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LC–MS/MS analysis of dextromethorphan metabolism in human saliva and urine to determine CYP2D6 phenotype and individual variability in *N*-demethylation and glucuronidation

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Abstract

In order to establish a fast screening method for the determination of the CYP2D6 metabolic phenotype a sensitive LC–MS/MS assay to quantify dextromethorphan (DEX) and its *O*-demethylated metabolite dextrorphan (DOR) in human saliva was developed with limits of quantitation of 1 pmol/ml. Saliva was provided by 170 medical students 2 h after oral ingestion of 30 mg (81 μmol) dextromethorphan hydrobromide. Individual ratios of the concentrations DEX/DOR (metabolic ratio, $MR_{DEX/DOR}$) varied more than 25,000-fold (0.03–780). Two groups comprising 156 'Extensive' and 14 'Poor Metabolizers' were clearly distinguished. For the investigation of individual differences in *N*-demethylation and glucuronidation, four additional metabolites of DEX, 3-methoxymorphinan (MOM), 3-hydroxymorphinan (HOM), and the two *O*-glucuronides (DORGlu and HOMGlu) were measured by LC–MS/MS analysis of 6-h urine of 24 volunteers. The *N*-demethylation reactions DEX-to-MOM and DOR-to-HOM defined by the respective MR were significantly correlated. The same holds for the glucuronidation pathways ($MR_{DOR/DORGlu}$ versus $MR_{HOM/HOMGlu}$). The three poor CYP2D6 metabolizers excreted relatively high amounts of the parent compound DEX (up to 7 μmol), but only low amounts of glucuronides (DORGlu: 0.4–1.0 μmol; HOMGlu: 0.2–0.7 μmol). For the 21 'Extensive Metabolizers', the two glucuronides were the most abundant, with relatively little interindividual variation (DORGlu: 10–44 μmol; HOMGlu: 5–17 μmol). For the excretion of the glucuronides, two normal distributions provided the best fit, indicating that the determination of the glucuronides alone could allow assignment of the CYP2D6 metabolic phenotype.

Keywords: Dextromethorphan; Metabolism; Phenotyping; CYP2D6; Monooxygenase; Demethylation; Glucuronosyltransferase; Individual; Statistics; Models

1. Introduction

The cytochrome P4502D6 enzyme is involved in the oxidative metabolism of a variety of drugs, including antiarrhythmics, antidepressants, neuroleptics, β-blockers, opioids and others. Enzyme activity is known to vary enormously between individuals due to genetic differences. CYP2D6 genotyping studies revealed a large number of alleles [1,2]. Their occurrence and frequency vary depending on the ethnic origin of the population group. In the European population, 5–10% are homozygous for inactivating mutations and lack the respective enzyme activity [3]. They are designated as 'Poor Metabolizers' for CYP2D6 (PM) as opposed to 'Extensive Metabolizers' (EM).

In PM, elimination of respective drugs is reduced and repeated ingestion can lead to accumulation. If the therapeutic index is small adverse effects are more frequent than in EM, which is seen for instance with tricyclic antidepressants [4]. In an attempt to apply this knowledge to clinical practice, dose recommendations were suggested for PM patients [5]. Both genotyping and phenotyping can be used for classification and the results are usually concordant [6,7]. In particular

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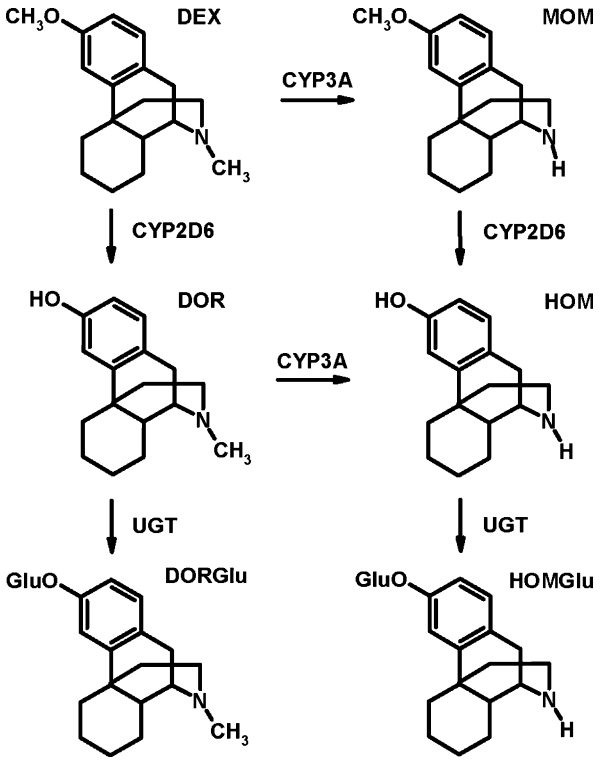

Fig. 1. Pathways for dextromethorphan (DEX) metabolism investigated in this study showing the metabolites dextrorphan (DOR), 3-methoxymorphinan (MOM), 3-hydroxymorphinan (HOM), dextrorphan glucuronide (DORGlu) and 3-hydroxymorphinan glucuronide (HOMGlu).

situations, however, phenotyping is advantageous because it provides direct information on the actual level of enzyme activity. For instance, concomitant medication can convert an EM genotype into a PM phenotype [8–10]. Knowing a person's metabolic capacity could therefore make drug treatment safer and more efficient and allow for personalized medicine [11].

The antitussive agent dextromethorphan (DEX) is normally used as a probe drug for phenotyping [12]. Fig. 1 shows the metabolic pathways in humans. The *O*-demethylation of DEX to dextrorphan (DOR) is catalyzed by CYP2D6. DEX and DOR can undergo *N*-demethylation to give 3-methoxymorphinan (MOM) and 3-hydroxymorphinan (HOM), respectively. CYP3A is known to be involved in this pathway [13]. For the *O*-demethylation of MOM to HOM CYP2D6 is also involved. Finally DOR and HOM are glucuronidated to give dextrorphan glucuronide (DORGlu) and 3-hydroxymorphinan glucuronide (HOMGlu). For phenotyping for CYP2D6 activity, a metabolic ratio (MR) is calculated by dividing the concentration (or amount) of the parent compound DEX in urine, plasma, or saliva by the concentration (or amount) of the metabolite DOR.

A variety of analytical methods to measure concentrations of DEX and DOR are in use. This includes HPLC with UV or fluorescence detection [14–19], gas chromatography with nitrogen–phosphorous detection [20], and mass spectrometric methods for urine [21] and plasma samples [22]. Two assays based on saliva have been described. They require either a high dose of 50 mg of DEX [14] or a large saliva volume of 5–10 ml [18]. Both methods use a time-consuming work-up by solid phase extraction or liquid/liquid extraction and a relatively insensitive LC-fluorescence measurement. Our first goal was to improve this assay by taking advantage of the high sensitivity and specificity of modern LC–MS/MS analysis. We report here on phenotyping groups of students for CYP2D6 activity with saliva samples of 0.25 ml taken 2 h after oral ingestion of 30 mg encapsulated DEX and minor sample work-up.

In order to investigate the interindividual variability not only for CYP2D6 but also for *N*-demethylation and glucuronidation we developed mass spectrometric procedures for the analysis of the *N*-demethylated metabolites MOM and HOM and of the two glucuronides DORGlu and HOMGlu. Different isoforms of CYP3A contribute to the *N*-demethylation of DEX and DOR in vitro [13] and in vivo [17,23] and a number of genetic polymorphisms and variants have been described [24,25]. However, the relevance of these genetic differences for interindividual variability in drug metabolism has been questioned [26]. Furthermore, CYP3A activity is readily modulated by numerous inducers and inhibitors [27]. Glucuronidation of DOR and HOM has only indirectly been investigated, by subtracting the concentration of free DOR from the total DOR concentration obtained after glucuronidase treatment [28,29]. No data are available on the question of polymorphisms or interindividual variability for the glucuronidation of DOR and HOM, since neither DORGlu nor HOMGlu had been measured in human samples before. Therefore, elucidation of individual differences in this pathway was our second goal. Saliva could not be used for this purpose, because the concentrations of DORGlu and HOMGlu were at the limit of quantitation in EM and not detectable in PM. In urine, on the other hand, all six compounds shown in Fig. 1 could reproducibly be measured by LC–MS/MS, so that this type of sample was taken for the investigation of the pathways of *N*-demethylation and glucuronidation.

2. Experimental

2.1. Chemicals

Dextromethorphan hydrobromide, dextrorphan tartrate salt, levallorphan tartrate salt (LEV) and morphine 3-β-D-glucuronide (M3Glu) were from Sigma. 3-Methoxymorphinan hydrobromide and 3-hydroxymorphinan hydrobromide were a gift from Roche, Basel, Switzerland. Water Rotisolv HPLC gradient grade was from Roth, Karlsruhe, Germany and acetonitrile HPLC gradient grade from Fluka, Taufkirchen, Germany.

2.2. Isolation of glucuronides from rat urine

Animal experiments were performed according to local standards and licensing guidelines. A female F344 rat was injected s.c. 10 mg of DEX hydrobromide in 0.5 ml Hanks solution. Urine was collected during the next 24 h. The glucuronides were separated from the other components by HPLC with UV detection. For sample preparation 0.5 ml MeOH was added to 0.5 ml urine, vortexed, incubated on ice for 30 min and centrifuged at 8000 × *g* for 10 min. Twenty microliters of the supernatant were injected. The column and the gradient were the same as for LC–MS/MS analysis of DEX, DOR, MOM and HOM (see below). The eluant was collected between 1.5 and 3.5 min, lyophilized and dissolved in 10 μl H_2O to give a stock solution. This procedure was repeated 10 times in order to collect sufficient material.

The concentrations of DORGlu and HOMGlu were determined by cleaving the glucuronides and quantifying their parent compounds (DOR and HOM) as described previously for bisphenol A [30]. The stock solution was diluted 1:100 with H_2O and 0.25, 1 and 5 μl were diluted further to 10 μl. Forty microliters NaAc-buffer (0.2 M adjusted to pH 4.7 with concentrated HCl) and 100 μl of β-glucuronidase solution (9 μl β-glucuronidase, Sigma 127,300 U/ml, diluted with 891 μl of NaAc-buffer) were added and the mixture was incubated at 37 °C for 20 h. To each sample 147 μl MeOH and 3 μl LEV (2.3 nmol/ml) internal standard were added. After incubation on ice for 15 min the sample was centrifuged at 8000 × *g* for 10 min and the supernatant was analyzed. Concentrations of DORGlu and HOMGlu were 15.9 and 3.8 nmol/ml in the 1:100 diluted solution. Completeness of cleavage was verified in that neither DORGlu nor HOMGlu were detectable by LC–MS/MS after glucuronidase treatment. To further confirm the identity of the glucuronides mass spectra were recorded on a QTrap instrument.

2.3. Sample preparation

2.3.1. Saliva

The saliva samples were centrifuged for 15 min at 10,000 × *g* at 4 °C. To 250 μl of the supernatant 10 μl internal standard LEV (0.23 nmol/ml H_2O) were added before evaporation to dryness in a Speedvac concentrator. One hundred microliters of MeOH/H_2O 60:40 were added to the residue. The sample was mixed and kept overnight at 4 °C for dissolving. After centrifugation at 8000 × *g* for 10 min the supernatant was analyzed.

2.3.2. Urine

To 250 μl urine 5 μl internal standard LEV (2.3 nmol/ml H_2O) and 250 μl MeOH were added. The samples were kept on ice for 30 min, centrifuged at 8000 × *g* for 10 min and the supernatant was analyzed. For measuring the glucuronides 5 μl M3Glu internal standard (10 nmol/ml H_2O) were added to 50 μl urine and 445 μl H_2O. The samples were centrifuged at 8000 × *g* for 10 min and the supernatant was analyzed. Total amounts of analytes excreted in urine were calculated by multiplying the concentrations with the amount of urine.

2.4. Liquid chromatography–mass spectrometry

For LC an Agilent 1100 G1312A pump with an Agilent 1100 Autosampler and a Hypersil 3 μ C8-BD column, 100 mm × 2 mm with a corresponding guard cartridge (Phenomenex, Aschaffenburg, Germany) were used. The mobile phase consisted of (A) 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The flow rate was 240 μl/min and the injection volume 10 μl. For the analysis of DEX, DOR, MOM and HOM a linear gradient from 20 to 50% B within 5 min, followed by 50% B isocratic for 8 min was used. For the analysis of the glucuronides the gradient started with 3% B isocratic for 4 min, followed by a linear increase to 50% B within 2 min and 50% B isocratic for 8 min. The column was coupled to a triple stage quadrupole mass spectrometer, MDS Sciex API 3000 instrument with a Turbo Ionspray source, Applied Biosystems, Darmstadt, Germany. Turbospray parameters were: IS 4000 V, TEM 400 °C with N_2 as curtain (CUR = 15), nebulizer (NEB = 12) and collision gas (CAD = 4). The compound specific parameters for DEX, DOR, MOM, HOM, LEV and M3Glu were obtained by infusion of the standards using the quantitative optimization function of Analyst software 1.3.1. For DORGlu and HOMGlu the same parameters as determined for M3Glu were used. Analytes were recorded by multiple reaction monitoring in the positive ion mode (+MRM). The transitions, declustering potentials and collision energies used are given in Table 1. For retention times see Figs. 3 and 5 for saliva and urine, respectively.

Enhanced product ion spectra of DORGlu and HOMGlu were recorded on a QTrap instrument with a Turbo Ionspray source (Applied Biosystems, Darmstadt, Germany) with the following parameters: IS 4000 V, TEM 400 °C; N_2 as curtain gas (40), gas 1 (45), gas 2 (65) and collision gas (CAD = 4).

Table 1
MS/MS-transitions, declustering potential (DP) and collision energy (CE)

Matrix	Compound	Transition (*m/z*)	DP (V)	CE (V)
Urine	DEX	272 → 215	61	35
	DOR	258 → 157	41	55
	MOM	258 → 215	46	31
	HOM	244 → 157	56	49
	LEV	284 → 157	46	59
	DORGlu	434 → 258	31	45
	HOMGlu	420 → 244	31	45
	M3Glu	462 → 286	31	45
Saliva	DEX	272 → 171	61	61
	DOR	258 → 157	41	55
	LEV	284 → 157	46	59

2.5. Assay validation

Peak areas were determined using Analyst software 1.3.1 or 1.3.2. Matrix effects were evaluated by analyzing the 24 blank saliva and urine samples collected before the oral application of DEX. In saliva, six calibration standards were prepared by adding 5 μl of a solution containing DEX and DOR in H_2O to 250 μl of blank saliva to give final concentrations ranging from 1 to 400 pmol/ml saliva. In urine, six calibration standards were prepared by spiking 250 μl blank urine with 5 μl of a solution containing DEX, DOR, MOM and HOM in H_2O to give concentrations from 5 pmol/ml to 2 nmol/ml urine. Standard curves were generated with LEV as internal standard. Best fit for accuracy (83–118% in saliva; 86–114% in urine) for the standard curves was achieved with a linear regression and $1/x$ weighting. For the glucuronides in urine, five calibration standards were prepared by spiking blank urine with the glucuronides isolated from rat urine (see above) to give concentrations from 0.8 to 80 nmol/ml urine for DORGlu and 0.19 to 19 nmol/ml urine for HOMGlu. Standard curves were generated with M3Glu as internal standard. Best fit for accuracy (89–115%) for the standard curves was achieved with a quadratic regression and $1/x$ weighting.

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined as the concentrations with a signal-to-noise ratio of 5 and 10, respectively. Quality control samples were prepared by spiking blank urine or saliva samples to give concentrations at the limit of quantitation. The assay was repeated 10 times.

2.6. Study protocol for CYP2D6 phenotyping in saliva (170 individuals)

In a laboratory course in pharmacology and toxicology 183 medical students of the Würzburg University (samples #25–207) registered for this study that had been approved by the "Ethik-Kommission der Medizinischen Fakultaet der Universitaet Wuerzburg". Each person gave informed written consent. Nine individuals were phenotyped but were not included in this analysis, because of possible interactions with other drugs. In four individuals DEX and DOR were below the limit of detection. This resulted in 170 samples used for this study. Participants swallowed a 30 mg (81 μmol) dextromethorphan hydrobromide capsule (Hustenstiller-ratiopharm™, Ratiopharm GmbH Ulm, Germany) with 100 ml water. Two hours later a 1 ml saliva sample was collected, frozen immediately and stored at −20 °C until analysis.

2.7. Study protocol for the analysis of DEX and metabolites in urine and saliva (24 individuals)

Twenty-four healthy individuals volunteered to ingest a 30 mg capsule Hustenstiller-ratiopharm™. Urine samples were collected before the application of DEX (=blank) and for the following 6 h. The amount of urine was measured and an aliquot stored at −20 °C until analysis. In addition saliva specimens before (=blank) the application, after 2 and 6 h were collected (samples #1–24).

2.8. Statistics

R was used for statistical analyses. *R* is a language for statistical computing and graphics. It is available as Free Software under the terms of the Free Software Foundation's GNU General Public License in source code form. It compiles and runs on a wide variety of UNIX platforms and similar systems, Windows and Mac OS. Download is available at http://www.r-project.org. The logarithm of MR was analyzed using model-based clustering. Normal mixtures with one, two or three groups with unequal variances were fitted using maximum likelihood. To compare the three fits, the Bayesian information criterion (BIC) was used, as recommended for this case. For correlation analyses the Pearson correlation coefficient was calculated. Confidence intervals were derived using the *z*-transformation (variance stabilizing transformation for the correlation coefficient).

3. Results and discussion

3.1. Analytical procedures

Analytical methods were optimized for fast sample preparation, chromatographic separation and reproducible quantitation in urine and saliva. Although urine was injected without prior extraction of the analytes and saliva was only concentrated by lyophilizing, no interference with matrix components was observed. Good accuracy and precision were obtained as shown in Table 2. The limit of detection and limit of quantitation for DEX and DOR were different for urine and saliva. However, based on the amount of analyte on column they were identical (LOD 10 fmol, LOQ 25 fmol). An LC–MS/MS method in urine using liquid/liquid extraction had been published by Vengurlekar et al. [21]. They found a LOQ of 1 ng/ml for DEX and MOM, 60 ng/ml for DOR and 100 ng/ml for HOM, while our method has a LOQ of 5 pmol/ml, corresponding to approximately 1.3 ng/ml for all these analytes.

DORGlu and HOMGlu were not commercially available. Therefore, they were isolated from rat urine after treatment with DEX. Identity was confirmed by glucuronidase treatment and concentration was determined by quantitation of the parent compounds DOR and HOM. We do not have standard purity data, but contamination by endogenous glucuronides did not affect our results in consideration of much higher concentrations of DORGlu and HOMGlu in the urine and in view of the use of specific mass transitions. Product ion spectra are shown in Fig. 2. They showed the characteristic loss of *m/z* 176 from the molecular ion indicative for glucuronides and the fragments *m/z* 199, 157 and 133 representative for DOR and HOM.

Table 2
Analytical data of LC–MS/MS analysis of dextromethorphan and metabolites

Matrix	Compound	LOD (pmol/ml)	LOQ (pmol/ml)	Range of calibration standards (pmol/ml)	Accuracy % ($n = 10$)	Precision % ($n = 10$)
Urine	DEX	2	5	5–2000	99–115	4.9
	DOR	2	5	5–2000	92–110	6.5
	MOM	2	5	5–2000	90–109	5.7
	HOM	2	5	5–2000	91–105	4.3
	DORGlu	30	160	800–80,000	91–103	3.8
	HOMGlu	40	190	190–19,000	98–114	4.9
Saliva	DEX	0.4	1	1–400	90–111	5.2
	DOR	0.4	1	1–400	90–108	5.2

Theoretically, glucuronidation could also occur at the nitrogen atom, which could not be distinguished from *O*-glucuronidation with our mass spectrometric analysis. Metabolites of DEX have only been analyzed after glucuronidase treatment, which did not allow distinction between *O*- and *N*-glucuronidation. *N*-glucuronides of the structurally related morphine have not been described [31], indicating that this reaction may not be favoured with DEX either.

3.2. Saliva analysis

For the phenotyping of 170 students for P4502D6 activity, concentrations of DEX and DOR were measured in saliva, 2 h after ingestion of 30 mg dextromethorphan hydrobromide. Concentrations ranged from 1.1 to 880 pmol/ml for DEX and 0.3–150 pmol/ml for DOR. Fig. 3 shows the chromatographic separations of DEX and DOR in saliva. Panel A exemplifies the peak heights seen with an EM, showing similar levels of DEX and DOR (MR = [DEX]/[DOR] = 1.24). Panel B shows the corresponding separation for a PM, with the concentration of DEX much higher than the concentration of DOR (MR = 280).

$MR_{DEX/DOR}$ values spanned from 0.03 to 780, i.e. showed a more than 25,000-fold variation. Fig. 4 shows the frequency distributions on a log scale. For panel A, the MRs were determined on the basis of standard curves, for panel B, MRs were based on chromatographic peak areas. With both methods, a clear separation of the groups of EM and PM was observed, comprising 156 and 14 individuals, respectively. A bimodal

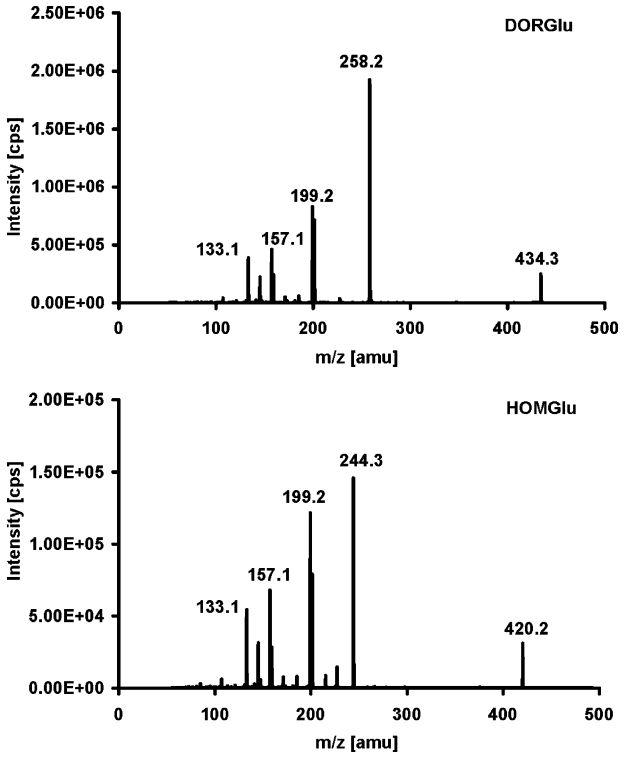

Fig. 2. Product ion mass spectra of dextrorphan glucuronide (DORGlu) and 3-hydroxymorphinan glucuronide (HOMGlu).

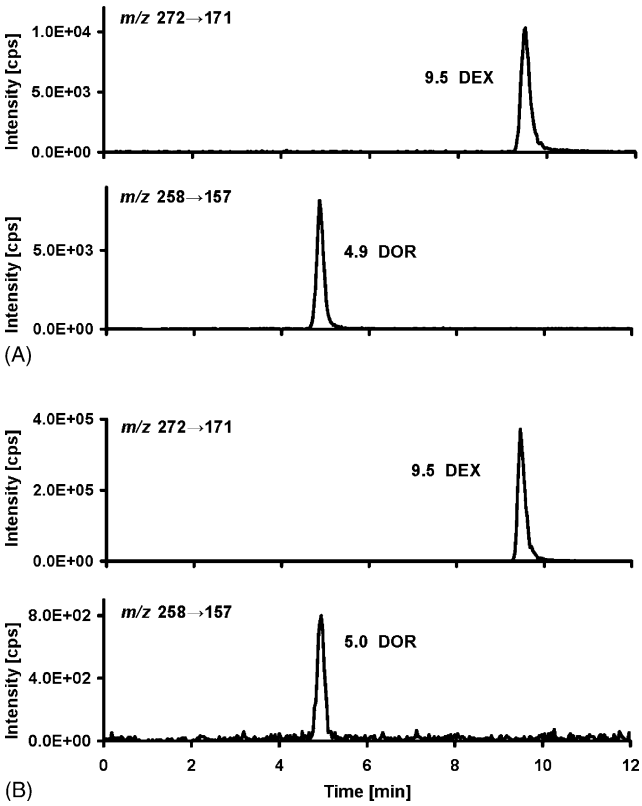

Fig. 3. Chromatograms of LC–MS/MS analyses of dextromethorphan (DEX) and dextrorphan (DOR) in saliva of an 'Extensive Metabolizer' (panel A) and a 'Poor Metabolizer' (panel B). Saliva was sampled 2 h after ingestion of a 30 mg capsule dextromethorphan hydrobromide. Note the different peak intensities in B for DEX and DOR, respectively.

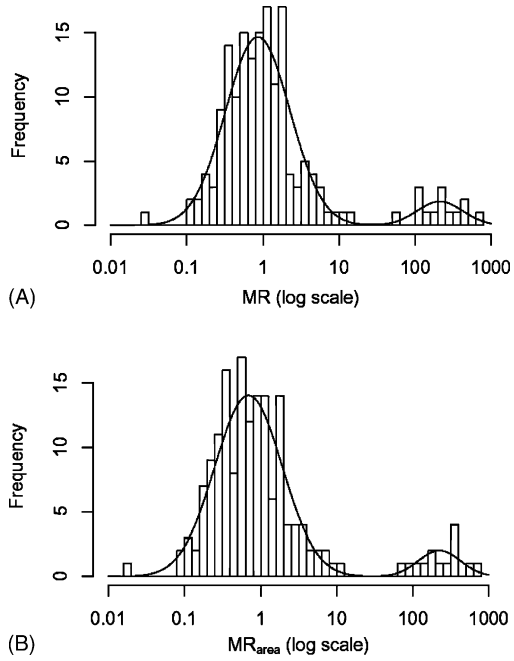

Fig. 4. Frequency distributions and best-fitting normal distributions of $MR_{DEX/DOR}$ based on concentrations determined on the basis of standard curves (panel A) or on peak areas obtained from LC–MS/MS analyses (panel B).

distribution was confirmed by model-based clustering; the BIC-value for 2 groups was higher than the BIC for 1 or 3 groups. For panel A, mean and standard deviation of log MR for the EM group was -0.067 ± 0.424, the respective values for the PM group were 2.324 ± 0.301. This is equivalent to mean MRs of 0.86 for EM and 211 for PM. The antimode was at MR 27. It is interesting to note that the separation of EM and PM was slightly better when peak areas were used (panel B; mean and standard deviation of log MR = -0.159 ± 0.443 and 2.348 ± 0.276, for EM and PM, respectively), instead of using concentrations derived from standard curves. This is probably due to a variance introduced by the standard curves used for the analysis of different batches of samples, as indicated by the analysis of residuals obtained from a linear regression of MR versus MR_{area}.

3.3. Urine analysis

Saliva samples did not provide quantitative information on MOM or HOM or on the two glucuronides. For the investigation of interindividual differences for the pathways of *N*-demethylation or glucuronidation therefore, urine samples were analyzed in a group of 24 volunteers. Chromatographic separations of LC–MS/MS analyses of cumulative 6 h urine are shown for DEX, DOR, MOM, HOM and the internal standard LEV (Fig. 5, upper five charts). The small peak eluting in front of HOM originates from partial decay of HOMGlu to HOM in the source due to the declustering potential of 56. However with a declustering potential of 31 used for measuring DORGlu and HOMGlu there was no decay in the source.

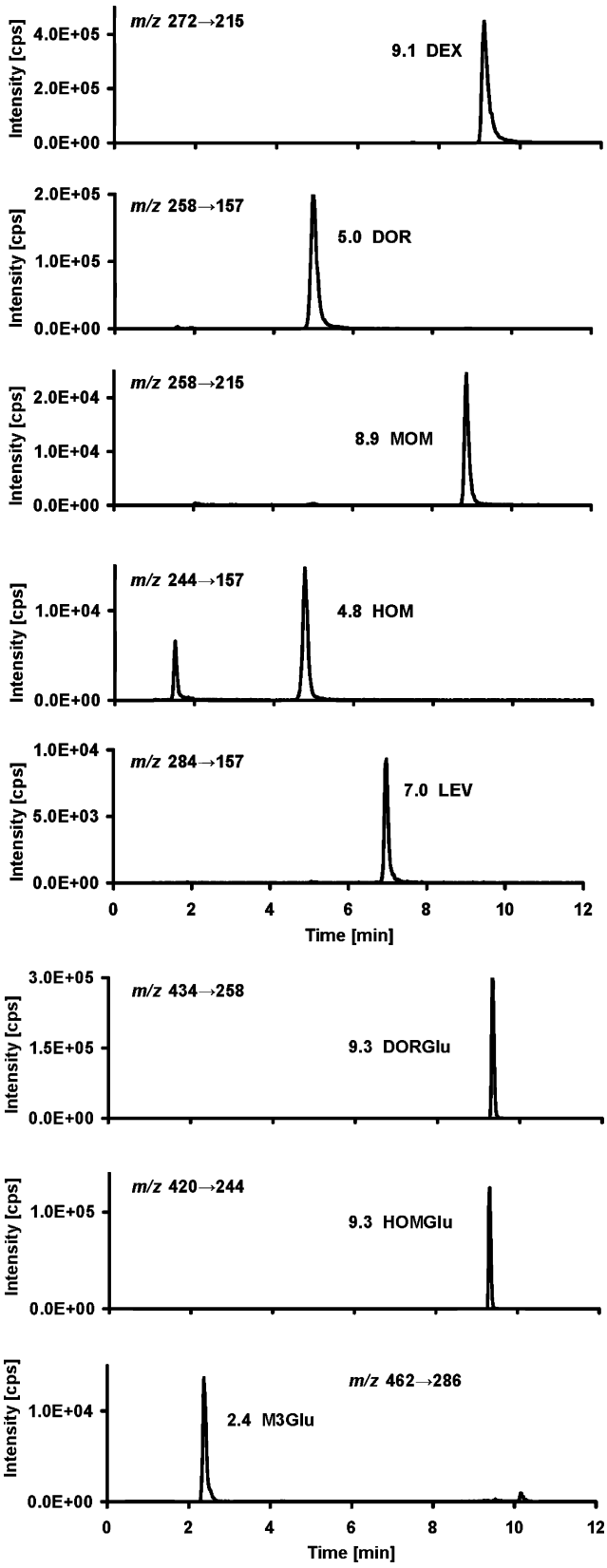

Fig. 5.

Table 3
Dextromethorphan and metabolites excreted in urine

Sample #	DEX	DOR	MR	Type	DORGlu	MOM	HOM	HOMGlu	Total
1	0.075	0.922	0.08	EM	43.87	0.005	0.241	17.40	62.51
2	0.135	0.512	0.26	EM	21.99	0.012	0.188	12.90	35.74
3	0.997	0.211	4.73	EM	10.54	0.057	0.240	5.33	17.38
4	0.088	0.853	0.10	EM	13.95	0.007	0.181	7.84	22.93
5	0.102	0.540	0.19	EM	28.63	0.008	0.296	14.62	44.20
6	7.174	0.051	140.67	PM	0.94	0.114	0.008	0.71	9.00
7	0.259	0.534	0.49	EM	20.52	0.016	0.227	9.01	30.57
8	0.041	0.988	0.04	EM	23.90	0.003	0.308	10.10	35.34
9	1.222	0.019	64.32	PM	0.36	0.030	0.005	0.19	1.83
10	0.016	0.186	0.09	EM	28.30	0.002	0.155	13.17	41.82
11	0.535	0.401	1.33	EM	25.83	0.032	0.264	13.20	40.26
12	0.236	0.421	0.56	EM	29.34	0.011	0.182	13.82	44.02
13	0.003	0.086	0.03	EM	20.86	0.001	0.129	7.05	28.13
14	1.600	0.981	1.63	EM	13.56	0.045	0.142	4.97	21.30
15	0.084	0.333	0.25	EM	18.95	0.001	0.136	9.93	29.43
16	0.261	0.366	0.71	EM	19.10	0.020	0.161	9.86	29.77
17	0.356	1.199	0.30	EM	26.25	0.014	0.351	8.25	36.42
18	1.375	0.048	28.65	PM	1.00	0.064	0.010	0.30	2.79
19	0.056	0.356	0.16	EM	27.45	0.007	0.281	8.12	36.27
20	0.043	0.722	0.06	EM	19.91	0.005	0.299	11.46	32.44
21	0.423	0.385	1.10	EM	12.42	0.043	0.260	8.49	22.02
22	0.541	1.419	0.38	EM	16.73	0.019	0.214	7.41	26.34
23	0.809	0.708	1.14	EM	11.06	0.045	0.160	4.40	17.19
24	0.018	0.362	0.05	EM	16.22	0.002	0.132	6.66	23.40

Total amounts (μmol) of dextromethorphan (DEX) and metabolites dextrorphan (DOR), 3-methoxymorphinan (MOM), 3-hydroxymorphinan (HOM), dextrorphan glucuronide (DORGlu) and 3-hydroxymorphinan glucuronide (HOMGlu) excreted in urine within 6 h after ingestion of 30 mg (81 μmol) dextromethorphan hydrobromide. MR, metabolic ratio = DEX/DOR. Phenotype: EM, extensive metabolizer, PM, poor metabolizer.

For the three glucuronides DORGlu, HOMGlu and the internal standard M3Glu, different chromatographic conditions were used (Fig. 5, lower three charts).

Table 3 shows the amounts of the parent compound DEX and the metabolites DOR, DORGlu, MOM, HOM, HOMGlu excreted in urine within 6 h after oral ingestion of 81 μmol DEX. In addition, the metabolic ratio MR = DEX/DOR is given, followed by the type designation of extensive versus poor metabolizer (EM versus PM). The three PM ranked top for urinary excretion of the parent compound DEX (up to 7 μmol). Excretion of CYP2D6-dependent metabolites was low, including the glucuronides (DORGlu: 0.4–1.0 μmol; HOMGlu: 0.2–0.7 μmol). For the 21 EM, on the other hand, the two glucuronides were the most abundant and showed relatively little individual variation (DORGlu 10–44 μmol; HOMGlu 5–17 μmol). MOM was the least abundant of all (0.001–0.114 μmol). Total urinary excretion of all six compounds combined ranged from 2 to 63 μmol (last column of Table 3). This is equivalent to 2.5–78% excretion of the ingested dose within 6 h.

Fig. 5. Chromatograms of LC–MS/MS analyses of dextromethorphan and its metabolites dextrorphan (DOR), 3-methoxymorphinan (MOM), 3-hydroxymorphinan (HOM), and the internal standard levallorphan (LEV), as well as dextrorphan glucuronide (DORGlu), 3-hydroxymorphinan glucuronide (HOMGlu) and the internal standard morphine-3-glucuronide (M3Glu) in urine after ingestion of 30 mg dextromethorphan hydrobromide. Different chromatographic conditions were used for the analysis of the glucuronides; see Section 2.4.

Panel A of Fig. 6 shows the histogram of the 24 MR values and the best fit of a bimodal distribution. A clear separation of two groups of 21 EM and three PM is seen. The classification was in agreement with the classification on the basis of saliva data, and the correlation between log MR for urine and log MR for saliva was 0.957 (data not shown).

Exploratory data analysis of Table 3 indicates that the investigation of the CYP2D6 phenotype does not necessitate the calculation of a metabolic ratio, but could be based on the amount of a single metabolite. For instance, panel B of Fig. 6 shows the histogram for the amount of DORGlu excreted in urine (column 6, Table 3). A 10-fold difference was observed for urinary DORGlu excretion between the PM with the highest value (#18, 1.00 μmol) and the EM with the lowest value (#3, 10.54 μmol). This difference was even larger than the difference between the $MR_{DEX/DOR}$ (EM #3, 4.73 versus PM #18, 28.65). A similar situation is shown for HOMGlu (panel C). The discriminative power is somewhat lower than for DORGlu, probably because the additional *N*-demethylation step increases the interindividual variability.

3.4. Correlation analyses for O-demethylation, N-demethylation and glucuronidation

The $MR_{DEX/DOR}$ has been shown to discriminate between EM and PM, indicating that this *O*-demethylation is controlled by a polymorphic enzyme. In view of the understanding that demethylation of MOM to HOM is controlled by the same enzyme [17,32], $MR_{DEX/DOR}$ would have to be

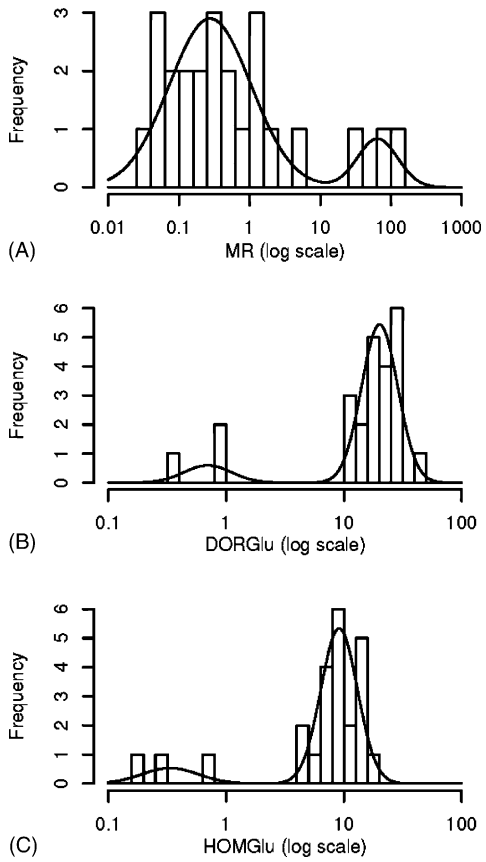

Fig. 6. Frequency distribution and best-fitting bimodal normal distributions for the metabolic ratio $MR_{DEX/DOR}$ (panel A), for dextrorphan glucuronide (DORGlu, μmol, panel B), and for 3-hydroxymorphinan glucuronide (HOMGlu, μmol, panel C). Amounts excreted in urine within 6 h after ingestion of 30 mg dextromethorphan hydrobromide.

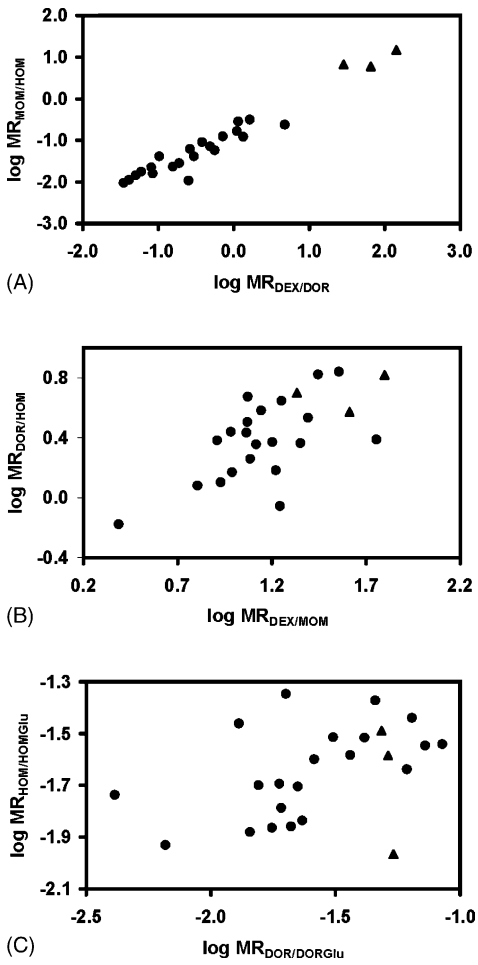

Fig. 7. Correlations of the MR for the metabolic pathways of *O*-demethylation (DEX to DOR vs. MOM to HOM; panel A), *N*-demethylation (DEX to MOM vs. DOR to HOM; panel B) and glucuronidation (DOR to DORGlu vs. HOM to HOMGlu; panel C). The respective MR were determined on the basis of the amounts excreted in urine within 6 h after ingestion of 30 mg dextromethorphan hydrobromide. Classification for CYP2D6 phenotype: EM (●), PM (▲).

correlated with $MR_{MOM/HOM}$. Panel A of Fig. 7 shows good correlation with a correlation coefficient of 0.97 and the 21 EM are clearly separated from the three PM not only on the *x*-axis ($MR_{DEX/DOR}$), but also on the *y*-axis ($MR_{MOM/HOM}$). Bimodal distributions provided better fits than unimodal or trimodal distributions also for the $MR_{MOM/HOM}$ (no histogram shown), confirming the CYP2D6 involvement also for this *O*-demethylation reaction.

Panel B of Fig. 7 shows the same type of analysis for the pathway of *N*-demethylation, for which CYP enzymes of the 3A subfamily are considered to be primarily responsible. The correlation for log $MR_{DEX/MOM}$ versus log $MR_{DOR/HOM}$ was not as strong as for the *O*-demethylation, but still significant ($r = 0.66$; 95% confidence interval 0.35–0.84). Individual differences were much smaller (span by a factor of 26 and 10, respectively), and the three PM, indicated by the triangular symbol, did not show any outstanding behavior. There was no indication of a multimodal distribution: The BIC was not higher for two or more groups.

Panel C of Fig. 7 shows the correlation for the glucuronidation pathway. The correlation for $MR_{DOR/DORGlu}$ versus $MR_{HOM/HOMGlu}$ was just significant with $r = 0.43$ and a 95% confidence interval of 0.03–0.71. Individual differences were the smallest among the three pathways, with a span by a factor 20 and 4 for $MR_{DOR/DORGlu}$ and $MR_{HOM/HOMGlu}$, respectively. Again, there was no indication of a multimodal distribution.

One major reason for the lower correlation in B and C is the smaller span of values (2 and 1.5 orders of magnitude for B and C, respectively, as opposed to 5 orders of magnitude for A on the *x*-axis). For C, the correlation is further reduced due to precursor variability not associated with the glucuronidation step.

3.5. Conclusions

For drugs that are eliminated predominantly by CYP2D6, 'Poor Metabolizers' run a higher risk of overdose toxicity than 'Extensive Metabolizers', particularly for long-term treatment with drugs that have small therapeutic indices. On the other hand, for drugs that require metabolic activation

by CYP2D6 (e.g., the formation of morphine from codeine), PM might not experience the full therapeutic effect. It is therefore important for 'personalized medicine' to establish fast assays that allow to phenotype large groups. Using modern LC–MS/MS techniques we established two non-invasive assays that allow a clearcut assignment, using the over-the-counter anti-cough agent dextromethorphan. While the assay based on saliva gives the basic information about the CYP2D6 polymorphism, urinary samples provide additional information on *N*-demethylation and glucuronidation. So far, we did not observe any significant deviation from a unimodal distribution in this study, but the relatively small group size limited the statistical power. Since glucuronidation is an important metabolic phase II reaction in humans, it might be worthwhile to investigate individual differences in more detail in further studies, including drugs where this pathway contributes substantially to their elimination.

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