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Dextromethorphan as an in vivo probe for the simultaneous determination of CYP2D6 and CYP3A activity

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Abstract

Dextromethorphan (DM) is O-demethylated into dextrorphan (DEX) in humans by the cytochrome P450 designated as CYP2D6 and N-demethylated into 3-methoxymorphinan (3MM) via CYP3As. Clinically, DM has been successfully used as an index of CYP2D6 and this paper describes analytical and clinical data that will help evaluate the use of DM hydrobromide as a probe of CYP3A activity. DM and its three demethylated metabolites were measured in a 4-h spot urine sample using a HPLC method employing solid-phase extraction (C_{18}), analysis on a phenyl column [mobile phase, methanol–acetonitrile–phosphate buffer (10 m*M*, pH 3.5, 20:25:55, v/v)] and fluorescence detection (excitation at λ=228 nm, no emmission cut-off filter). The urinary molar ratio DM–DEX was used to assess CYP2D6 activity while DM–3MM was used for CYP3As. The DM–3MM ratios were sensitive to the co-administration of selective CYP3A inhibitors grapefruit juice and erythromycin. In addition, in healthy volunteers and cancer patients, the N-demethylation of DM correlated with the CYP3A-mediated metabolism of verapamil and tamoxifen. DM appears to be a promising way to simultaneously phenotype patients for CYP2D6 and CYP3As.

Keywords: Dextromethorphan; Dextrorphan; 3-Methoxymorphinan; Cytochromes

1. Introduction

There are wide inter-individual variations in drug efficacy and toxicity. To a great extent, these differences are the result of differences in the metabolism, distribution and elimination of the therapeutic agents. While clinical factors affecting distribution (body mass, albumin levels, etc.) and elimination (kidney function) are relatively easy to measure, the assessment of metabolic capacity is not a routine procedure. Part of this is due to the fact that metabolizing enzymes exhibit a large degree of inter-individual variability in their levels of expression [1]. Some metabolic abnormalities are understood at the DNA level (genotype) but for the majority, the underlying mutations have not been identified [2]. In such cases, metabolic phenotyping remains the only way to assess metabolic irregularities through the identification of specific metabolite patterns produced by test compounds or 'probe drugs' [1].

In probe drug phenotyping, urine or blood samples are collected before and after administration of the probe and if the enzyme(s) responsible for the production of a particular metabolite is (are) known, metabolite to parent ratios can be used to derive phenotypes. Individuals who are deficient in their ability to breakdown the probe drug, relative to the mean rate and extent of metabolism, are called 'slow' or 'poor' metabolizers. Those who have a normal or greater than average metabolism are called 'fast' or 'extensive' metabolizers.

While the genotype prescribes the native levels of

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enzymatic activity, changes in the relative levels and activities of metabolizing enzymes can be produced by drug interactions [3] and/or clinical conditions such as disease progression or malnutrition [4]. Thus a relatively healthy patient will not necessarily have the same reaction to a drug when that person's health has degenerated. This phenomenon has been suggested in AIDS patients for the activity of the enzyme N-acetyltransferase 2 (NAT2). In this case, the NAT2 phenotype appears to change in AIDS patients from fast to slow [5] while the genotype remains constant [6].

The key barrier in the routine incorporation of metabolic activity assessments in clinical treatment is the lack of rapid, inexpensive and 'user friendly' methods for these measurements. An example of an acceptable approach is the determination of NAT2 phenotypes from the urinary excretion pattern of caffeine metabolites [7,8]. As little as a single cup of coffee and a 4-h urine sample can be used to determine if a person has a fast or slow NAT2 phenotype.

The objective of this study is to expand this approach to another class of metabolizing enzymes, the cytochromes P450 (CYPs); in particular CYP3A. CYPs constitute a superfamily of heme-containing monooxygenases catalyzing the metabolism of multiple endogenous and exogenous compounds [1,2]. The CYP3A subfamily includes CYP3A4 and CYP3A5 and represents the most abundantly expressed CYPs in human liver [9] and upper gut [10]. In some human livers, CYP3A4 can represent up to 60% of total CYP content [11]. CYP3A5 is only detectable in 10–20% of human livers [12].

It is thought that CYP3A is the most important subfamily for the metabolism of xenobiotics and that CYP3A4 is the most important isoform. It has been shown that CYP3A5 has similar catalytic properties as CYP3A4 but, unlike CYP3A4, this isoform is non-inducible [13,14]. Although the number of drugs predominantly metabolized by CYP3As is expanding rapidly, a safe and accurate probe drug of CYP3A activity has not been described.

In human microsomes, dextromethorphan (DM), a non-narcotic drug commonly found in non-prescription cough syrups, was found to be O-demethylated into dextrorphan (DEX) via CYP2D6 [15] and N-demethylated into 3-methoxymorphinan (3MM) via CYP3As [16–19] (Fig. 1). More importantly, DEX is not formed via CYP3As and 3MM is not produced

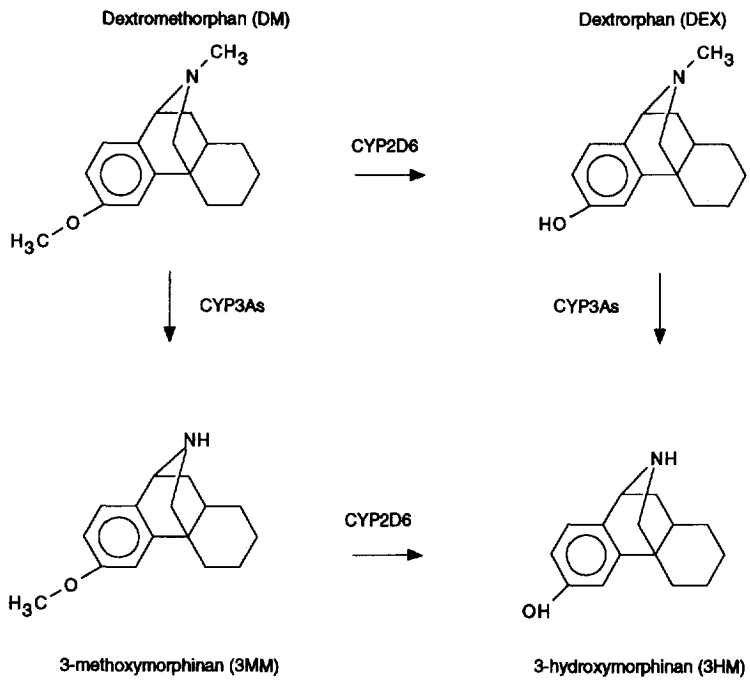

Fig. 1. Chemical structures of DM and of its O- and N-demethylated metabolites.

via CYP2D6 [18–20]. Both DEX and 3MM are further demethylated into 3-hydroxymorphinan (3HM). The secondary metabolite 3HM is thought to be formed from DEX via CYP3As and from 3MM via CYP2D6 [16,20].

The genetics of CYP2D6 have been extensively studied and this enzyme has been shown to be polymorphic [21]. Humans can be divided into extensive and poor metabolizers with a population-based distribution which can range from only seven poor metabolizers out of 695 Chinese subjects (1.01%) to a poor metabolizer frequency of 6.82% in a population of white Swedish subjects [22]. Many of the large population studies and several clinical protocols [22–24] have used the urinary excretion of DEX relative to DM as the determinate of poor and extensive CYP2D6 activity. This is possible because DM and its metabolites are significantly eliminated by the kidney [25].

This paper describes a 'user friendly' protocol which employs DM to assess relative CYP3A acitivity and the analytical and clinical data that will help validate this approach. In this method, a single oral dose of 30 mg of DM hydrobromide was administered and molar ratios of metabolite over parent were measured in 4-h urine samples. Studies were conducted in healthy subjects, in volunteers who ingested known CYP3A inhibitors and in patients and volunteers treated with threapeutic agents known to be CYP3A substrates. Urine samples were collected from all of the subjects and analyzed for DM and its three demethylated metabolites by HPLC. The results of this study are presented below.

2. Experimental

2.1. Analytical procedures

2.1.1. Chemicals and reagents

Dextromethorphan hydrobromide, dextrorphan tartrate, 3-methoxymorphinan hydrobromide, 3-hydroxymorphinan and levalorphan (internal standard, I.S.) were kindly provided by Hoffmann LaRoche (Nutley, NJ, USA). β-Glucuronidase was purchased from Boehringer-Mannheim (Montreal, Canada). Organic solvents and potassium phosphate were of HPLC grade while all other chemicals were of analytical grade (BDH, Montreal, Canada; Fisher Scientific, Montreal, Canada).

2.1.2. Chromatography

The chromatographic system was composed of a Spectra-System P1000 pump (Thermo Instruments, Mississauga, Canada), a SP 8880 autosampler (Thermo Instruments) with a 100-μl injection loop (Rheodyne, Cotati, CA, USA) and a Spectroflow 980 fluorescence detector (ABI Analytical, Kratos Division, Ramsey, NJ, USA). As previously reported by Guttendorf et al. [26], with this detector configuration, the optimum response was obtained with an excitation wavelength of λ=228 nm and no cut-off filter for emission. Chromatograms were recorded on a Datajet integrator (Thermo Instruments) connected to a an electronic data collection system using the Winner on Windows software on a 386 computer (Thermo Instruments).

As per Park et al. [27], analytes were separated on a phenyl column, 5 μm, 25 cm×4.6 mm I.D. (Regis Technologies) at a flow-rate of 1.0 ml/min. The mobile phase consisted of methanol, acetonitrile and 10 m*M* potassium phosphate buffer (pH 3.5, 20:25:55, v/v). The solution was degassed under vacuum and filtered through 0.45-μm membranes (Millipore, Fisher Scientific). The mobile phase was prepared daily and never recirculated. The system was operated at room temperature.

2.1.3. Sample preparation

Urine samples were deconjugated and extracted according to the method developed and validated by Wenk et al. [28] although in this study the extraction method was slightly modified. Urine samples (1 ml) were incubated at 37°C for 18 h with 20 μl of β-glucuronidase and 1 ml of 0.1 *M* sodium acetate buffer (pH 5). Bond-Elut 1 ml C_{18} solid-phase extraction columns (Analytichem International, Harbor City, CA, USA) were pre-conditioned with methanol (6 ml) and water (6 ml). Deconjugated urine samples (250 μl) were deposited onto the columns, spiked with 100 μl of I.S. solution (20 μg/ml in water) and diluted with 2 ml of 0.1 *M* sodium carbonate buffer (pH 9.2). Volumes were aspirated under a light pressure and columns were sequentially washed with water (2 ml) and acetonitrile (1 ml). Analytes were eluted with a mixture of 3 ml of methanol–acetonitrile–2% phosphoric acid

(50:30:20, v/v) and evaporated to dryness under a stream of nitrogen. Residues were reconstituted in 500 μl of 0.01 *M* hydrochloric acid and 100 μl were injected onto the HPLC system.

2.1.4. Calibration curves

Drug-free urine was spiked with all four analytes at 0.10, 0.50, 1.00, 5.00, 8.00, 10.00 and 25.00 μg/ml. Calibration curves were generated by least-square regression of the analyte/I.S. peak-area ratio against the analyte concentration. To minimize analytical variability, a new standard curve was analyzed with each batch of clinical samples and concentrations were derived according to the same day standard curve.

2.1.5. Recovery

The recovery of DM and its metabolites from urine was estimated in triplicate at two different concentrations (0.50 and 10.00 μg/ml). Blank deconjugated urine samples spiked with the appropriate concentrations of analytes and 100 μl of I.S. were extracted ($n = 5$) and compared with blank deconjugated urine extracts subsequently spiked with the same amounts of analytes and I.S. ($n = 5$). The two sets of extracts were evaporated to dryness and reconstituted in hydrochloric acid. Recovery was assessed by comparing the peak-area ratios of analyte/I.S. in the two sets of extracts. The recovery of the I.S. was estimated by comparing the peak area after extraction with that of a direct injection of the same amount of drug ($n = 10$).

2.1.6. Precision and accuracy

Intra-day variability was assessed from replicate measurements ($n = 5$) of two concentrations selected within the range of the standard curves (0.10 and 10.00 μg/ml). Accuracy was determined by comparing the estimated amount with the known drug concentration of spiked samples at 0.50, 1.00 and 8.00 μg/ml ($n = 3$).

2.1.7. Limit of detection and quantitation

Sequentially diluted solutions of DM and its three metabolites were injected directly onto the HPLC system to estimate the minimum amount detectable with a signal-to-noise ratio of 3 (limit of detection, LOD). For each analyte, the limit of quantitation (LOQ) was set at the lowest concentration of the calibration curve.

2.1.8. Sample reanalysis

When the concentrations of the analytes fell outside the range of the standard curve, the samples were assayed using the following protocol: (1) if the values fell below the LOQ, 500 ml of the deconjugated urine was extracted; (2) if the values fell above the maximum limit of quantitation, 125 ml of the deconjugated urine was extracted. If the concentration of an analyte fell below the LOD, the LOQ 0.10 mg/ml was used to derive the molar ratios.

2.2. Metabolic phenotyping protocol

2.2.1. General protocol

All protocols were approved by the Montreal General Hospital Ethics Committee and all subjects, carefully informed of the study, signed the consent forms. On the day of the test, subjects answered a short questionnaire designed to collect relevant information regarding their ethnic origin, smoking and drinking habits, and medication profile, to ensure that factors potentially affecting drug metabolism were considered. Subjects who had taken cough syrup during the past two weeks were excluded from the study. DM cough syrup (Robitussin DM) was kindly provided by Whitehall Robins (Mississauga, Canada).

After collecting a pre-dose morning void urine, healthy volunteers received a single oral dose of 10 ml of Robitussin DM containing 30 mg of DM hydrobromide or 23 mg of DM base. The dose of DM was selected to be equivalent to a usual antitussive dose. At 4 h after DM administration, the subjects were asked to provide another urine sample (the 4-h spot urine). Using this sample, the CYP2D6 phenotype was determined by the urinary molar ratio of DM over DEX and the CYP3A phenotype by the urinary molar ratio of DM over 3MM.

2.2.2. Intra-day variability

Four subjects received a single dose of DM and spot urines were obtained at 2, 4, 6, and 8 h post dose.

2.2.3. Inter-day variability

Eight subjects were phenotyped using the 4-h spot urine protocol on days 1 and 50 ($n = 6$) and on days 1, 50 and 90 ($n = 3$), always in the morning.

2.2.4. Total number of subjects

In all, 65 healthy volunteers (57 male, 8 female; 36±19 years old; 78±11 kg) were phenotyped with DM using the 4-h spot urine protocol. To minimize analytical variability, the urine samples were batched and kept frozen at −20°C until analysis. All subjects were unrelated non-smoking caucasians.

2.3. Validation of DM as a probe of CYP3A activity

2.3.1. Grapefruit juice protocol

On day 1, following the collection of a morning pre-dose blank urine sample, 5 healthy volunteers (3 male, 2 female; 37±10 years old, 70±17 kg), all CYP2D6 extensive metabolizers, took 250 ml of water with 10 ml of Robitussin DM. One hour later, they drank another glass of water. At 4 h, a second spot urine sample was collected. Subjects carefully recorded their food and drink intake on specifically designed case report forms.

On day 4, the subjects were instructed to eat the same breakfast. After the collection of a morning urine sample, the subjects ingested 10 ml of cough syrup with 250 ml of grapefruit juice (Old South, Lykes Pasco, Dade City, FL, USA). The grapefruit juice was prepared from concentrate and all volunteers drank from the same batch to ensure comparable flavonoid intake. One hour later, subjects drank another glass of grapefruit juice and 4 h later they provided a spot urine sample.

2.3.2. Erythromycin protocol

On day 1, following the collection of a morning pre-dose blank urine sample, 6 healthy volunteers (2 male, 4 female; 31±6 years old; 65±15 kg), all CYP2D6 extensive metabolizers, ingested 10 ml of Robitussin DM and provided a 4-h spot urine sample. Immediately after, they began a 3-day treatment of erythromycin estolate (Novorythro, Novopharm), equivalent to 1500 mg per day of erythromycin base. On day 4, approximately 12 h after the last erythromycin dose, subjects ingested 10 ml of cough syrup. Four hours later, a spot urine sample was collected.

2.3.3. Tamoxifen study

Fifteen patients (8 male, 7 female; 49±10 years old; 1.75±0.19 m^2) suffering from recurrent high-grade gliomas and to be treated with high-dose tamoxifen (120 mg/m^2 BID) were phenotyped with a single dose of 10 ml of Robitussin DM prior to starting their treatment. Patients were not on chemotherapy. Concomitant medications were allowed but were kept constant for a week prior to study entry and for four weeks thereafter. A blank urine and a 4-h spot urine sample were obtained. Before and after four weeks of oral tamoxifen, plasma levels of the parent compound and of N-desmethyltamoxifen were measured by a previously reported validated HPLC method [29] which separates and quantitates tamoxifen and its major plasma metabolites. After their chromatographic separation, analytes were converted into highly fluorescent phenanthrene derivatives with a photochemical reactor. The method is highly specific and biological samples did not contain interfering peaks.

2.3.4. Verapamil study

Following six days of oral verapamil at 120 mg TID, verapamil N-demethylation was assessed in 15 healthy males (22±1 years old; 79±3 kg) by calculating the area under the plasma concentration vs. time curve of norverapamil (mean $AUC_{0\text{-}24\ h}$±SEM, ng/ml per h). Plasma levels of verapamil and norverapamil were analyzed according to a previously reported validated HPLC method [30] and AUCs were calculated using the trapezoidal rule [31]. Before starting verapamil, volunteers were phenotyped with a single dose of 10 ml of Robitussin DM using the 4-h spot urine protocol. All subjects were non-smoking and were not taking any medications.

2.4. Statistical analysis

Metabolic phenotyping experiments repeated on the same individuals were compared using a one-way ANOVA for repeated measures. Metabolic phenotyping experiments repeated on the same individuals before and after a single intervention (grapefruit juice and erythromycin protocols) were compared

using a Student's t-test for paired data (if the distribution was normal) or a Wilcoxon signed rank test (if the distribution was not normal). Metabolic phenotyping ratios between individuals (extensive vs. poor CYP2D6 metabolizers) were compared using a Mann-Whitney rank sum test (since ratios were not normally distributed). Relationships were investigated using linear regression. The level of statistical significance was set at 0.05.

3. Results

3.1. Analytical methodology

The analytical method was modified from those of Park et al. [27] and Wenk et al. [28]. Park et al. used two separate liquid–liquid extractions, one to simultaneously extract DM and 3MM and the other one for DEX and 3HM, on 2-ml urine samples. Lam and Rodriguez [32] developed a single extraction procedure for both DM and DEX but did not include an internal standard. In our hands, a liquid–liquid extraction did not effectively clean the samples and resulted in a significantly decreased sensitivity. Jacqz-Aigrain et al. [33], then Wenk et al. [28], improved sensitivity by developing a solid-phase extraction but measured only DM and DEX. Our method is the first one combining a solid-phase extraction and fluorescence detection for the determination of all four analytes.

Representative chromatograms of extracts obtained from drug-free urine and spiked urine are presented in Fig. 2 and Fig. 3. The total run time was 12 min and showed good resolution of DM from its metabolites. All calibration curves were linear over a wide concentration range. On the day of the assay validation, the regression equation for DM was $y =$

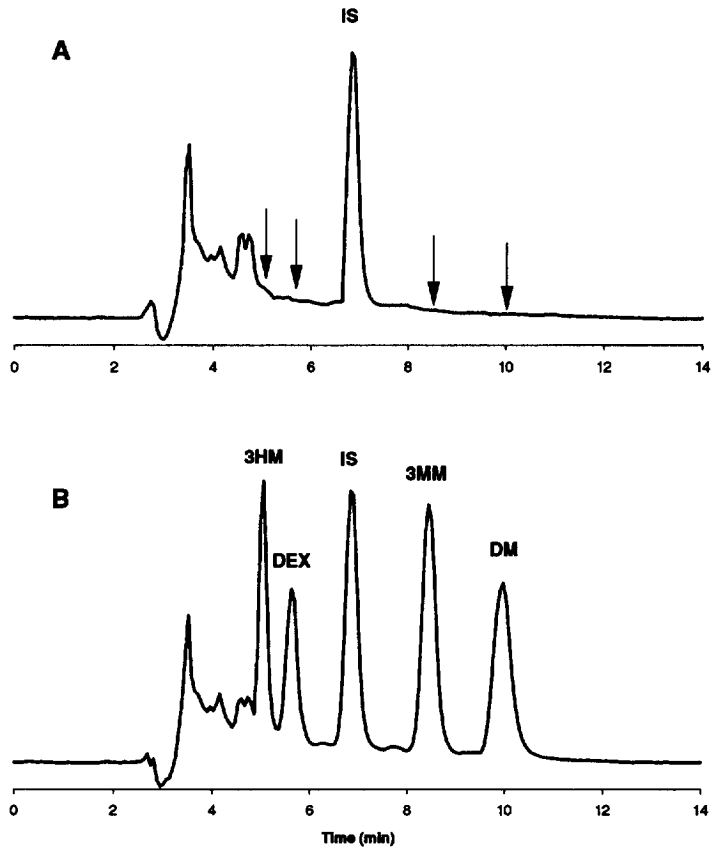

Fig. 2. Representative chromatograms of drug-free urine spiked with 2 μg of I.S. (A) and urine spiked with 5 μg of each analyte and 2 μg of I.S. (B). Arrows indicate the analyte retention times.

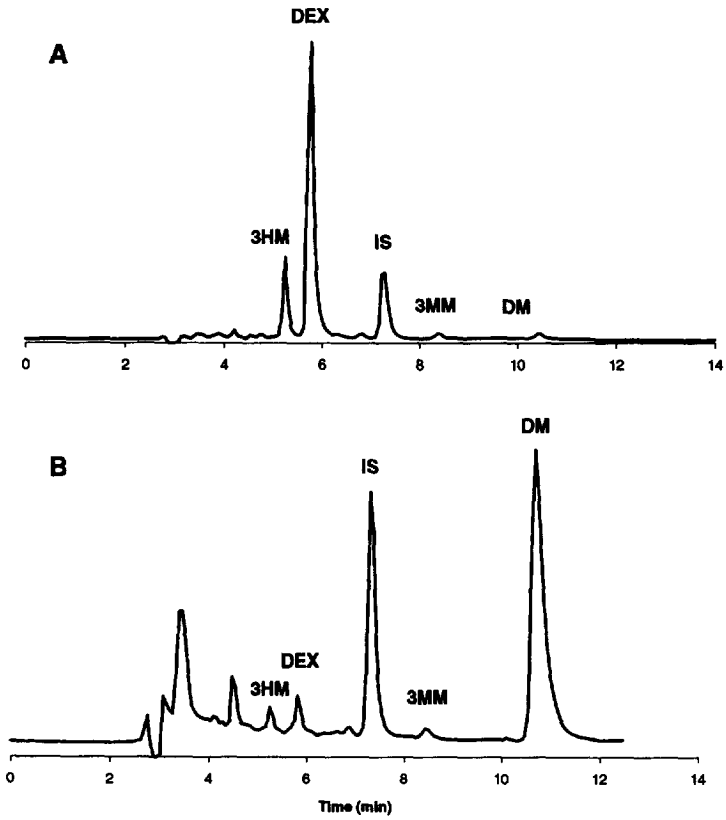

Fig. 3. Representative chromatograms of 4-h spot urine samples from an extensive (A) and a poor (B) metabolizer of CYP2D6.

$0.3116x-0.0396$ ($r^2 = 0.994$). For DEX, the regression equation was $y = 0.1582x-0.0062$ ($r^2 = 0.993$). For the other two metabolites, the regression equations were $y = 0.3438x-0.0558$ ($r^2 = 0.992$) for 3MM and $y = 0.2013x-0.0203$ ($r^2 = 0.995$) for 3HM.

For all 4 analytes, the intra-day assay variability ($n = 5$) was always lower than 12%: for DM, the coefficients of variation (C.V.)=8.9% (at 0.10 μg/ml), 5.6% (at 10.00 μg/ml); DEX, C.V.=10.5% (0.10 μg/ml), 4.9% (10.00 μg/ml); 3MM C.V.=3.1 (0.10 μg/ml), 5.7% (10.00 μg/ml); 3HM C.V.= 6.9% (0.10 μg/ml), 5.1% (10.00 μg/ml).

When the inter-day assay variability was determined, the C.V. values varied from approximately 10% at the high concentrations to 25% at the low concentrations, and an acceptable inter-day validation was not achieved. Therefore, since the intra-day reproducibility was within acceptable limits, the samples were batched into single day runs. Concentrations were derived according to the same day standard curve.

The results from the recovery experiments indicated that for DM and its metabolites, there was no signficant differences between the recoveries obtained at the 0.50 μg/ml and 10.00 μg/ml concentration. The calculated recoveries were: 89±2.2% (DM), 84±1.2% (DEX), 89±0.8% (3MM), 81±0.2% (3HM) and 98±11% (I.S.).

The accuracy of the assay was investigated using standards at concentrations of 0.50, 1.00 and 8.00 μg/ml. For DM the means±S.D. (%C.V.) for the 0.50, 1.00 and 8.00 μg/ml samples were 0.43±0.02 (5.03%), 0.86±0.06 (6.94%), 8.08±0.35 (4.32%), respectively. For 3MM the results were 0.47±0.05 (10.35%), 0.85±0.09 (6.86%), 8.06±0.32 (3.93%); and for DEX the results were 0.69±0.18 (26.04%), 0.99±0.05 (5.37%), 8.29±0.18 (2.19%). While the accuracy for DEX at the 0.50 μg/ml concentration was outside of the acceptable limits, the urinary

concentrations of this metabolite were never less than 5.00 μg/ml and no further attempt was made to reduce the variability at the lowest level. Thus, for DM and 3MM the LOQ was set at 0.10 μg/ml and the LOQ for DEX was set at 1.00 μg/ml. The LOD was 0.06 μg/ml for all of the analytes.

3.2. Phenotyping experiments

3.2.1. General procedures: choosing a 4-h spot urine

Since the objective of the study was to establish an assay which could be used in a large clinical population, it was anticipated that there would be a large inter-subject variability in urinary output. Thus, a spot urine sample could not be used to compare the absolute urinary excretion of each analyte. However, urinary molar ratios of parent over metabolites could be reliably compared within and between volunteers.

The intra-day hourly variation in the urinary ratios of parent over metabolites was investigated using four healthy volunteers. The subjects were phenotyped with a single oral dose of 30 mg of DM hydrobromide and urine samples were collected before DM administration and at 2, 4, 6 and 8 h post dosing. The urinary molar ratios of DM/DEX and DM/3MM were determined for each sample and for the 0–8 h period.

Previous studies utilizing DM to assess the CYP2D6 phenotype have determined the molar ratios of DM/DEX using 0–8 h urinary samples (18). The results presented in Table 1 indicate that the DM/DEX molar ratios determined in the 4-h urine sample were in agreement with those determined for the 0–8 h period, Eventhough the 4-h DM/DEX ratio for subject 1 is only 73% of the 0–8 h value, the subject would still be classified as an 'extensive' DM metabolizer relative ot the activity of CYP2D6.

The DM/3MM urinary molar ratios determined in the 4-h urine samples were also in agreement with those determined for the 0–8 h period (Table 1). Since the genes responsible for CYP3A activities have not been identified nor has a polymorphic phenotype been established, the significance of the comparison between the 4 h and 0–8 h urine molar ratios of DM/3MM cannot be assessed. However, if the objective of this method is to compare DM conversion to 3MM (i.e. CYP3A activity) with the metabolism of therapeutic agents, two criteria are necessary: (1) the measurements must be reproducible over time; (2) the measurements must reflect changes in CYP3A activity. The results from experiments designed to address these issues are presented below.

Table 1
Intra-day variations in urinary molar ratios of dextromethorphan (DM) and its O-demethylated (DEX) and N-demethylated (3MM) metabolites as determined from sequential urine samples

Subject	2-h Urine	4-h Urine	6-h Urine	8-h Urine	0–8-h Urine
DM/DEX (molar ratios×10^2)					
1	2.69	2.01	0.80	0.50	0.54
2	ND[a]	1.71	2.48	0.72	1.26
3	0.73	1.12	0.71	0.91	0.84
4	0.54	0.50	1.03	0.39	0.54
DM/3MM (molar ratios)					
1	0.189	0.294	2.326	2.083	0.418
2	ND[a]	0.334	5.263	0.420	0.541
3	0.314	0.422	1.010	1.563	0.559
4	0.735	1.086	0.201	0.218	0.337

[a]No urine sample was provided and the values were not determined (ND).

The inter-day variations in data obtained from the 4-h spot urine procedure was investigated using eight healthy volunteers over 50 ($n = 8$) and 90 ($n = 2$) day periods. The results showed no significant changes in DM/DEX and DM/3MM molar ratios over time, as shown in Fig. 6. All subjects were fast CYP2D6 metabolizers and retained the same classification at all times. Mean ratios of DM/DEX were 0.017±0.011, 0.018±0.006 and 0.022±0.013 on day 1, 50 and 90, respectively. All values were well below 1, the cutoff point separating extensive from poor metabolizers. Similarly, for CYP3As, ratios remained within the same order of magnitude. For DM/3MM, mean ratios were 1.03±0.61, 1.39±0.67 and 0.82±0.13 on day 1, 50 and 90, respectively.

3.2.2. Phenotyping studies

Sixty-five healthy volunteers were phenotyped with a single oral dose of 30 mg of DM hydrobromide. To allow visualization of all ratios on the same graph, the logarithms of DM/DEX and DM/3MM were plotted (Fig. 4). On the basis of previously reported data [15,23] and from the visual inspection of the frequency distribution curve (Fig. 5), four subjects were classified as CYP2D6 poor

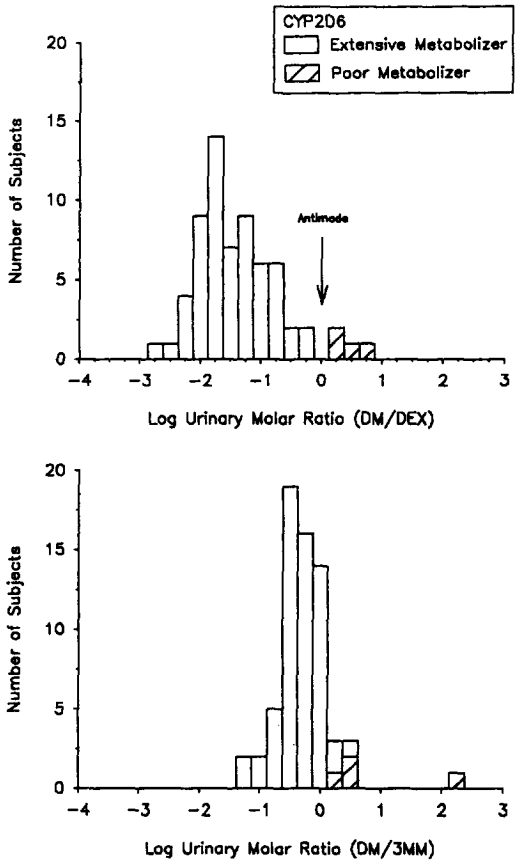

Fig. 4. Intra-day variability of metabolic phenotyping for CYP2D6 (DM/DEX urinary molar ratios) and CYP3As (DM/3MM urinary molar ratios). Each symbol represents a different subject.

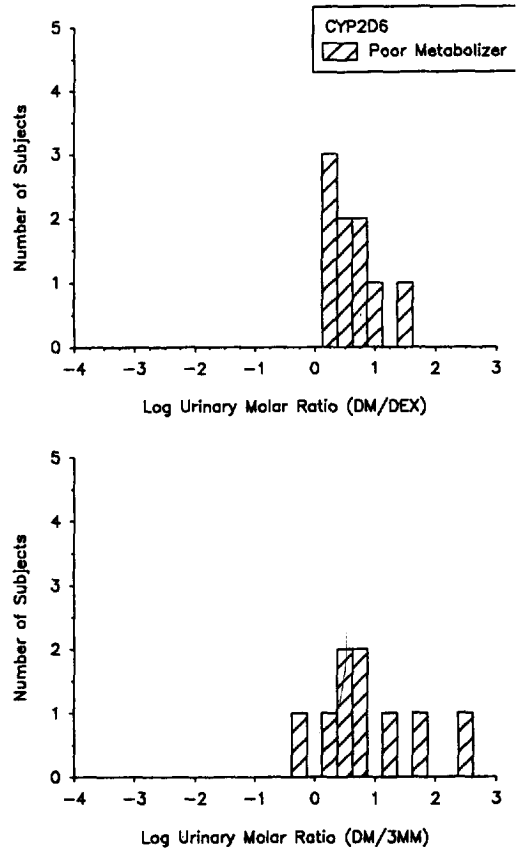

Fig. 5. Frequency distribution of the DM/DEX and DM/3MM urinary molar ratios in a 4-h spot urine sample of 65 healthy volunteers who received a single oral dose of 30 mg of DM hydrobromide.

metabolizers (log DM/DEX higher than zero) while 61 were classified as CYP2D6 extensive metabolizers (log DM/DEX lower than zero).

The mean values of all urinary molar ratios in both groups are presented in Table 2. For DM/DEX, ratios varied from 0.002 to 0.74 in CYP2D6 extensive metabolizers and from 2.79 to 9.28 in CYP2D6 poor metabolizers. For DM/3MM, ratios ranged from 0.07 to 4.15 in CYP2D6 extensive metabolizers and from 2.07 to 196.18 in CYP2D6 poor metabolizers. As seen in Fig. 5, one volunteer was a clear outlier for the DM/3MM ratio. Without this patient, DM/3MM ratios in CYP2D6 poor metabolizers ranged from 2.07 to 3.92, for a mean of 3.07±0.94, which is comparable to the mean value obtained in CYP2D6 extensive metabolizers. The DM/3MM ratios for the three other poor metabolizers were distributed within the population of CYP2D6 extensive metabolizers.

In addition to the four CYP2D6 poor metabolizers

Table 2
Urinary molar ratios of dextromethorphan (DM) and its metabolites (mean±S.D.) in a 4-h spot urine sample following the administration of a single oral dose of 30 mg of dextromethorphan (DM) hydrobromide to 65 healthy volunteers

	CYP2D6	
	Extensive metabolizers (n = 61, 94%)	Poor metabolizers (n = 4, 6%)
DM/DEX	0.10±0.15	4.71±3.07*
DM/3MM	0.82±0.67	51.35±96.56*
DEX/3HM	2.61±1.49	1.71±1.04
3MM/3HM	0.22±0.18	0.96±0.59*

*$P < 0.05$, Mann-Whitney rank sum test, extensive vs. poor CYP2D6 metabolizers.

Table 3
Urinary molar ratios of dextromethorphan (DM) and its metabolites (mean±S.D.) in a 4-h spot urine sample following the administration of a single oral dose of 30 mg of dextromethorphan (DM) hydrobromide to 9 poor metabolizers of CYP2D6

	CYP2D6, poor metabolizers ($n = 9$)
DM/DEX	5.27±5.96
DM/3MM	31.00±64.33
DEX/3HM	1.74±1.16
3MM/3HM	1.09±0.58

found in our study population, five others were phenotyped (Table 3, Fig. 6). Among the 9 poor metabolizers, ratios varied from 1.25 to 19.80 for DM/DEX and from 0.66 to 196.18 for DM/3MM.

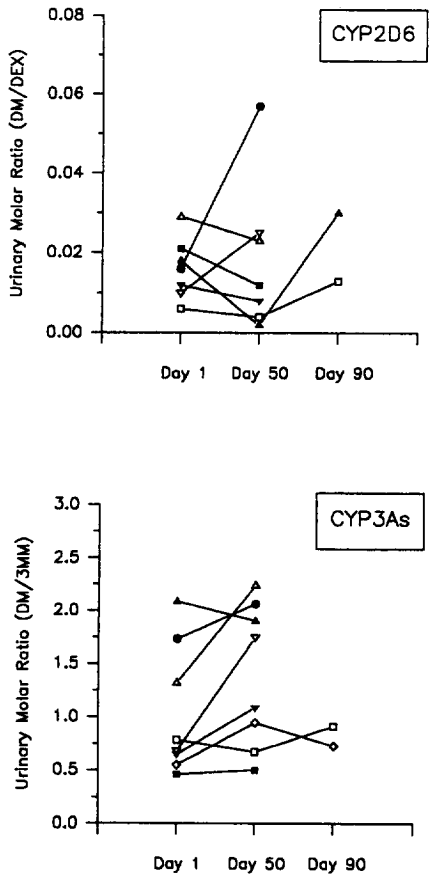

Fig. 6. Frequency distribution of the DM/DEX and DM/3MM urinary molar ratios in a 4-h spot urine sample of 9 healthy volunteers, CYP2D6 poor metabolizers, who received a single oral dose of 30 mg of DM hydrobromide.

Three poor metabolizers had DM/3MM ratios higher than the average extensive metabolizers. Their values were 196.18 (as previously indicated), 55.13 and 13.67 (the three subjects with log values higher than 1 in Fig. 5). In all volunteers, poor or extensive metabolizers, DM/3MM and DM/DEX molar ratios were not correlated ($r^2 = 0.2280$ in 61 extensive metabolizers and $r^2 = 0.2420$ in 9 poor metabolizers).

3.2.3. Inhibitor studies

In vitro, grapefruit juice has been shown to be an inhibitor of CYP3A isoforms [35]. In vivo, it has been reported to inhibit the metabolism of CYP3A substrates such as nifedipine or cyclosporine [36,37]. The effect of grapefruit juice on the metabolism of DM was investigated in five healthy subjects using a sequentially designed experiment where: on day 1 the DM was administered with water and on day 4 with grapefruit juice. The results are presented in Fig. 7 and indicate that coadministration of grapefruit juice had no effect on the calculated DM/DEX molar ratios, 0.014±0.005 (day 1) vs. 0.015±0.004 (day 2) ($P = 0.916$). In contrast, DM/3MM ratios were increased 15 fold with coadministration of grapefruit juice, 2.18±0.63 (day 1) vs. 33.14±24.63 (day 2) ($P = 0.046$). Other ratios involving the secondary metabolite 3HM did not change significantly ($P = 0.321$ and 0.197).

In vitro, erythromycin is a known inhibitor of CYP3As [38]. In vivo, erythromycin interacts with several CYP3A substrates such as midazolam [39] or terfenadine [40]. Five healthy subjects were phenotyped with DM before (day 1) and after (day 4) a 3-day treatment with erythromycin and the results are presented in Fig. 8. The DM/DEX ratios remained unchanged, 0.05±0.08 (day 1) vs. 0.03±0.04 (day 4) ($P = 0.438$) while DM/3MM ratios increased from 1.36±0.60 (day 1) to 3.12±3.00 (day 4) ($P = 0.031$). DEX/3HM and 3MM/3HM were not altered by erythromycin treatment ($P = 0.193$ and 0.313, respectively).

3.2.4. In vivo comparison of DM metabolism with metabolism of tamoxifen and verpapmil

The hepatic microsomal metabolism of tamoxifen (TMX) has been investigated using characterized human liver microsomes and CYP3As have been identified as the major isoforms involved in the

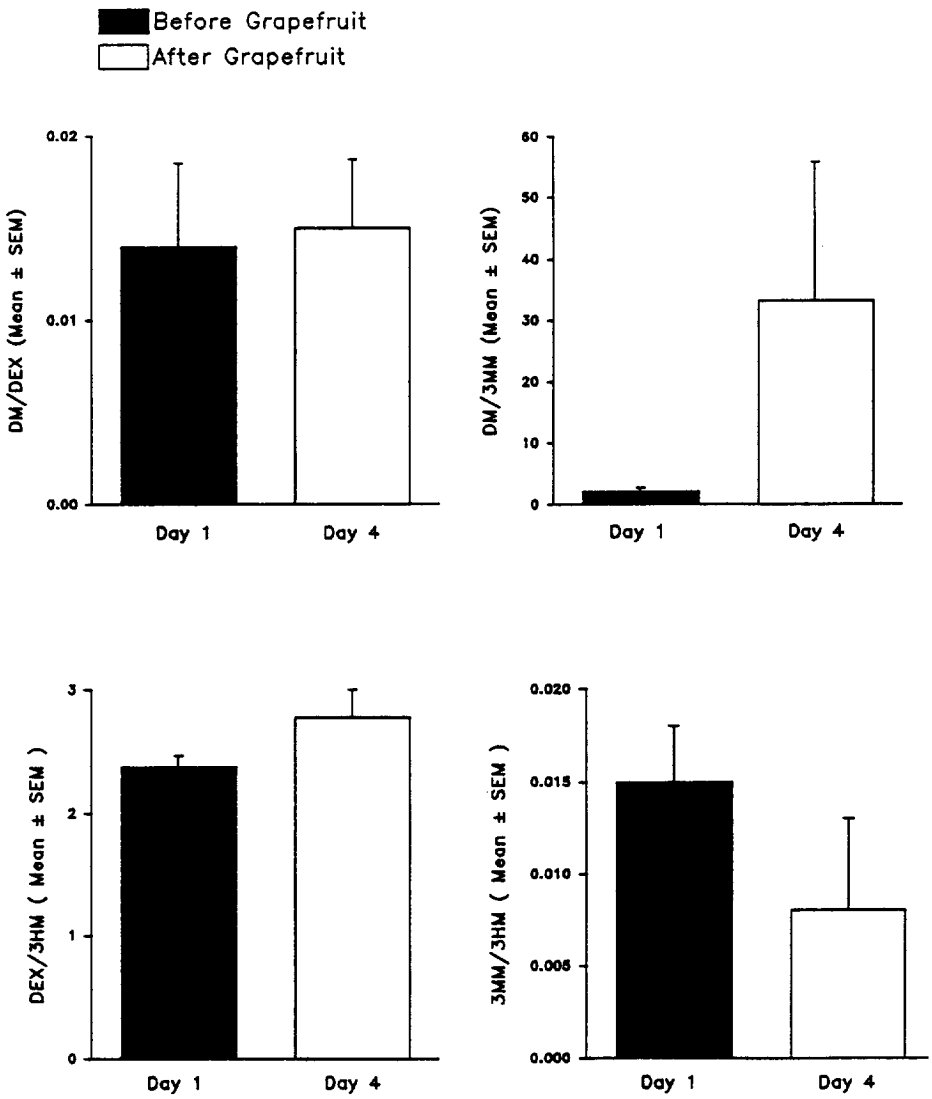

Fig. 7. In vivo effect of grapefruit juice on the urinary molar ratios of DM/DEX, DM/3MM, DEX/3HM and 3MM/3HM in a 4-h spot urine sample of 5 healthy volunteers.

formation of N-desmethyltamoxifen (DES). DES is the major circulating metabolite of TMX in steady-state clinical samples [41,42]. The steady-state plasma concentrations of TMX and DES were determined in fifteen cancer patients who were receiving high-dose TMX for treatment of advanced cerebral astrocytomas. The patients were also phenotyped with DM. The steady-state molar ratios of DES were significantly correlated with the urinary molar ratios of DM/3MM ($r^2 = 0.6775$, Fig. 9), the faster N-demethylators of DM being the faster N-demethylators of TMX. There was no correlation between the formation of DES and the O-demethylation of DM ($r^2 = 0.1415$).

In human liver microsomes, CYP3As are also the main isoforms involved in the formation of norverapamil, one of the two main metabolites of verapamil [43]. In fifteen healthy volunteers phenotyped with DM, the plasma AUCs of norverapamil were significantly correlated with the urinary molar ratios of DM/3MM ($r^2 = 0.2922$, Fig. 10). The AUCs of norverapamil were not correlated

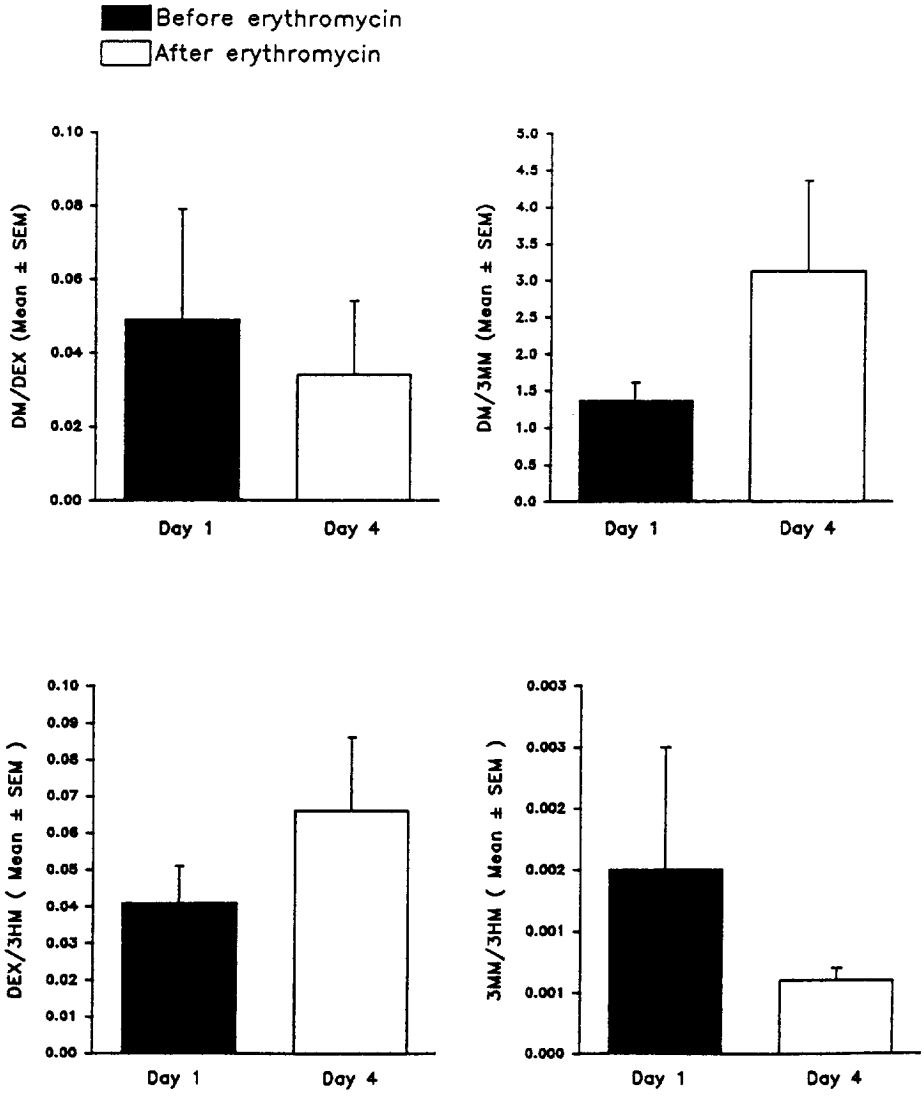

Fig. 8. In vivo effect of erythromycin on the urinary molar ratios of DM/DEX, DM/3MM, DEX/3HM and 3MM/3HM in a 4-h spot urine sample of 6 healthy volunteers.

with the urinary molar ratios of DM/DEX (r^2 = 0.0500).

4. Discussion

Numerous in vitro studies have investigated the microsomal metabolism of DM. Researchers from independent laboratories have confirmed the involvement of CYP2D6 in the O-demethylation of DM and 3MM and of CYP3As in the N-demethylation of DM and DEX [15–20,44].

The O-demethylation of DM was inhibited by CYP2D6 substrates or competitive inhibitors, such as 4-hydroxydebrisoquin, quinidine, *rac*-perhexiline, dextropropoxyphene and *rac*-methadone [20,44]. The O-demethylation of 3MM was also inhibited by the same compounds [20]. Conversely, the N-demethylations of DM or DEX were not inhibited by quinidine nor *rac*-methadone [19,20].

The N-demethylation of DM was inhibited by

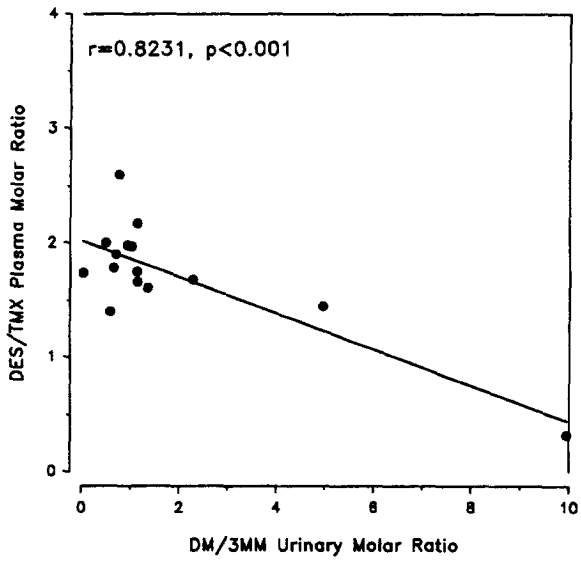

Fig. 9. Correlation between the N-demethylation of DM and the N-demethylation of tamoxifen in 15 cancer patients.

CYP3A substrates or inhibitors, such as midazolam and gestodene [19]. Significant correlations were also observed between the formation of 3MM and the human liver microsomal sample's erythromycin N-demethylase or testosterone 6β-hydroxylase activities and immunoquantified levels of CYP3A4 [19]. In foetal liver microsomes, which possess

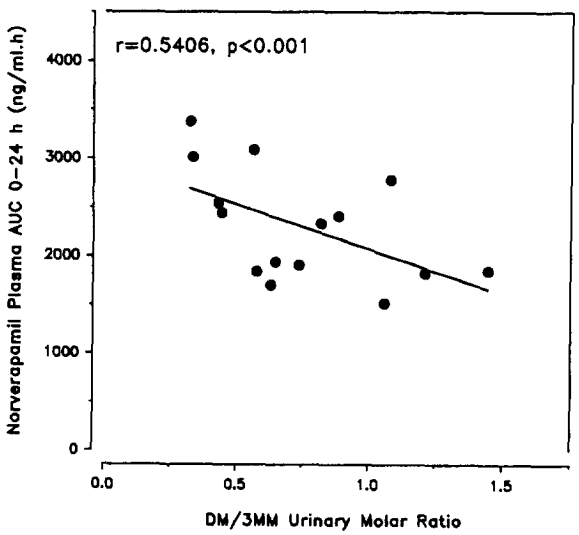

Fig. 10. Correlation between the N-demethylation of DM and the formation of norverapamil, as expressed by the AUC vs. time curve over 24 h, in 15 healthy volunteers.

CYP3A activity but not CYP2D6, DM was N-demethylated but not O-demethylated [16,17]. In addition, lymphoblastoid cells expressing CYP3A4 catalyzed the formation of 3MM [19].

Therefore, in vitro studies provided a sound rationale for the investigation of DM as an in vivo probe of CYP2D6 and CYP3A activities [18,19]. Since DEX is not formed via CYP3As and 3MM is not produced via CYP2D6 [18–20], molar ratios of DM/DEX would reflect CYP2D6 activity while DM/3MM would represent CYP3As. The molar ratios of DEX/3HM could also reflect CYP3A activity and 3MM/3HM could be indicative of CYP2D6. However, since these ratios involve a secondary metabolite, they are largely influenced by the formation of the primary metabolite, making them less specific indicators of enzyme activity.

The clinical pharmacokinetics of a single oral dose of DM have been previously investigated. Since 90 to 95% of caucasians are extensive metabolizers of CYP2D6 [24,47,48], most pharmacokinetic studies were undertaken in fast metabolizers of DM. Hence, DM was rapidly metabolized into DEX, and its plasma or urine concentrations rapidly fell below detectable levels. Accordingly, most pharmacokinetic studies followed DEX levels. Following the administration of single oral doses ranging from 30 to 60 mg of DM hydrobromide to healthy volunteers [49] or to patients with pathological cough [50], DEX half-life approximated 3 h. In patients suffering from ALS receiving higher doses of 2.5 mg/kg of DM in a syrup formulation, DEX elimination half-life ranged from 1.4 to 3.9 h [51]. Silvasti et al. [46] obtained plasma half-lives for both the parent and the main metabolite, with values ranging from 3.2 to 3.6 h for DM and from 2.7 to 4.0 h for DEX.

Since after seven half-lives more than 99% of the dose is eliminated from the body [52], approximately 28 h are necessary for the complete urinary excretion of DM or DEX. In this study, we always ensured that a previous dose of DM was entirely eliminated before a subsequent administration. The grapefruit juice and erythromycin protocols were carried out in CYP2D6 extensive metabolizers and a 3-day period separated a two phenotyping episodes. All subjects were not allowed to have cough syrup in the 2 weeks preceding the phenotyping testing, since in slow metabolizers, the plasma half-life of DM can be prolonged up to 45 h [53]. The blank urine sample

collected on the morning of the test confirmed that DM and its metabolites were not detected.

Having established that both the analytical method and the phenotyping procedures led to reproducible results and did not compromise phenotype assignment, 65 healthy volunteers were phenotyped with a single oral dose of DM. Using a 4-h spot urine sample, we were able to confirm the bimodal distribution of DM/DEX ratios, with an antimode of zero separating the log of the ratios of extensive (94% of the study population) and poor metabolizers (6%) [8,24,34]. The absolute values of the ratios were in good agreement with those obtained by other investigators with total urinary collections over 4 h [8,24], 8 h [18,54] or 10 h [55,56]. The collection of a 4-h spot urine sample adds considerable simplicity to existing metabolic phenotyping procedures and lends itself to the execution of phenotyping protocols involving a large number of subjects.

In CYP2D6 extensive metabolizers, the lowest ratios were those of DM/DEX, pointing to DEX as the predominantly excreted metabolite. In CYP2D6 poor metabolizers, ratios implicating DM were significantly higher compared to those involving only metabolites, suggesting a significanlty higher excretion of unchanged DM. This was confirmed by Jacqz-Aigrain et al. [18] who administered a single oral dose of 40 mg of DM to 155 French caucasians while collecting their urinary output for the following 8 h. Since they measured total urinary excretion of each analyte, they determined that the higher ratios of DM/3MM in CYP2D6 poor metabolizers were due to an increased elimination of unchanged DM. Although they noted that 3MM urinary excretion was slightly higher in CYP2D6 poor metabolizers, they did not detect any difference in the partition of DM/3MM ratios between poor and extensive CYP2D6 metabolizers. This was also corroborated in vitro, where the Michaelis–Menten constant for the N-demethylations of DM into 3MM and of DEX into 3HM did not differ between microsomes obtained from CYP2D6 extensive and poor metabolizers [20]. In addition, in our study and that of Jacqz-Aigrain et al. [18], for all volunteers, poor or extensive metabolizers, DM/3MM and DM/DEX molar ratios were not correlated.

In order to be used as a measure of enzymatic activity, the metabolism of the probe drug must change with alterations in the activity or expression of the target enzyme, i.e. increase with induction of the enzyme and decrease with it inhibition. In this study, the administration of the CYP3A inhibitors grapefruit juice and erythromycin signficiantly increased the DM/3MM molar ratios. This was not a general effect since the DM/DEX ratios (a reflection of CYP2D6 activity) were unaffected. Thus, the in vivo N-demethylation of DM was sensitive to the inhibitory effects of grapefruit juice and erythromycin, indicating that the DM/3MM urinary molar ratios are indeed sensitive to changes in CYP3A activity.

One of the ultimate applications of a metabolic probe drug is the prediction of individual clinical response, i.e. how will the patient respond to this medication. For example, there is a correlation between a slow NAT2 phenotype and hypersensitivity to the drug sulfamethoxazole [57]. Thus, a simple probe drug phenotyping before the initial administration of the drug may prevent an unwanted toxic response.

A significant problem in the development of a probe drug-therapeutic agent relationship is the fact that drug metabolism often involves multiple enzymes and a perfect correlation is often impossible. However, if the enzymatic activity being probed plays a significant role in the metabolism of the drug, then the metabolic patterns of the probe and target should be related.

In this study, the N-demethylation of DM, a probe of CYP3A activity, was compared to the N-demethylation of tamoxifen and verapamil, predominantly CYP3A-mediated pathways. The results indicate that there was indeed a correlation between the probe and the therapeutic agents. For example, the N-desmethyltamoxifen/tamoxifen ratios were correlated with DM/3MM but not with DM/DEX. This is consistent with published in vitro data excluding CYP2D6 in tamoxifen N-demethylation [41,42,45]. Linear regression showed that 68% of the variability in N-desmethyltamoxifen/tamoxifen ratios could be explained by DM/3MM ratios (Fig. 9).

Similarly, 29% of the variability in the AUCs of norverapamil could be explained by DM/3MM ratios. In vitro, the formation of norverapamil was correlated with the expression of CYP3As with an r of 0.58 [43], which is very similar to the 0.54 we obtained in vivo. The lower coefficient of correlation can be explained by the fact that in vitro studies have suggested that norverapamil may also be formed via CYP1A [43]. However, the involvement of CYP1A

has never been demonstrated in vivo and current studies in our laboratory suggest that the observed variations may be due to further CYP3A and CYP2C8 mediated conversions of norverapamil.

Other probes of CYP3A activity have been proposed but they have been used with only limited success. Although caffeine has been suggested [58], it is our experience that phenotyping with caffeine can be difficult: (1) Blank urine samples are almost impossible to obtain and metabolite ratios can be biased by caffeine ingestion during the sampling period. (2) The excretion of the various metabolites is extremely dependent upon urine flow and since caffeine has diuretic properties, metabolite/parent ratios are extremely variable. (3) Urine samples have to be acidified to avoid the ex vivo conversion of AFMU into AAMU. (4) Finally, the determination of a CYP3A phenotype might be complicated by the fact that CYP2E1 and CYP4B1 also contribute to caffeine 8-hydroxylation [59,60].

Other tests have been examined, such as the erythromycin breath test [61], dapsone N-hydroxylation [62] or cortisol 6β-hydroxylation [63]. Although dapsone was once thought to be a promising probe of CYP3A activity [64], it is now being challenged [65]. The erythromycin breath test, involving the i.v. administration of radioactive erythromycin, is quite invasive and has limited clinical applicability. When the three tests were carried out in the same subjects, there was no significant correlation between any of the trait measurements [66]. In additon, since CYP3A4 is found in significant concentrations in the upper gut, it is doubltful that an i.v. administered probe can be used to predict the fate of an orally administered agent.

The results of these studies suggest that DM may be an appropriate probe for orally administered medications. This agent has been incorporated in a number of protocols involving breast cancer and AIDS patients, and with controls, the number of subjects will reach 500. The data from these studies should confirm or reject the use of DM as a marker of CYP3A activity.

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