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Determination of dextromethorphan and its metabolite dextrorphan in human urine using high performance liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry: a study of selectivity of a tandem mass spectrometric assay

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Abstract

Analytical method for the simultaneous determination of dextromethorphan (**1**) and dextrorphan (**2**) in urine, based on solid-phase extraction of drug from acidified hydrolyzed biological matrix, were developed. The analytes (**1** and **2**) and the internal standard (levallorphan, **3**, IS) were detected by high-performance liquid chromatography–mass spectrometry (HPLC–MS/MS) in positive ionization mode using a heated nebulizer (HN) probe and monitoring their precursor → product ion combinations of *m/z* 272 → 215, 258 → 201, and 284 → 201 for **1**, **2**, and **3**, respectively, in multiple reaction monitoring mode. The analytes and IS were chromatographed on a Keystone Prism reverse phase (50 mm × 2.0 mm) 5 μm column using a mobile phases consisting of a 35/65 or 27/73 mixtures of methanol/water containing 0.1% TFA adjusted to pH 3 with ammonium hydroxide pumped at 0.4 ml/min for **1** and **2**, respectively. The limits of reliable quantification of **1** and **2** were 2 and 250 ng/ml, respectively, when 1 ml of urine was processed. The absence of matrix effect was demonstrated by analysis of neat standards and standards spiked into urine extracts originating from five different sources. The linear ranges of the assay were 2–200 and 250–20,000 ng/ml for **1** and **2**, respectively. Assay selectivity was evaluated by monitoring the "cross-talk" effects from other metabolites into the MS/MS channels used for monitoring **1**, **2**, and **3**. In addition, an interfering peak originating from an unknown metabolite of **1** into the quantification of dextromethorphan was detected, requiring an effective chromatographic separation of analytes from other metabolites of **1**. The need for careful assessment of selectivity of the HPLC–MS/MS assay in the presence of metabolites, and the assessment of matrix effect, are emphasized.

Keywords: Dextromethorphan; Matrix effect; Dextrorphan

1. Introduction

Dextrorphan (*O*-desmethyl dextromethorphan, DT, **2**, Fig. 1), is a major metabolite of dextromethorphan (DTM, **1**, Fig. 1), an over-the counter antitussive. DT is mainly excreted in human urine as its *O*-glucuronide. Studies have shown that the formation of DT is primarily mediated by cytochrome P4502D6 (CYP2D6) [1]. Many compounds like tricyclic anti-depressants and β-blockers are metabolized by this enzyme. Two phenotypes of the enzyme are expressed in the population and characterized as extensive metabolizers (EMs) and poor metabolizers (PMs). Extensive metabolizers express this enzyme to various degrees while poor metabolizers do not. The Caucasian population is comprised of approximately 10% of poor metabolizers [1]. One method of determining the CYP2D6 phenotype of an individual involves the administration of an oral dose of **1** and the determination of the ratio of **1** to **2** in a post-dose urine sample following treatment of the sample with β-glucuronidase to hydrolyze the DT-glucuronide. Concentrations of **2** in urine following enzymatic hydrolysis are generally in the μg/ml

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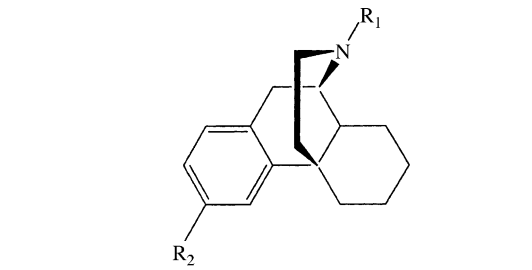

Fig. 1. The chemical structures of **1**, **2**, and **3**.

range in EMs, whereas, concentrations of **1** in these samples usually fall in the low ng/ml range. Hence, a highly sensitive method is required for the determination of **1**. Additionally, the fact that the concentrations of **1** and **2** differ by several orders of magnitude makes the reliable determination of the **1/2** ratio highly challenging.

A number of methods have been described in the literature for the determination of **1** and **2** in urine including direct fluorescence spectrometry [2], high-performance liquid chromatography (HPLC) with fluorescence detection [3–7], HPLC with ultraviolet detection [8], and gas chromatography with flame ionization [9] and mass spectrometric detection [10]. In most of these methods, the limit of quantification of **1** was insufficient for the accurate determination of the **1/2** ratio. Numerous endogenous peaks were present under the chromatographic conditions specified in several of these methods, complicating the quantification of **1** or **2**. Additionally, the sample preparation procedures described in these methods generally suffered from low analyte recoveries. Finally, the selectivity of these methods in the presence of a number of other metabolites of **1** was not established.

Two methods [11,12] utilizing HPLC–MS/MS detection for the determination of **1** and **2** in plasma have been published. The feasibility of adapting one of these methods to analysis of **1** and **2** in urine was examined. The first method [11] relied on a "dilute and shoot approach", which provided high recoveries of analytes but samples containing many endogenous interfering peaks were analyzed. This approach is equivalent to a direct injection technique with a high potential for matrix effects in urine. The second method [12] used a liquid–liquid extraction with a back extraction for the isolation of drug, but extremely short HPLC runtime of 1 min was utilized. These chromatographic conditions did not allow for the separation of metabolites that may interfere with the quantitation of the analyte. The HPLC–MS/MS method for **1** and **2** [13] and other metabolites in urine was also reported and it was based on a simultaneous analysis of **1**, **2**, and three other metabolites using a single compound (an analog) as an internal a standard for all four analytes, gradient HPLC, and turbo ion spray (TISP) interface for HPLC–MS/MS analysis. The absence of matrix effect and assay selectivity in the presence of other metabolites was not studied and the selectivity

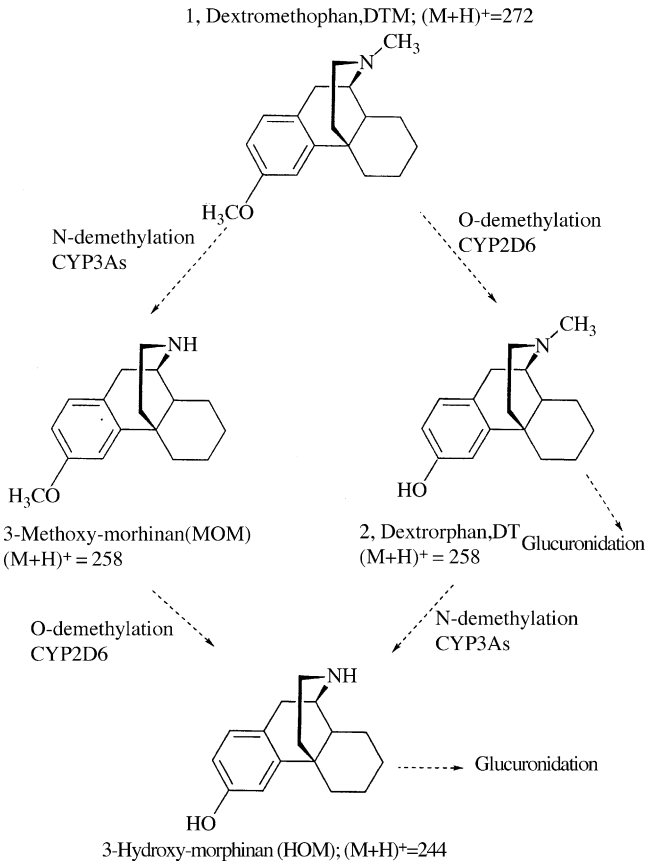

Fig. 2. The metabolic pathway of dextromethorphan.

of the method in urine samples originating from different subjects was not evaluated. Methods based on HPLC–MS/MS utilizing TISP interface and an analog as internal standard were shown to exhibit a significant matrix effect [14–16]. All these effects may adversely affect the determination of **1**, **2,** and other metabolites. In addition to **2**, dextromethorphan (**1**) is metabolized to 3-methoxy-morphinan (MOM), and both **2** and MOM are further metabolized to 3-hydroxy-morphinan (HOM) (Fig. 2). Both **2** and HOM are also found in urine in the form of their respective glucuronides. A number of other oxidative metabolites may also be formed, and they may all interfere with the quantification of **1** and **2**. Due to the potential for an "in-source" fragmentation of these metabolites, the selectivity of MS/MS detection without an efficient HPLC separation may not be guaranteed, and both matrix effect and cross-talk, due to "in-source" fragmentation, between channels used for monitoring the analytes in the presence of metabolites needs to be carefully assessed. Therefore, a need existed for a more reliable, sensitive and selective assay for the determination of **1** and **2** in human urine.

In order to overcome the shortcomings associated with prior methods, selective HPLC–MS/MS methods for the simultaneous determination of **1** and **2** (Fig. 1) in urine samples were developed. The assessment of matrix effect and assay selectivity was carried out in detail. It was demonstrated that the method developed was highly selective, free from matrix

effect complications, and selective in the presence of the N-oxide metabolite of **1** that was identified during the course of these studies.

2. Experimental

2.1. Materials

Dextromethorphan (**1**) was received from Sigma (St. Louis, MO). Dextrorphan (**2**), and levallorphan (**3**) (Fig. 1) were received from Research Biochemicals International (Natick, MA). All solvents were HPLC or analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ). The different lots of control urine originated from laboratory personnel. Nitrogen (99.999%) and argon (99.999%) were purchased from West Point Supply (West Point, PA).

2.2. Instrumental

A Perkin-Elmer (PE) Sciex (Thornhill, Canada) API III$^+$ tandem mass spectrometer equipped with a heated nebulizer (HN) interface, a PE 200 autoinjector, and a PE 200 Quaternary pump were used for all HPLC–MS/MS analyses. The data were processed using MacQuan software (PE Sciex) on a MacIntosch Quadra 900 microcomputer.

2.3. Standard solutions

A stock solution of 100 μg/ml for standard **1** was prepared in water. The stock solution was serially diluted with water to standard concentrations of 0.02, 0.05, 0.1, 0.5, 1.0, 2.0 μg/ml. A stock solution of 1 mg/ml for standard **2** was also prepared in water. This solution was then serially diluted with water to give a series of working standards of 2.5, 5.0, 10.0, 50.0, 100.0, and 200.0 μg/ml. The IS (**3**) was also prepared as a stock solution of 100 μg/ml in water. It was serially diluted with water to yield a working standard of 10.0 μg/ml.

2.4. Chromatographic conditions

Chromatographic separation of the analytes was performed on a Keystone Scientific's Prism reverse phase (50 × 2.0 mm 5 μm, Keystone Scientific, Bellefonte, PA) analytical column. For the determination of **1**, a mobile phase was composed of a mixture of 35/65 methanol/water containing 0.1% TFA adjusted to pH 3.0 with ammonium hydroxide pumped at 0.4 ml/min. The retention times for **1** and **3** were 2.1 min (capacity factor, $k' = 7.4$) and 1.3 min ($k' = 4.2$), respectively, with an overall runtime of 5 min. A separate injection using a different mobile phase was required for the determination of **2**. In this case, a mobile phase composed of 27/73 methanol/water containing 0.1% TFA adjusted to pH 3.0 with ammonium hydroxide pumped at 0.4 ml/min was utilized. Under these conditions, the retention times for **2** and **3** were 1.8 min ($k' = 6.2$) and 2.9 min ($k' = 10.6$), respectively, with an overall HPLC runtime of 10 min. The "long" 10 min HPLC runtime allowed for all metabolites and **1** to elute off the column and not interfere with the quantification of **2** in subsequent injections.

2.5. HPLC–MS/MS conditions

A PE Sciex triple quadrupole mass spectrometer (Sciex API III$^+$) was interfaced via a Sciex HN probe with the HPLC system. The HN probe was maintained at 500 °C and gas phase chemical ionization was effected by a corona discharge needle (+4 μA) using positive ion atmospheric pressure chemical ionization (APCI). The nebulizing gas (N_2) pressure was set for the HN interface at 80 psi. The auxiliary flow was 2 l/min, the curtain gas flow (N_2) was 0.9 l/min, and the sampling orifice was set at +50 V. The dwell time was 400 ms, and the temperature of the interface heater was set at 60 °C. Mass analyzers Q1 and Q3 were operated at unit mass resolution. The mass spectrometer was programmed to admit the protonated molecules $[M+H]^+$ at *m/z* 272 for **1**, 258 for **2**, and 284 for **3** via the first quadrupole filter (Q1). Collision induced fragmentation at Q2 (collision gas argon, 275×10^{13} atoms cm^{-2}) yielded the product ions at Q3 of *m/z* 215, 201, and 201 for **1**, **2**, and **3**, respectively. Peak area ratios obtained from selective reaction monitoring of the analytes (*m/z* 272 → 215)/(284 → 201) for the quantification of **1** and (*m/z* 258 → 201)/(284 → 201) for the quantification of **2** were utilized for the construction of calibration lines, using weighted ($1/x^2$) linear least-square regression of the plasma concentrations and measured peak area ratios. Data collection, peak integration, and calculations were performed using MacQuan PE-Sciex software.

2.6. Sample preparation

Seven different standard lines containing **1** and **2** in water and human urine were constructed to evaluate the assay accuracy, precision, recovery, and the absence or presence of the matrix effect. The first standard line (line 1) was constructed to evaluate the MS/MS response for neat standards of all three analytes injected in water. The second line (line 2) was constructed in urine extracts originating from a single urine source and spiked after extraction. A separate set (set 1) of five standard lines was analyzed in urine originating from five different sources. The urine samples in set 1 were spiked before extraction. The first line of set 1 was constructed in the same urine as used during the construction of line 2 (samples). By comparing the absolute areas of peaks **1**, **2**, and **3,** the peak areas ratios, and slopes of the standard lines between lines 1, 2 and standard lines constructed in five different urine sources (set 1), the absence or presence of "absolute" matrix effect [16] was assessed. In addition, precision and accuracy of the method and recoveries of analytes were also determined.

Line 1: This standard line was constructed by placing 100 μl of the appropriate standards (**1** or **2** in water) and

100 μl of IS (**3**) in H_2O into 15 ml centrifuge tubes. One hundred microliters of a 27/73 (v/v) mixture of methanol/water containing 0.1% TFA adjusted to pH 3.0 with ammonium hydroxide was added to the tubes. After mixing, 200 μl was transferred to the autosampler vials and 20 μl was injected into the HPLC–MS/MS system.

Line 2: This standard line was constructed in a single source of urine by adding 1 ml of urine to a 15 ml centrifuge tube followed by the addition of 300 μl of water. The urine was acidified by adding 1 ml of pH 5.0 acetate buffer containing 1500 units β-glucuronidase and incubated in a 37 °C water bath for 18 h. A SPE Waters Oasis HLB 3 cc cartridge was attached to a vacuum manifold and activated by eluting 2 ml of methanol followed by 2 ml of water. The urine sample was then loaded onto an activated cartridge and the cartridge was washed with 2 ml of a 25/75 (v/v) mixture of methanol/water. The analytes were eluted off the column with 2 ml of methanol. The methanol eluant was split into two parts. One part contained a volume of 1.8 ml for the dextromethorphan (**1**) assay and the other fraction of 200 μl was used for the dextrorphan (**2**) assay. The eluants were evaporated to dryness and spiked with 100 μl of the appropriate standards of **1** or **2**, and **3** and diluted in a 27/73 mixture of methanol/water containing 0.1% TFA adjusted to pH 3.0 with ammonium hydroxide, using volumes of 100 and 300 μl for a total volume of 300 and 500 μl for the (**1**) and (**2**) assays, respectively. Part of the extract (20 μl) was injected separately into the HPLC–MS/MS system for the analysis of (**1**) and (**2**).

Set 1: Five standard lines were constructed in urine from different sources. Standard line 1 of set 1 was constructed in the same urine as the one used for the construction of line 2 (analytes spiked after extraction). One millilitre of urine was placed in a 15 ml centrifuge tube to which 100 μl of appropriate standards and 100 μl of IS were added. The control (blank) tubes had 1 ml of urine to which 300 μl of water was added. The urine was acidified by adding 1 ml of pH 5.0 acetate buffer containing 1500 units β-glucuronidase and incubated in a 37 °C water bath for 18 h. Similar solid-phase extraction as described for line 2 was followed, except **1**, **2**, and **3** were added to urine prior to loading onto the activated SPE cartridge. The dried extracts were reconstituted in 300 and 500 μl of a 27/73 mixture of methanol/water containing 0.1% TFA adjusted to pH 3.0 with ammonium hydroxide for the analysis of **1** and **2**, respectively. Twenty microliters of the reconstitution solvent was injected separately into the HPLC–MS/MS system for the analysis.

2.7. Precision, accuracy and recovery

The precision of the method was determined by the replicate analyses of **1** and **2** ($n = 5$, set 1) in different human urine samples at all concentrations utilized for the construction of calibration curves. The linearity of each standard curve was confirmed by plotting the peak area ratio of the drug to IS (**3**) versus drug concentration. The unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by weighted ($1/x^2$) linear regression of the standard line. The accuracy of the method was expressed by [(mean observed concentration)/(spiked concentration)] × 100. The recovery was determined by comparing the mean peak areas of **1** and **2** obtained in set 1 to those observed during the construction of line 2.

2.8. Assessment of matrix effect

The assessment of matrix effect is critical when analogs rather then stable isotope-labeled analytes are used as internal standards [14]. Undetected co-eluting endogenous impurities may affect the ionization efficiency of the analytes. By comparing the peak areas of analytes in different lots of urine, the peak area ratios of analytes to an internal standard, and by analyzing urine samples spiked before and after extraction, the recovery and ion suppression or enhancement associated with a given lot of urine was assessed.

2.9. Assessment of assay selectivity

The assay selectivity was assessed by analyzing extracts from five lots of urine originating from different sources. In addition, the "cross-talk" between MS/MS channels used for monitoring **1**, **2**, and **3** was evaluated by injecting separately each analyte at the highest concentration on the standard line and monitoring the responses in all other MS/MS channels at the LLOQ of the respective analyte.

2.10. Clinical sample preparation

Following administration of the investigational drug for 18 days, a single 60 mg oral dose of dextromethorphan was given to the subjects. Urine was collected for the measurement of **1** and **2** for the time period 0–8 h post-dose. The urine samples were stored at −20 °C until time of analysis. A 1 ml aliquot was used for the analysis of all clinical samples. The clinical samples were prepared with a daily calibration line and quality control samples as outlined in Section 2.5.

2.11. Quality control sample preparation

Quality control samples were prepared from a pool of five different sources of urine at the concentrations of 5, 75 and 150 ng/ml for **1** and 500, 5000 and 15,000 ng/ml for **2**. A conjugated glucuronide quality control sample of **2** in urine was obtained from a human volunteer (analyst) taking a 30 mg dose of **1** orally. Urine was collected for a period of 0–8 h post-dose. The urine was thoroughly mixed. This sample was incubated with 1500 units β-glucuronidase and analyzed for the content of **1** and **2** immediately after collection. The analysis of this sample together with clinical samples served as a daily marker to ensure the complete cleavage of the glucuronide of **2**. The quality control samples were frozen at −20 °C.

3. Results

3.1. Assay validation

Full scan positive ion mass spectra of **1**, **2**, and **3** yielded predominately the protonated molecules at *m/z* 272, 258, and 284, respectively. The product ion mass spectra of these protonated molecules (Fig. 3) indicated the presence of intense product ions at *m/z* 215, 201, and 201 for **1**, **2**, and **3**, respectively. Multiple reaction monitoring mode was used for the quantification of **1**, **2**, and **3** utilizing the precusor → product ion combinations of *m/z* 272 → 215, 258 → 201, and 284 → 201, respectively.

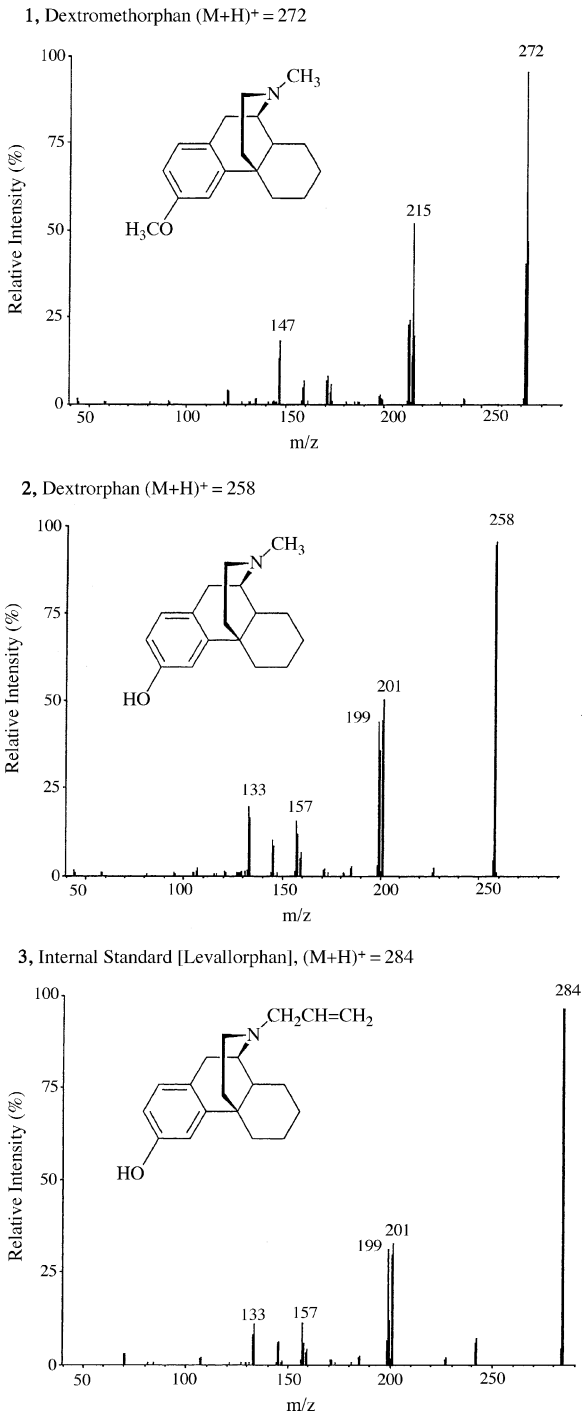

Fig. 3. The product ion mass spectra of **1**, **2**, and **3**.

The isolation of **1**, **2**, and **3** was based on a simple solid-phase extraction, dividing the extracts into two portions, evaporation of the extracts to dryness, reconstitution of the residues, and injection of samples into the HPLC–MS/MS system under two different HPLC conditions.

The assay was validated for **1** and **2** in human urine in the concentration range of 2–200 and 250–20,000 ng/ml, respectively. The difference between the nominal standard concentration and the back-calculated concentration from the weighted linear regression line was less than 7% for each point on the standard curve indicating that the linear regression analysis applied ($1/x^2$) provided an adequate fit of the data. The correlation coefficients for the mean standard curve of five different lots of urine (set 1) were 0.999 and 0.998 for **1** and **2,** respectively. Typical equations for the calibration curves for **1** and **2** were $y = 0.001236x + 0.00051$ and $y = 0.001176x - 0.00021$, respectively. The correlation coefficients for **1** and **2** were greater than 0.99 for daily runs. The intra-day accuracy ranged from 95 to 103% with precision values of less than 6.2% for both analytes indicating excellent accuracy and precision of the assay. The intra-day accuracy and precision data are summarized in Tables 1 and 2.

3.2. Assay selectivity

It is important during the development of any HPLC–MS/MS method to confirm assay selectivity in the presence of metabolites. If metabolites are not chromatographically separated from the parent drug, they can fragment in the ionization region of the mass spectrometer to give the same protonated molecular ion as the one originating from the drug [17–20]. These ions having the same *m/z* values could produce common product ions detected by the MS/MS

Table 1
Intra-day precision and accuracy of replicate analysis ($n = 5$) of dextromethorphan (**1**) in five different sources of human control urine

Nominal concentration (ng/ml)	Mean concentration[a] (ng/ml)	Precision[b] C.V. (%)	Accuracy[c] (%)
2	2.0	6.9	100
5	4.8	5.9	96
10	10.2	2.1	102
50	47.4	0.8	95
100	102.7	2.1	103
200	206.7	3.1	103

[a] Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.

[b] Expressed as coefficient of variation (C.V.%).

[c] Expressed as [(mean observed concentrations)/(nominal concentration) × 100] ($n = 5$).

Table 2
Intra-day precision and accuracy of replicate analysis ($n = 5$) of dextrorphan (**2**) in five different sources of human control urine

Nominal concentration (ng/ml)	Mean concentration[a] (ng/ml)	Precision[b] C.V. (%)	Accuracy[c] (%)
250	253	4.4	101
500	494	2.4	99
1000	981	4.0	98
5000	4928	3.4	99
10000	10224	2.1	102
20000	20241	3.7	101

[a] Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.
[b] Expressed as coefficient of variation (C.V.%).
[c] Expressed as [(mean observed concentrations)/(nominal concentration) × 100] ($n = 5$).

system. The urinary metabolites of **1** (**2** and MOM) have identical molecular weights and quite possibly could produce common product ions. Thus, in an effort to ensure assay selectivity, initial chromatographic conditions were developed to separate **1**, **2**, and **3**, MOM and HOM. Using a Supelcosil ABZ$^+$ 75 mm × 4.6 mm, 3 μm column and a mobile phase composed of 48:52 methanol/water containing 0.05%TFA pumped at 1 ml/min, baseline separation of **1**, **2**, and **3** from other known metabolites and endogenous urine background was achieved. However, under these chromatographic conditions, a peak corresponding to an unreported metabolite was observed to elute as a shoulder on peak **1** in chromatograms of post-dose urine samples. It was postulated that this peak corresponded to the N-oxide of **1** (**1-NO**). **1-NO** could possibly be fragmented in the heated nebulizer to **1**, and thus would be detected in the MS/MS channel used for monitoring **1**. In order to test this hypothesis, **1-NO** was synthesized ex vivo by reacting **1** with hydrogen peroxide. Injection of the reaction mixture confirmed that the product of the oxidation reaction eluted at the same retention time as the shoulder observed on the peak **1** in post-dose samples. Under ion-spray ionization conditions, the product of oxidation reaction gave a protonated molecular ion at *m/z* 288 implying the formation of the oxidized **1**. On the other hand, a Q1 scan of **1-NO** injected through the heated nebulizer showed a protonated molecular ion at *m/z* 272, indicating that **1-NO** was reduced to **1** in the heated nebulizer interface of the MS/MS system. Similar thermal decomposition of **1-NO** to **1** may occur in TISP interface utilized in method [9]. Using the chromatographic conditions for the analysis of **1** (Keystone Prism reverse phase (5 μm, 50 mm × 2 mm) column with a mobile phase composed of a mixture of 35/65 methanol/water containing 0.1% TFA adjusted to pH 3.0 with ammonium hydroxide pumped at 0.4 ml/min) a 300 ng injection of the **1-NO** using the heated nebulizer probe was made. The primary response was in the channel *m/z* 272 → 215 used for monitoring **1**, clearly confirming the thermal conversion of **1-NO** to **1** in the HN probe and the need for HPLC separation of **1-NO** from **1**.

The results of these experiments clearly demonstrated the need for more efficient HPLC separation to ensure assay selectivity and separation of **1-NO** from **1**. Therefore, additional HPLC analytical columns were evaluated for the analysis of **1** and **2**. The Keystone Prism reverse phase (5 μm, 50 mm × 2 mm) column was selected for its excellent peak symmetry, sufficient retention of the analytes and baseline separation of **1** and **2** from **3**, metabolites, and extraneous matrix peaks by using different HPLC mobile phases for the analysis of **1** and **2**. For the determination of **1**, a mobile phase was composed of a mixture of 35/65 methanol/water containing 0.1% TFA adjusted to pH 3.0 with ammonium hydroxide pumped at 0.4 ml/min. Under these chromatographic conditions, **1**, **2**, and **3** were baseline separated, but **2** could not be quantified due to interfering contribution from the MOM metabolite that co-eluted with **2** into the channel *m/z* 258 → 201 used to monitor **2**. A second mobile phase that was composed of 27/73 methanol/water containing 0.1% TFA adjusted to pH 3.0 with ammonium hydroxide pumped at 0.4 ml/min was used to quantify **2**. Although using this mobile phase separation of **1**, **2**, and**, 3** was also observed, the peak **1** was broadened leading to the reduction of the LLOQ of **1** beyond the acceptable limit. Therefore, it was necessary to use two mobile phases for the quantification of **1** and **2**.

Endogenous peaks at the retention time of the analytes of interest were not observed in all urine samples evaluated. Representative chromatograms are presented in Figs. 4 and 5. Fig. 6 clearly shows the absence of any MS/MS response from the analytes into internal standard channel and vice versa.

3.3. Assessment of the matrix effect and the recovery of analytes

The matrix effect and the possibility of ionization suppression or enhancement for **1**, **2**, and **3** in different urine samples were closely examined. As seen in Tables 3 and 4, the coefficients of variation (C.V.s, %) of the mean peak areas of **1**, **2**, and **3** (set 1) at any given concentration in five different urine lots were small (≤11%), strongly indicating little or no difference in ionization efficiencies and consistent recoveries of the analytes from different urine lots. In addition, any small changes in peak areas of **1** or **2** were compensated for by a similar change in the internal standard (**3**) peak areas. The C.V.s of the peak area ratios of **1/3** and **2/3** were generally smaller than the C.V.s of peak areas of **1** and **2** (Tables 3 and 4), confirming the desired compensating effect of the presence of internal standard on the precision and reliability of quantification of **1** and **2**. In addition, by comparing peak areas of all analytes for samples spiked after extraction from urine with the analogous peak areas obtained by injecting neat standards directly (Tables 5 and 6), the extent of the "absolute" matrix effect [16] could be estimated. The values >100% indicate ionization enhancement in urine versus neat standards, whereas values <100% indicate ionization suppression. As it is seen from the data in Table 5, a small ionization enhancement (20 and 6% for **1** and **3**,

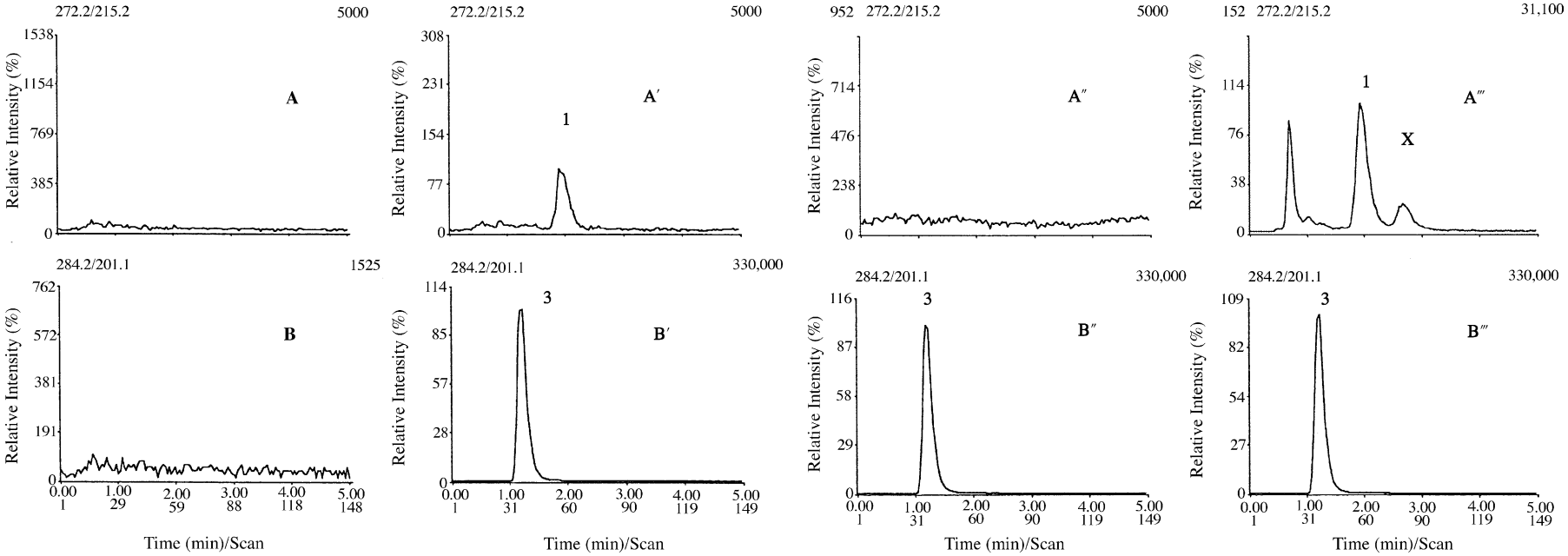

Fig. 4. Representative chromatograms of **1** and **3** spiked into urine and monitored at *m/z* 272 → 215 (channel A) and 284 → 201 (channel B), respectively. Chromatograms A and B: blank urine; chromatograms A′ and B′: blank urine spiked with 5 ng/ml of **1** and 1000 ng/ml of **3**; chromatograms A″ and B″: subject pre-dose urine spiked with 1000 ng/ml of **3**; Chromatograms A‴ and B‴: subject 0–8 h post-dose urine spiked with 1000 ng/ml of **3** (calculated concentration of **1** is 65.7 ng/ml) and the peak marked ***x*** is the **1-NO**. The values in the upper right hand corner represents peak heights expressed in arbitrary units.

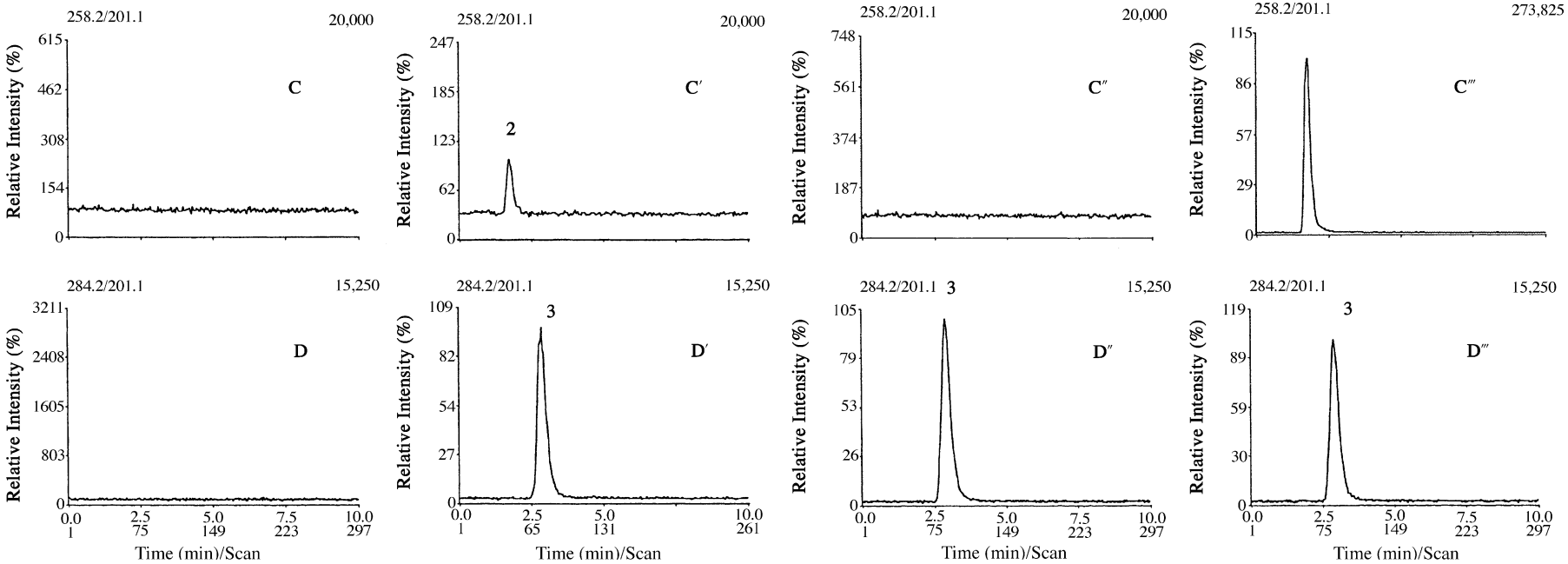

Fig. 5. Representative chromatograms of **2** and **3** spiked in urine and monitored at *m/z* 258 → 201 (channel C) and 284 → 201 (channel D), respectively. Chromatograms C and D: blank urine; chromatograms C′ and D′: blank urine spiked with 250 ng/ml of **2** and 1000 ng/ml of **3**; chromatograms C″ and D″: subject pre-dose urine spiked with 1000 ng/ml of **3**; chromatograms C‴ and D‴: subject's 0–8 h post-dose urine spiked with 1000 ng/ml of **3** (calculated concentration of **2** is 10,682 ng/ml). The values in the upper right hand corner represents peak heights expressed in arbitrary units.

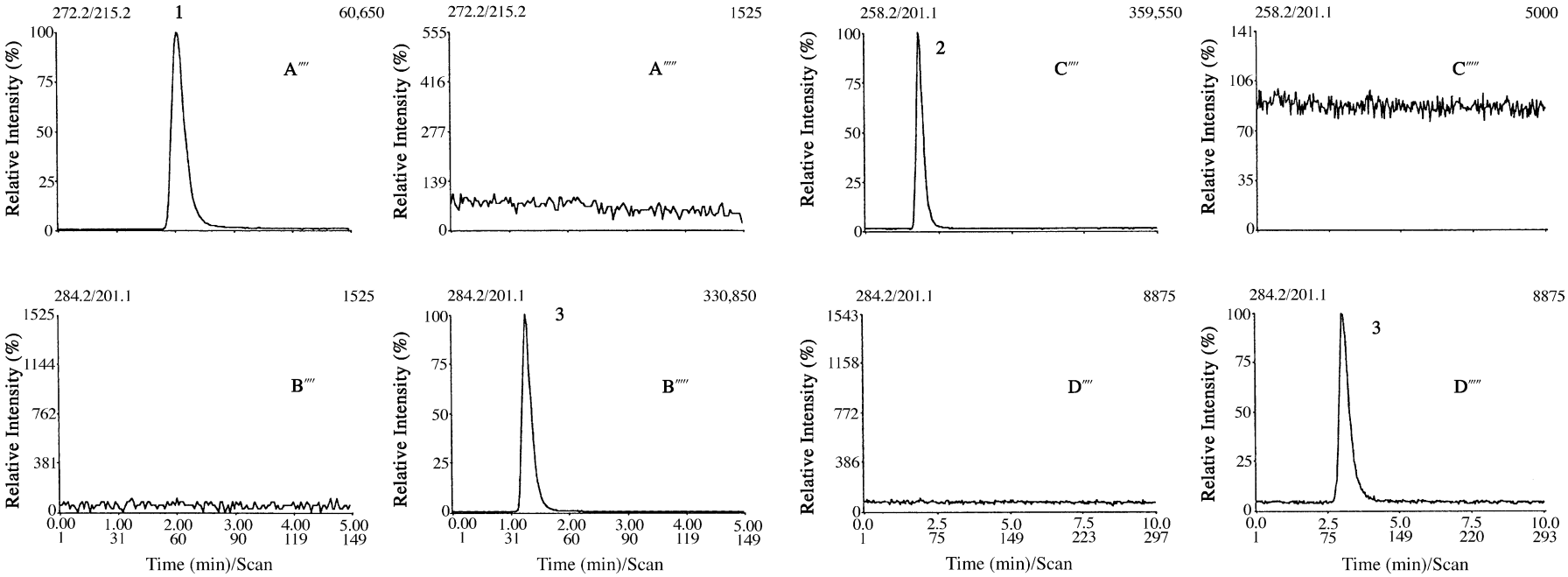

Fig. 6. Representative chromatograms A′′′′ and B′′′′: blank urine spiked with 200 ng/ml of **1**; chromatograms A′′′′′ and B′′′′′: blank urine spiked with 1000 ng/ml of **3**; chromatograms C′′′′ and D′′′′: blank urine spiked with 20,000 ng/ml of **2**; chromatograms C′′′′′ and D′′′′′: blank urine spiked with 1000 ng/ml of **3**. The values in the upper right hand corner represents peak heights expressed in arbitrary units.

Table 3
Mean[a] peak areas of **1** and **3** spiked into five different sources of urine before extraction (set 1) and the precision of determination of peak area ratios of analytes **1** to the internal standard (**3**)

Nominal concentration (ng/ml)	Peak area of **1**[a]	Precision C.V. (%)	Peak area of **3**[a]	Precision C.V. (%)	Peak area ratios of **1/3**	Peak area ratios of **1/3**, C.V. (%)
2	9611	7.9	3270400	5.6	0.0029	6.9
5	22412	5.7	3300739	3.3	0.0068	5.9
10	48222	3.3	3354369	4.6	0.0144	2.1
50	222753	2.5	3352789	2.9	0.0664	0.8
100	472321	5.9	3284536	5.2	0.1438	2.1
200	975899	1.6	3374493	2.6	0.2893	3.1
Slope[b]					0.00140	

[a] $N = 5$.
[b] Mean ($n = 5$) slope (m) calculated from the equation $y = mx + b$, where x is the concentration of the analyte, y is the peak area ratio, and b is an intercept.

Table 4
Mean[a] peak areas of **2** and **3** spiked into five different sources of urine before extraction (set 1) and the precision of determination of peak area ratios of analytes **2** to the internal standard (**3**)

Nominal concentration (ng/ml)	Peak area of **2**[a]	Precision C.V. (%)	Peak area of **3**[a]	Precision C.V. (%)	Peak area ratios of **1/3**	Peak area ratios of **1/3**, C.V. (%)
250	49963	3.3	168692	6.5	0.2968	4.4
500	111087	7.3	173481	8.7	0.6411	2.4
1000	227406	5.4	170801	6.1	1.3227	4.0
5000	1132517	10.8	163010	10.7	6.9500	3.4
10000	2286957	7.7	158078	9.4	14.4874	2.1
20000	4790492	8.0	166918	9.1	28.7412	3.7
Slope[b]					0.001423	

[a] $N = 5$.
[b] Mean ($n = 5$) slope (m) calculated from the equation $y = mx + b$, where x is the concentration of the analyte, y is the peak area ratio, and b is an intercept.

Table 5
Peak areas of neat standards of **1** and **3** (line 1) and the same standards spiked into of urine after extraction (line 2)[a]

Nominal concentration (ng/ml)	Peak area[b] of **1** (**A**)	Peak area[c] of **1** (**A′**)	Matrix effect[d] **A′**/**A** × 100	Peak area[b] of **3** (**B**)	Peak area[c] of **3** (**B′**)	Matrix effect[d] **B′**/**B** × 100	Peak area ratios[e] of **1/3**	Peak area ratios[f] of **1/3**
2	7558	10260	136	3560550	3714824	104	0.0021	0.0028
5	18270	22981	126	3647062	3741370	103	0.0050	0.0061
10	43069	51475	120	3543516	3790695	107	0.0122	0.0136
50	221728	250449	113	3632740	3845995	106	0.0610	0.0651
100	476410	541046	114	3639872	3852273	106	0.1309	0.1404
200	966426	1066458	110	3495566	3936830	113	0.2765	0.2709
Mean			120			106		
S.D.[g] (±)			9.8			3.5		
C.V.%[h]			8.2			3.3		
Slope							0.001369	0.001309

[a] HPLC mobile phase composed of 35:65 MeOH/H_2O containing 0.1% TFA adjusted to pH 3 with ammonium hydroxide was utilized.
[b] Neat standards: standard line 1.
[c] Standards spiked after extraction: standard line 2.
[d] Matrix effect (%) expressed as the ratio of the mean peak area of an analyte spiked into urine post-extraction (**A′** and **B′**) to the mean peak areas of the same analyte standards (**A** and **B**) multiplied by 100. A value >100% indicates ionization enhancement, and the value <100% indicates ionization suppression.
[e] Peak area ratios obtained from neat standards.
[f] Peak area ratios obtained from spiking analytes after extraction of urine.
[g] Standard deviation.
[h] Coefficient of variation.

Table 6
Peak areas of neat standards of **2** and **3** (line 1) and the same standards spiked into urine after extraction (line 2)[a]

Nominal concentration (ng/ml)	Peak area[b] of **2** (**A**)	Peak area[c] of **2** (**A′**)	Matrix effect[c] **A′**/**A** × 100	Peak area[b] of **3** (**B**)	Peak area[c] of **3** (**B′**)	Matrix effect[d] **B′**/**B** × 100	Peak area ratios[e] of **2/3**	Peak area[f] ratios of **2/3**
250	61799	55601	90	200882	202132	101	0.3076	0.2751
500	131609	131609	93	213883	199581	93	0.6153	0.6110
1000	264435	264435	99	200904	207657	103	1.3162	1.2641
5000	1433525	1433525	95	204583	196860	96	7.0071	6.9427
10000	2921176	2921176	98	211116	198484	94	13.8368	14.4410
20000	5748956	5748956	100	196368	200355	102	29.2763	28.7459
Mean			96			98		
S.D.[g] (±)			3.9			4.4		
C.V.%[h]			4.1			4.5		
Slope[b]							0.000647	0.000674

[a] HPLC mobile phase composed of 35:65 MeOH/H_2O containing 0.1% TFA adjusted to pH 3 with ammonium hydroxide was utilized.
[b] Neat standards: standard line 1.
[c] Standards spiked after extraction: standard line 2.
[d] Matrix effect (%) expressed as the ratio of the mean peak area of an analyte spiked into urine post-extraction (**A′** or **B′**) to the mean peak areas of the same analyte standards (**A** or **B**) multiplied by 100. A value >100% indicates ionization enhancement, and the value <100% indicates ionization suppression.
[e] Peak area ratios obtained from neat standards.
[f] Peak area ratios obtained from spiking analytes after extraction of urine.
[g] Standard deviation.
[h] Coefficient of variation.

Table 7
Representative standard line slopes for **1** and **2** spiked into five different lots of control urine

Calibration line	**1**	**2**
1	0.001440	0.001383
2	0.001389	0.001416
3	0.001376	0.001439
4	0.001385	0.001427
5	0.001408	0.001450
Mean	0.001400	0.001423
S.D. (±)	0.000025	0.000026
C.V.%	1.8	1.8

respectively) was observed, under the chromatographic conditions used for the analysis of **1**. The data in Table 6 shows a small ionization suppression for **2** and **3** (4 and 2%, respectively) under the chromatographic conditions used for the analysis of **2**. In both cases, the observed small ionization enhancement or suppression practically did not affect the ratios of **1**/**3** and **2**/**3** that were used for constructing the standard curves. In addition, comparison of the mean slopes of the lines (line 2 versus line 1) indicated that they were different by less than 5% for both **1** and **2**.

Representative slopes of the standard lines constructed in five different lots of urine for both **1** and **2** are presented in Table 7. The "relative" matrix effect [16], based on peak area ratios and/or slopes of the standard lines in different urine sources was not observed as indicated by small coefficient of variation (<2%) of the slopes of standard lines spiked into different sources of urine.

The extraction recovery (%) was calculated by comparing the mean peak areas of analytes spiked before extraction (set 1) divided by the areas of analytes spiked after extraction (line 2) and multiplied by 100. The mean recoveries of **1** and **2** were 102 and 86%, respectively, and the calculated mean recovery of **3** for the assays of **1** and **2** was 90%.

Table 8
Dextromethorphan and dextrorphan urine concentrations (ng/ml, 0–8 h collection period) of subjects receiving a single 60 mg oral dose of **1** on day 18, 1 h following dosing with a substance P inhibitor

Subject #	**1** (ng/ml)	**2** (ng/ml)
1	65.7	10683
2	25.5	15113
3	231.5	15851
4	877.5	6323
5	185.1	11493
6	9.4	15547
7	142.5	32101
8	241.1	15164

3.4. Freeze-thaw stability

Freeze-thaw stability was examined by exposing quality control sample to three freeze-thaw cycles (freezer nominal temperature of −20 °C). By comparing the initial mean values at three different concentrations of quality control samples after one freeze thaw cycle to the similar mean values after subsequent freeze thaw cycles, the effect of freeze thawing on the stability of **1** and **2** in plasma was determined. There were no significant differences (<10%) in the assay concentrations following multiple freeze-thaw cycles, thus indicating analyte/sample stability.

3.5. Post-dose samples

The method was used to assay **1** and **2** from a clinical trial investigating an effect of a novel substance P inhibitor

on cytochrome P450 system specifically the CYP3A4 and CYP2D6. Individual concentrations of **1** and **2** are presented in Table 8.

4. Conclusion

A sensitive and selective HPLC–MS/MS method for the determination of **1** and **2** in human urine was developed but required two different chromatographic conditions to ensure separation of **1** and **2** from all interfering metabolites. The paper demonstrates the clear need for the careful evaluation of the selectivity of quantification of analytes in the presence of metabolites in post-dose samples. The selectivity of the method presented was confirmed in the presence of the N-oxide metabolite. It is clear from this work and previously reported studies [14–16] that the evaluation of an assay based only upon analytes spiked into control biological matrices may not be sufficient for reliable determination of analytes in "real world" post-dose samples. The assessment of contribution to the analyte channels from metabolites should constitute an integral part of all HPLC–MS/MS method validation.

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