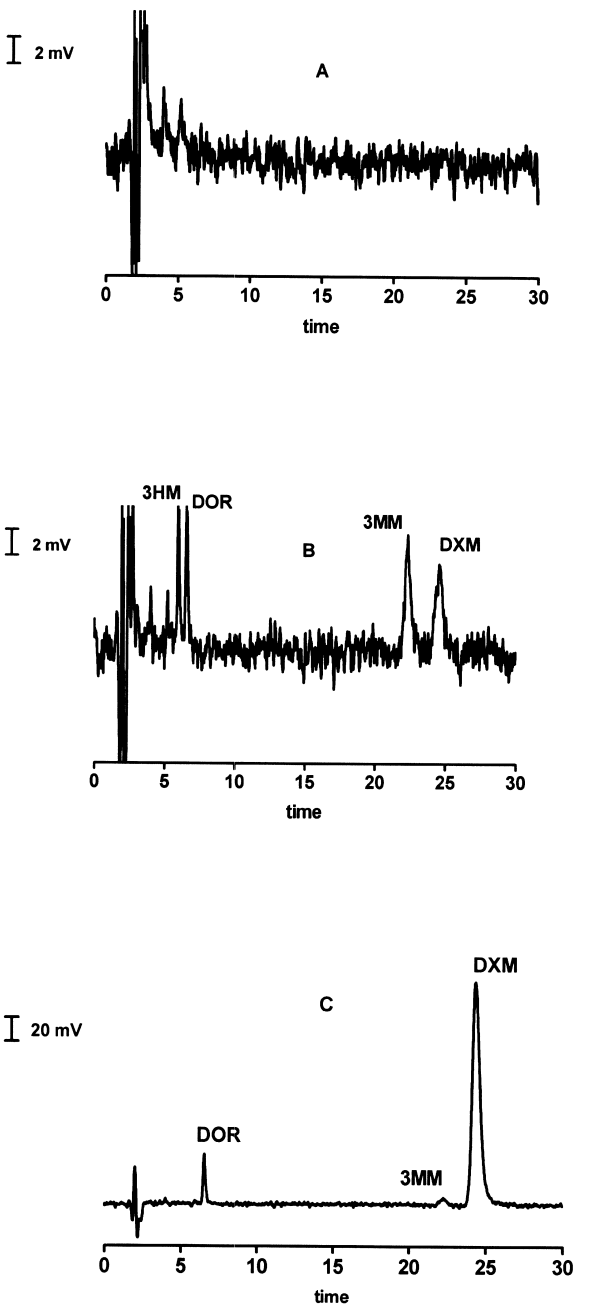

Fig. 2. Chromatogram of (A) serum blank, (B) serum containing known concentrations of 3-methoxymorphinan (3HM), dextrorphan (DOR), 3-methoxymorphinan (3MM), and dextromethorphan (DXM), and (C) a serum sample 15 min after an 18 mg/kg i.p. dose of DXM. Samples were subjected to extraction prior to chromatographic analysis as described in the text.

Table 1
Precision of extraction recovery for dextromethorphan and metabolites from rat serum

nmol/mL	Extraction recovery from serum[a]			
	3HM	DOR	3MM	DXM
1.25	82±6%	83±3%	70±2%	77±4%
0.85	81±2%	81±4%	75±4%	73±6%
0.25	60±11%	89±4%	79±10%	77±6%

[a] Values are mean±SD ($N=3$).

samples varied depending on the time the sample was taken, it was confirmed that increasing the serum volume to 600 μL did not adversely affect standard curve slopes.

3.1.2. Precision and bias

Extraction recoveries from serum at three quality control concentrations are shown in Table 1. Excellent precision was obtained as indicated by the standard deviation obtained for all four analytes.

Table 2 shows the accuracy and precision at the lower limits of quantitation (LLOQ) for all four compounds in 100 μL of serum. The bias was better than 15% for all four analytes. For an analytical method to pass validation for pharmacokinetic studies in our laboratory, the bias must be better than ±20% at the LLOQ so that the accuracy of the pharmacokinetic parameters is not compromised.

The inter-day and intra-day precision at three concentrations is shown in Table 3. The limit of detection (LOD) for 3-hydroxymorphinan, dextrorphan, 3-methoxymorphinan, and dextromethorphan was 0.06 nmol/mL for 3-hydroxymorphinan and dextrorphan, 0.14 nmol/mL for 3-methoxymorphinan, and 0.11 nmol/mL for dextromethorphan in 100 μL of serum. Poorer LODs for 3-methoxymorphinan and dextromethorphan were due to peak broadening, which caused poorer signal-to-noise ratios relative to 3-hydroxymorphinan and dextrorphan.

Table 2
Precision and accuracy at the lower limits of quantitation (LLOQ) for dextromethorphan and its metabolites

Compound	Nominal[a] (nmol/mL)	Predicted (nmol/mL)	RSD (%)	% Bias
3HM	0.25	0.23	8	−7
DOR	0.19	0.17	5	−12
3MM	0.27	0.23	18	−14
DXM	0.22	0.22	10	−2

[a] Values are means ($N=4$).

Table 3
Inter-day and intra-day precision of analysis within the dynamic range of the method

Compound	nmol/mL	RSD (%)	
		Intra-day ($N=4$)	Inter-day ($N=3$)
3HM	1.23	1	1
	0.86	1	3
	0.25	4	8
DOR	0.92	2	5
	0.65	5	1
	0.18	5	10
3MM	1.35	2	1
	0.95	4	4
	0.27	10	4
DXM	1.10	1	11
	0.77	5	1
	0.22	11	9

3.1.3. Preliminary pharmacokinetics in rats

The utility of the method for pharmacokinetic analysis in single animals was then demonstrated. Although the sensitivity was inferior to LC–MS–MS methodologies reported in the literature, we have demonstrated that LC with fluorescence detection offers a low-cost approach to determination of these analytes in small animals. Rats were challenged with a dextromethorphan i.p. dose which represented an abused dose in humans. This approach was adequate for the evaluation of concentration–time profiles for dextromethorphan and free dextrorphan. Fig. 3 shows concentration–time profiles for dextromethorphan and dextrorphan in Sprague–Dawley rats ($N=3$) following i.p. administration of 18 mg/kg dextromethorphan. 3-Hydroxymorphinan was not detected in any of the pharmacokinetic samples. 3-Hydroxymorphinan is likely in the glucuronide conjugate form and it is not surprising that it was not detected as free metabolite. Dextromethorphan and dextrorphan concentrations were determined in as little as 40 μL of serum. The elimination half-life was 70 and 200 min for dextromethorphan and dextrorphan, respectively. These values for dex-

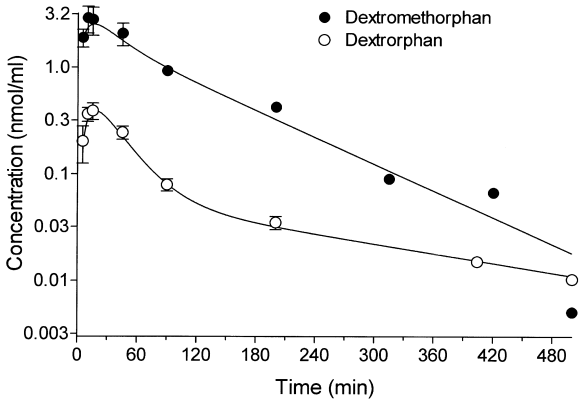

Fig. 3. Serum concentration (log scale) of dextromethorphan (●) and free dextrorphan (○) at various times after an i.p. injection of 18 mg/kg of dextromethorphan in rats. Individual concentration data points from three determinations were sorted to the nearest common time. Data in triplicate at each time up to 90 min, duplicate at 200 and 420 min (dextromethorphan only), all other points after 300 min based on a single determination. Data were fit to a tri-exponential equation ($1/y$ weighting).

tromethorphan are comparable to values reported by Wu et al. [8]

4. Conclusions

An inexpensive, rugged, and reproducible assay for the determination of dextromethorphan and its metabolites in microliter volumes of rat serum was developed. The method was robust and enabled the determination of reproducible pharmacokinetic profiles in individual laboratory rats following a dose analogous to those in human abuse cases. Serum concentration limits of detection are 0.5 nmol/ml for 3-methoxymorphinan in a 50 μL serum sample and 0.01 nmol/ml for dextrorphan in a 600 μL serum sample. Although the data were not presented here, levallorphan and pentazocine were both evaluated as internal standards. Levallorphan was an acceptable internal standard but marginally poorer precision was obtained relative to the external standard approach. Pentazocine was not acceptable due to the poor stability of this compound under the assay conditions.

Reversed-phase solid-phase extraction (SPE) using a 96-well plate format as a means of sample cleanup was investigated as a means of improving sample throughput (data not shown). In our hands, the reproducibility of this extraction technique was not acceptable to the external standard approach taken here for quantitation. No significant improvement in sample clean-up or sample throughput was obtained using this approach either. Evaporation of the SPE elution solvent was the rate-limiting step with this technique. This was not a significant hindrance using our liquid extraction technique as the sample was concentrated during the back-extraction step. We routinely processed 24 samples at one time using our approach. Incorporation of an internal standard (such as a stable isotope of dextromethorphan) and tandem mass spectrometric detection to improve the lower limits of quantitation are valid solutions to these limitations of this SPE format; however, a high-purity stable isotope for dextromethorphan would have to be synthesized in-house (>99.98% isotopic purity). In addition, tandem mass spectrometry would be more costly compared to fluorescence detection.

Preliminary pharmacokinetic parameters were in close agreement with those published previously [8]. Neither 3-hydroxymorphinan nor 3-methoxymorphinan were detected in the pharmacokinetic experiments. While these are certainly metabolites of dextromethorphan, they may not be relevant to a study of the abuse liability of dextromethorphan. Dextrorphan, which was detected in these preliminary pharmacokinetic studies, is considered the active metabolite of dextromethorphan and has been implicated in the abuse liability of dextromethorphan. The fact that 3-hydroxymorphinan and 3-methoxymorphinan were not detected at significantly high concentrations, relative to dextrorphan, provides support that female Sprague–Dawley rats are viable models for investigating the abuse potential of dextromethorphan in humans.

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