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# Quantification of dextromethorphan and metabolites: a dual phenotypic marker for cytochrome P450 3A4/5 and 2D6 activity

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**Abstract**

A sensitive and selective liquid chromatographic procedure using fluorimetric detection was developed to quantify dextromethorphan (DTM), 3-methoxymorphinan (3MM), dextrorphan (DT), 3-hydroxymorphinan (3OH) and two internal standards, codeine (COD) and ethylmorphine (ETM), in urine. Precision and accuracy of the assay were determined over a concentration range of 5–3200 ng/ml urine for DTM, 5–400 ng/ml urine for 3MM, 400–40 000 ng/ml urine for DT and 200–16 000 ng/ml urine for 3OH, by assaying freshly prepared calibration standards and replicates of six quality control (QC) samples on separate days. All of the inter-day and intra-day coefficients of variation (C.V.s) were less than 20% except for a low QC for 3MM. The inter-day and intra-day accuracies were less than 20% for the low QCs, less than 15% for the medium QCs and less than 12% for the high QCs, for all compounds. The limit of quantification (LOQ) was 2 ng/ml urine for DTM and 3MM, 250 ng/ml urine for DT, and 100 ng/ml urine for 3OH. Absolute recovery was 76% for DTM, 74% for 3MM, 77% for DT, 46% for 3OH, 73% for ETM, and 57% for COD. The frequency distribution of the CYP2D6 metabolic ratio (DTM/DT) illustrated a bimodal distribution whereas, the CYP3A metabolic ratio (DTM/3MM) exhibited a unimodal distribution in overnight urine samples of volunteers who ingested 30 mg dextromethorphan hydrobromide. The CYP2D6 metabolic ratio significantly correlated with 3MM/3OH ($r$=0.82) and DTM/3OH ($r$=0.95) but did not correlate with the CYP3A metabolic ratio ($r$=0.27).

*Keywords:* Dextrometorphan; 3-Methoxymorphinan; Dextrorphan; 3-Hydroxymorphinan

## 1. Introduction

Dextromethorphan (DTM), an over-the-counter antitussive, is metabolized in man to dextrorphan (DT) or 3-methoxymorphinan (3MM). These metabolites, in turn, are further glucuronidated or oxidized to 3-hydroxymorphinan (3OH) [1]. Previous studies have shown that the O-demethylation of DTM to DT is primarily mediated by cytochrome P450 2D6 (CYP2D6) [2]. This enzyme is involved in the metabolism of commonly prescribed compounds like $\beta$-blockers and tricyclic antidepressants, and is expressed in the population as two phenotypes, extensive metabolizers (EMs) and poor metabolizers (PMs) [2]. PMs do not express CYP2D6 and comprise about 10% of the Caucasian population, whereas EMs express this enzyme to varying degrees. The phenotypes can be determined by estimating a urinary ratio of DTM/DT after an oral dosage of DTM [2].

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The N-demethylation of DTM to 3MM is mediated primarily by enzymes in the human CYP3A subfamily in vitro [3]. The CYP3A subfamily comprises about 25% of the total cytochrome P450 in the adult human liver and consists of two proteins, CYP3A4 and CYP3A5 [4,5], which metabolize diverse substrates that include xenobiotics, e.g., macrolide antibiotics, calcium channel blockers, quinidine, cyclosporin A, terfenadine, midazolam and endogenous substrates, e.g., progesterone, estradiol, and cortisol [6–11]. There is marked interindividual heterogeneity in the expression of CYP3A genes and the potential utility of specific in vivo phenotyping probes for CYP3A is substantial [4]. Our laboratory has hypothesized that the urinary ratio of DTM/3MM may be used as a phenotypic probe for CYP3A4/5. In order to use this metabolic ratio (MR) a method was developed to quantify concentrations of DTM, 3MM, DT, and 3OH in urine using codeine (COD) or ethylmorphine (ETM) as the internal standard. This method was used to assess frequency distributions and correlations of MRs of CYP2D6 (DTM/DT) and CYP3A (DTM/3MM) in overnight urine of volunteers who had ingested DTM.

## 2. Experimental

### 2.1. Reagents

Sodium azide, β-glucuronidase (G-1512), triethylamine, dextromethorphan, codeine and ethylmorphine were obtained from Sigma Chemical Co., (St. Louis, MO, USA). HPLC-grade ethyl acetate, and ACS-grade hydrochloric acid were obtained from Mallinckrodt (Paris, KY, USA). ACS-grade glacial acetic acid, HPLC-grade hexane, ortho-phosphoric acid (85%) and ACS-grade sodium acetate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). ACS-grade glycine, ACS-grade sodium chloride and ACS-grade sodium hydroxide, were obtained from Matheson, Coleman and Bell (Norwood, OH, USA). Dextrorphan was obtained from Research Biochemicals International (Natick, MA, USA). 3-methoxymorphinan and 3-hydroxymorphinan were gifts from Hoffman-LaRoche (Basel, Switzerland).

Preparation of 1 *M* NaOH-glycine buffer, pH 11.3: Solution A: 1 *M* glycine and 1 *M* NaCl; solution B: 1 *M* NaOH; mix equal volumes of solutions A and B and adjust the pH to 11.3 by the addition of either solution A or solution B.

All buffers were washed with hexane prior to use.

### 2.2. Apparatus and chromatographic conditions

The chromatographic system consisted of a Beckman 114M solvent delivery module controlled by a Beckman 421 Controller, an autosampler (Waters 712 WISP) and a fluorescence detector (Applied Biosystems, Model 980 programmable fluorescence detector). Separation was obtained using a Waters radial compression module containing a Nova-Pak® CN HP Radial-Pak cartridge 100 × 8 mm I.D. which was preceded by a cyano 7-μm guard column (Bioanalytical Systems). The mobile phase used in the separation was acetonitrile–100 m*M* sodium acetate with 600 μl triethylamine/l adjusted to pH 3 with phosphoric acid (12:88, v/v) with a flow-rate of 1.0 ml/min. The fluorescence detector was programmed to change excitation wavelengths during each run with no emission filter. At the beginning of the run, the excitation wavelength was set to 190 nm which was changed to 235 nm at approximately 7 min. Then, the excitation wavelength was changed to 190 nm at approximately 15 min. Peak heights were determined using a Spectra-Physics ChromJet integrator. A nitrogen evaporator was used for solvent evaporation.

### 2.3. Solution preparation

Stock solutions of 3OH, DT, COD and ETM were prepared as 1 mg free base/ml methanol. 3MM and DTM stock solutions were prepared as 1 mg free base/ml acetonitrile. A second set of stock solutions was prepared for quality control samples. All stock solutions were stored at −20°C. Diluted stock solutions for standard curve and quality control samples were freshly prepared for each run in 12×75 mm culture tubes.

### 2.4. Standards and quality controls

Urine standards ranged from 100 to 20 000 ng 3OH/ml urine, 250 to 50 000 ng DT/ml urine, 2 to

500 ng 3MM/ml urine and 2 to 4000 ng DTM/ml urine. Six quality control (QC) samples were prepared, in duplicate, prior to the standard curve preparation. The QC concentrations ranged from 200 to 16 000 ng 3OH/ml urine, 400 to 40 000 ng DT/ml urine, 5 to 400 ng 3MM/ml urine and 5 to 3200 ng DTM/ml urine.

### 2.5. Sample preparation

For the standard curve samples, the methanolic standards were evaporated and 5 ml of urine were added to each screw-cap polypropylene tube. The caps were applied to the tubes and the tubes were rocked for 5 min. A 5 ml volume of each QC sample and each sample to be quantified were placed into a screw-cap polypropylene tube. Next, 1 ml of 0.2 *M* acetate buffer, pH 5, 50 $\mu$l of 0.6 *M* sodium azide, and 75 $\mu$l $\beta$-glucuronidase (1000 units/10 $\mu$l of 0.2 *M* acetate buffer, pH 5) were added to all samples. The caps were applied to the tubes and all samples were mixed. Then, the cap of each tube was loosened and the urine samples were hydrolyzed in a water-bath at 37°C for 18 h. Next, 1 ml NaOH–glycine buffer, pH 11.3 was added to all samples, and the internal standards, COD and ETM (1600 ng/ml urine; 160 $\mu$l of 50 ng/$\mu$l), were added to all urine samples except Standard 0. Then, 6 ml of hexane–ethyl acetate (50:50, v/v) were added to each tube, and the tubes were rocked for 15 min, then centrifuged at 700 *g* for 5 min. The hexane–ethyl acetate was transferred to a clean screw-cap polypropylene tube and another 6 ml of hexane–ethyl acetate were added to the tube containing the aqueous matrix. The tubes were rocked for 15 min, and centrifuged at 700 *g* for 5 min. Then, the organic phase was transferred and combined to that previously transferred. Next, 1 ml of 0.1 *M* HCl was added to each tube containing the hexane–ethyl acetate. The tubes were rocked for 15 min and centrifuged at 700 *g* for 5 min. The upper organic layer was aspirated and discarded. Next, 1 ml of 1 *M* NaOH–glycine buffer, pH 11.3 was added to the aqueous matrix and the tube was mixed for 5 s. Then, 8 ml of hexane–ethyl acetate were added to each tube and the tubes were rocked for 15 min then centrifuged at 700 *g* for 5 min. The hexane–ethyl acetate was transferred to a clean screw-cap polypropylene tube and evaporated to dryness using a nitrogen evaporator. Then, the sample was reconstituted with 150 $\mu$l of mobile phase and mixed for 15 s. Next, the mobile phase was transferred to a polypropylene tube inside a WISP vial and 5–120 $\mu$l were injected onto the HPLC system.

### 2.6. Calculations

Standard curves were estimated by linear regression of peak height ratios of 3OH, DT, 3MM and DTM to COD or ETM versus supplemented urine concentrations. Concentrations of each component in QC and unknown samples were estimated by applying the linear regression equation of the standard curve to the unknown sample peak-height ratio. Low standards were used to estimate the low QC concentrations (QC low 1, medium 1 and high 1). High standards were used to estimate the high QC concentrations (QC low 2, medium 2 and high 2). Acceptance of the standard curve was assessed by the accuracy of the QC samples. Acceptance criteria were 12% accuracy for the high QC samples, 15% accuracy for the medium QC samples and 20% accuracy for the low QC samples. The standard curve for a given compound was deemed unacceptable if two of six QC concentrations did not meet the acceptance criteria. In that situation the standard curve for that compound, including unknown samples, was discarded and the samples were reassayed for that compound.

### 2.7. Extraction efficiency

Absolute recovery was determined at urine concentrations of 5–3200 ng/ml urine for DTM, 5–400 ng/ml urine for 3MM, 400–40 000 ng/ml urine for DT and 200–16 000 ng/ml urine for 3OH by assaying five replicate samples at each of six concentrations. Recoveries of COD and ETM were evaluated at a concentration of 1600 ng/ml ($n$=30). Peak heights of extracted QC samples were compared to average peak heights of standards added to non-supplemented urine extracts ($n$=3).

### 2.8. Assay precision and accuracy

Precision and accuracy of the assay were assessed over the concentration range of 5–3200 ng/ml urine

for DTM, 5–400 ng/ml urine for 3MM and 400–40 000 ng/ml urine for DT by assaying ten, or eleven, freshly prepared calibration standards and five replicates of six QC samples on four separate days. For 3OH, precision and accuracy of the assay was determined over the concentration range of 200–16 000 ng/ml urine by assaying ten freshly prepared calibration standards and five replicates of six QC samples on two separate days. Precision was assessed using % coefficient of variation (C.V.) and accuracy was estimated using % accuracy [(mean/expected) × 100].

### 2.9. Subjects

The human study was approved by the Institutional Review Board of Indiana University. All volunteers were between 18 and 45 years of age. A 30-mg amount of DTM hydrobromide (Benylin® DM, Parke-David, Morris Plains, NJ, USA) were administered to each volunteer ($n=149$) and overnight urine was collected. All volunteers were advised to abstain from any known medications that would interfere with the enzyme of interest.

## 3. Results

### 3.1. Extraction procedure optimization

A 5-ml volume of urine was selected for extraction to obtain enough 3MM for detection in EMs of DTM. Many basic compounds were tested for suitable internal standards. These compounds included tricyclic antidepressants, benzodiazepines, opiods and opiod antagonists, and calcium channel blockers. None of these internal standards worked as well as COD or ETM. The latter two compounds were used to quantify DTM and metabolites in urine but only one was selected per run based on the outcome of the QC samples.

Hexane–ethyl acetate (50:50, v/v) was chosen as the solvent in the liquid–liquid extraction procedure because it afforded a better extraction of 3MM and DTM without loss of 3OH and DT. Hexane and ethyl acetate were assessed as extraction solvents alone but the overall recovery was markedly better with hexane–ethyl acetate (50:50, v/v).

Other changes that resulted in better recovery of each compound from urine included enzyme deconjugation compared to acid deconjugation and the use of polypropylene tubes compared to silanized glass tubes. Specifically, a more purified β-glucuronidase was used in the enzymatic hydrolysis of the conjugated metabolites because it resulted in cleaner chromatograms.

### 3.2. HPLC optimization

The chromatographic conditions were a modification of a previously published report [7]. The molarity and the pH of the aqueous component of the mobile phase were altered to improve the peak shape and the retention characteristics of DTM and metabolites. Specifically, the molarity of the aqueous component was varied from 20 m*M* to 100 m*M* sodium acetate and the pH was changed from 3 to 5. The mobile phase at the higher molarity and lower pH resulted in much better peak shape and retention characteristics for all compounds.

The excitation wavelength of the fluorescence detector was modified during each run to enhance detection of DTM and 3MM and to prevent the overload of the detector by DT and 3OH in EMs of DTM. Peak heights instead of peak areas were selected for peak detection because of decreased variability. Chromatograms of extracted urine from an EM and a PM are illustrated in Fig. 1.

### 3.3. Assay specificity

DTM, metabolites and internal standards were suitably resolved from endogenous urine components. Extracted urine samples, without drugs or internal standards, from fifteen individuals illustrated that the present assay is adequately specific. Three of the fifteen individuals exhibited peaks in the extracted urine that co-eluted with peaks of interest. One of these individuals, a PM of CYP2D6, had taken DTM two weeks previously, so the presence of 3MM and DTM in urine was not surprising. The other two individuals had peaks in the extracted urine that co-eluted with DT but were in such low concentrations that minimal interference was anticipated. Almost all individuals had small peaks that co-eluted with the internal standards. However, when

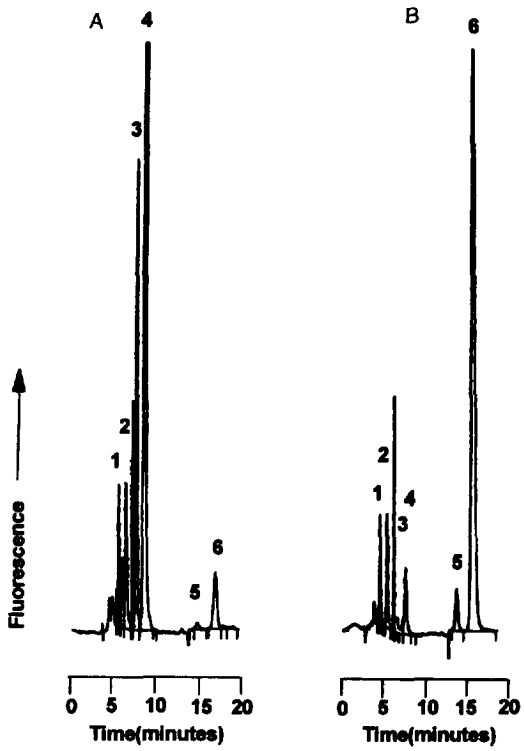


Fig. 1. Chromatograms of an extensive metabolizer (A) and a poor metabolizer (B) of CYP2D6. Peaks: 1=codeine; 2=ethylmorphine; 3=3-hydroxymorphinan; 4=dextrorphan; 5=3-methoxymorphinan; 6=dextromethorphan.

one subject was excluded from the study the average interferences to the normal internal standard concentrations were 3% for COD and 1% for ETM.

### 3.4. Extraction efficiency

Absolute recovery of DTM, 3MM, DT and 3OH, and of the two internal standards, COD and ETM, from urine was determined by quantifying five replicate samples at each of six concentrations. Peak heights of extracted QC samples were compared to average peak heights of standards added to non-supplemented urine extracts. The average (± S.D.) extraction efficiency was 57±10.9% for COD, 73±12.4% for ETM, 46±8.3% for 3OH, 77±14.1% for DT, 74±11.4% for 3MM and 76±17.3% for DTM.

### 3.5. Assay precision and accuracy

Assay precision and accuracy were evaluated based on the analysis of QC standards assayed on four separate days. All of the inter-day precision estimates were less than 20%, except for the low 1 QC of 3MM, which was 26% (Table 1). The inter-day accuracies (% accuracy), using all data points, were in the acceptable range for all compounds at all concentrations. The intra-day precision, based on the C.V., was less than 16% for all samples except for low 1 QC of 3MM, which was 25%. However, the intra-day accuracies were in the acceptable range for all compounds at all concentrations. The limit of quantification (LOQ) was 2 ng/ml urine for DTM and 3MM, 250 ng/ml urine for DT and 100 ng/ml urine for 3OH.

The assay was used to quantify DTM and metabolites in overnight urine of 149 volunteers who

Table 1
Intra-day and inter-day precision estimates of selected quality control samples

| Quality control | | 3OH | DT | 3MM | DTM |
|---|---|---|---|---|---|
| LOQ | Expected concentration (ng/ml) | 100 | 250 | 2 | 2 |
| | Intra-day C.V. (%) | 7.7 | 2.2 | 19.1 | 25.7 |
| L1 | Expected concentration (ng/ml) | 200 | 400 | 5 | 5 |
| | Inter-day C.V. (%) | 13.9 | 16.5 | 26.2 | 14.5 |
| L2 | Expected concentration (ng/ml) | 1600 | 4000 | 50 | 100 |
| | Inter-day C.V. (%) | 6.4 | 8.0 | 7.0 | 7.9 |
| H2 | Expected concentration (ng/ml) | 16000 | 40000 | 400 | 3200 |
| | Inter-day C.V. (%) | 6.6 | 5.7 | 3.4 | 7.9 |

Intra-day ($n$=5–6). Inter-day (3OH, $n$=2 days, 5 samples/day; DT, 3MM, DTM, $n$=4 days, 5 samples/day).

Table 2
Percentage of the dosage excreted as dextromethorphan and metabolites

| | | Percentage of the administered dose | | | |
|---|---|---|---|---|---|
| | | 3OH | DT | 3MM | DTM |
| Extensive metabolizers ($n$=135) | Mean | 8.95 | 28.15 | 0.03 | 0.22 |
| | S.D. | 4.80 | 11.52 | 0.04 | 0.37 |
| Poor metabolizers ($n$=12) | Mean | 0.64 | 0.95 | 0.19 | 2.19 |
| | S.D. | 0.77 | 0.93 | 0.14 | 1.57 |

ingested 30 mg of DTM hydrobromide. Table 2 lists the percentage of the total dosage excreted as dextromethorphan and metabolites in EMs and PMs. Fig. 2 illustrates the bimodal distribution of the CYP2D6 MR in a frequency distribution plot from urine of the 149 volunteers. The antimode was designated as 0.3 based on a previous publication [2]. CYP2D6 MRs ranged from 0.0002 to 128. Fig. 3 shows the unimodal distribution of the CYP3A MR in the same volunteers. The MRs of CYP3A ranged from 0.1–115. Fig. 4A illustrates the significant correlation of two O-demethylation pathways, DTM/DT and 3MM/3OH. These data suggest that the O-demethylations are performed by the same enzyme. Fig. 4B displays the significant correlation of the CYP2D6 MR and the ratio of DTM/3OH. These data show that 3OH formation is significantly controlled by CYP2D6. Fig. 5 shows the non-significant correlation of the CYP2D6 metabolic ratio compared to the CYP3A ratio. This non-significant correlation suggests that there are different enzymes mediating this reaction.

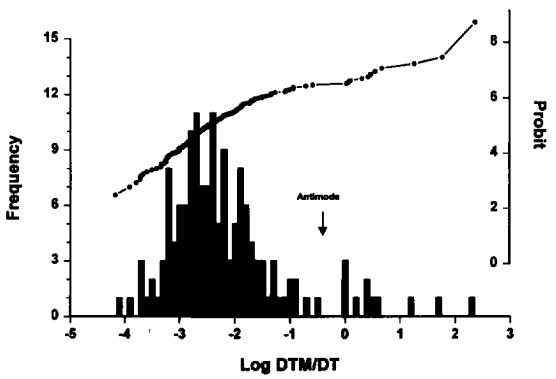


Fig. 2. Frequency distribution histogram and probit plot of the CYP2D6 metabolic ratio. Antimode=0.3.

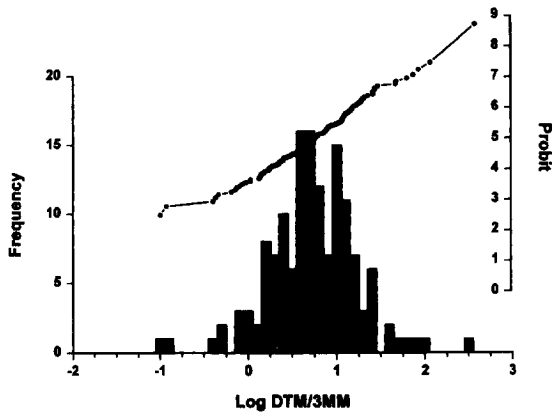


Fig. 3. Frequency distribution histogram and probit plot of the CYP3A metabolic ratio.

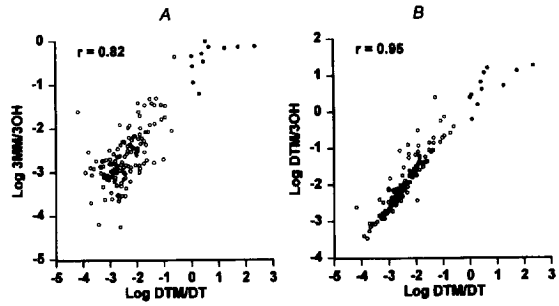


Fig. 4. Correlations between dextromethorphan urinary metabolic ratios. (A) Correlation between log 3MM/3OH and log DTM/DT. (B) Correlation between log DTM/3OH and log DTM/DT. (○)= EM of CYP2D6, (●)=PM of CYP2D6.

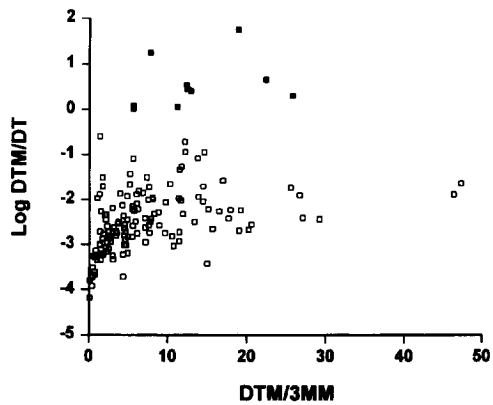


Fig. 5. Correlation between the CYP2D6 and CYP3A metabolic ratios. (□)=EM of CYP2D6, (■)=PM of CYP2D6.

## 4. Discussion

The current method is a modification of a previously published procedure [12,13]. EMs produce substantially more DT compared to 3MM after DTM administration [1]. Since EMs make up approximately 90% of the population, the previous methods were modified to selectively quantify DTM and 3MM without substantial loss of DT and 3OH in urine of volunteers who ingested 30 mg of DTM hydrobromide. The assay of DTM and metabolites was used to estimate MRs which reflect the O-demethylation and N-demethylation of DTM metabolic activity. The MRs were used to observe the frequency distribution of CYP2D6 and CYP3A in the volunteers. The CYP2D6 MR exhibited a predictable bimodal frequency distribution with commonly observed ranges (0.0002–128) of urinary ratios. In contrast, the CYP3A MR exhibited a unimodal distribution based on the frequency and probit plot. A previous study has suggested that CYP3A may have a multimodal frequency distribution in the population [14]. However, our results do not illustrate any polymorphisms of the CYP3A enzyme using the DTM/3MM MR.

In order to show similar enzymes affecting different pathways, correlation analyses were performed with the MRs. The CYP2D6 MR significantly correlated with the 3MM/3OH. These data show that the two O-demethylations occur by the same enzyme, which has been shown in a previous study [15]. The CYP2D6 MR also correlated with DTM/3OH. The CYP2D6 MR did not correlate with the CYP3A MR which suggests that they probably reflect different enzyme activities. Also, PMs of CYP2D6 are not distinguishable, as a group, based on their CYP3A MR, which illustrates that these two MRs reflect different enzyme activities.

In conclusion, we have developed an accurate and precise method to quantify DTM and metabolites in urine that can be used to estimate MRs which reflect in vivo CYP2D6 and CYP3A4/5 activities.

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