Journal of Chromatography B, 871 (2008) 349–356

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Metabolite profiling in human urine by LC–MS/MS: Method optimization and application for glucuronides from dextromethorphan metabolism☆

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ARTICLE INFO

Article history:
Received 18 February 2008
Accepted 10 April 2008
Available online 3 May 2008

Keywords:
Mass spectrometry
Liquid chromatography
Multivariate analysis
Drug metabolism
Metabolite profiling
Metabolomics
CYP2D6
Polymorphism
Humans
Urine
Phenotyping
Dextromethorphan

ABSTRACT

Analysis of human urine for specific compounds or metabolites is an established method for biomonitoring occupational or environmental exposures. Modern liquid chromatography–tandem mass spectrometry is not limited to single compounds but can simultaneously analyze whole classes of urine constituents with both high sensitivity and specificity. Individual differences in the composition of urine are very large in humans, which raises a number of problems that are not encountered in animal experimentation. In this report, we investigated whether analysis of glucuronides as a class could reflect differences between human individuals regarding the polymorphic activity of the cytochrome P450 enzyme CYP2D6. From a group of 152 students that had been classified for CYP2D6 activity, urine of 12 "poor metabolizers" and 35 "extensive metabolizers" was collected 90 min after ingestion of 10 mg of the antitussive drug dextromethorphan (DEX) and analyzed for glucuronides. Methods development included the following aspects: adjustment of urine samples to equal creatinine concentration to avoid differences between samples in retention times and ion suppression; on-line enrichment of low-level analytes by column switching; precursor ion scan vs. theoretical multiple reaction monitoring; use of quality control samples to check for reproducibility in large sample series; peak extraction and handling of null entries to build the data matrix; logarithmic data transformation and different scaling procedures; principal component analysis (PCA) vs. discriminant analysis. Our results show that an optimized procedure not only identified the known DEX metabolites as predictors of CYP2D6-specific metabolic pathways but also indicated the presence of additional, so far unknown path-specific glucuronide metabolites. We conclude that metabolite profiling of urine and other biofluids by modern mass spectrometric methodology may help characterize individual differences and become useful in drug development and personalized pharmacotherapy.

1. Introduction

Metabolite profiling of urine by LC–MS followed by multivariate analysis emerges as a powerful tool in the study of drug metabolism [1]. The first publications were related to investigations in animals [2,3], since human studies are complicated by a number of factors. The dose that can be administered in humans is limited by a large safety factor below putative toxicity, so that metabolite concentrations are smaller and sample enrichment may be required. Differences between human individuals are much larger than in animal experimentation. This is due not only to genetic differences, but also to gender, age, and lifestyle [4], nutrition [5,6], specific chemical exposures [7], or differences in the intestinal flora [8]. This results in a wide variety of urinary compositions, which may affect chromatographic and mass spectrometric behaviour of the analytes. This aspect requires particular attention in biofluids like urine, because differences in composition and concentration can influence the ionization process, a phenomenon known as ion suppression/enhancement [9,10]. Dilution of human urine due to increased water consumption is a major confounder. It can result in more than 10-fold variation in the concentration of urinary constituents. Concentration adjustment before the analysis might therefore, be required to harmonize retention times and ion yields for all samples and to avoid bias in the multivariate analyses that may be introduced by mathematical adjustment of the data.

LC–MS is a common tool for the analysis of specific compounds of known structure, and the respective methods provide good accuracy and precision if isotope-labelled internal standards can be used. In metabolomics this is not practicable because of the large number of analytes with unknown structure at the outset of the

☆ This paper is part of a special volume entitled "Hyphenated Techniques for Global Metabolite Profiling", guest edited by Georgios Theodoridis and Ian D. Wilson.

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doi:10.1016/j.jchromb.2008.04.018

analysis. In this situation it is crucial at least that each peak with a given mass and retention time represents the same compound in all samples and that the peak area is proportional to the amount present in all samples. Reproducibility of retention time and peak area is required not only between samples with different composition but also for series of measurements performed over many hours. Repeated measurement of the same sample ("quality control samples") is necessary to check the stability of the LC–MS analyses with time.

In the first part of this manuscript, we exemplify the problems addressed above and show how we solved them in the analysis of human urine for glucuronides. This is a major class of phase II metabolites of drugs, chemicals, and endogenous compounds and is well suited for specific and sensitive measurement by LC–MS/MS techniques [11]. In the second part, we investigated whether the optimized analytical methodology could be combined with multivariate analyses for (i) metabolite profiling, (ii) investigating differences between individuals and subpopulations, and (iii) finding so far unknown glucuronic acid metabolites. As probe drug, we used the antitussive agent dextromethorphan (DEX) for which two glucuronides, dextrorphan-*O*-glucuronide (DORGlu) and 3-hydroxymorphinan-*O*-glucuronide (HOMGlu) represent the major urinary metabolites. As illustrated in Fig. 1, formation of the *O*-demethylated metabolite DORGlu requires CYP2D6 and UGT activity, while HOMGlu in addition requires CYP3A activity for *N*-demethylation. A genetic polymorphism in the activity of CYP2D6 is known. Eight percent of the Caucasian population lack CYP2D6 activity [12] and are named poor metabolizers (PM). They can be distinguished from extensive metabolizers (EM) on the basis of the ratio of the concentrations DEX/DOR measured in plasma, urine, or saliva [13].

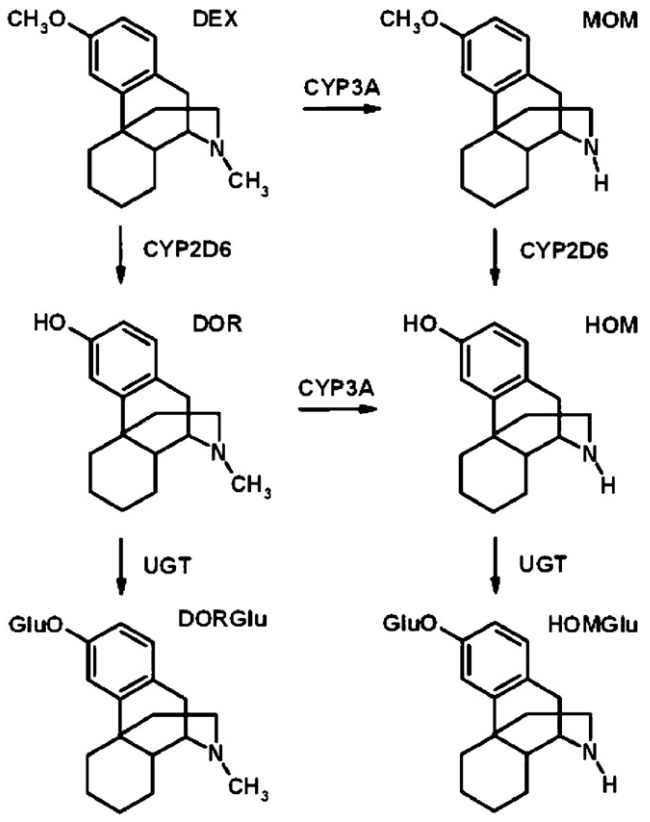

Fig. 1. Main pathways for dextromethorphan metabolism in humans. Abbreviations: DEX, dextromethorphan; DOR, dextrorphan; DORGlu, dextrorphan-*O*-glucuronide; MOM, 3-methoxymorphinan; HOM, 3-hydroxymorphinan; HOMGlu, 3-hydroxymorphinan-*O*-glucuronide; CYP2D6, cytochrome P450 2D6; CYP3A, cytochrome P450 3A subfamily; UGT, UDP-glucuronosyltransferase.

Statistical aspects are a focus in this part of the manuscript. Transformation of the raw data, scaling procedures, and principal component analyses vs. discriminant analyses are discussed. Finally, information in the loadings plot was used not only to locate the CYP2D6-specific glucuronide metabolites shown in Fig. 1 but also to find additional, so far unknown glucuronide metabolites. Their structural information was derived from information-dependent acquisition (IDA) of enhanced product ion (EPI) spectra using both negative and positive ionization.

2. Experimental

2.1. Chemicals

Rotisolv™ HPLC gradient grade water was from Roth, Karlsruhe, Germany, HPLC gradient grade acetonitrile from Fluka, Taufkirchen, Germany. DORGlu was from Toronto Research Chemicals Inc., Canada. HOMGlu was isolated as described [13]. All other chemicals were of analytical grade from Merck, Darmstadt, Germany. Dextromethorphan hydrobromide for human use was from FAGRON GmbH, Barsbüttel, Germany.

2.2. Urine sampling and phenotyping for CYP2D6

In a laboratory course in pharmacology and toxicology 152 students of the Würzburg University registered for the study that had been approved by the Regional Ethical Committee of the Würzburg University. Each person gave informed written consent. After voiding the bladder, participants ingested 10 mg dextromethorphan hydrobromide dissolved in 100 mL water. After 90 min urine samples were collected, divided into aliquots and stored at −20 °C. Blank urine samples were obtained from six female and six male volunteers and stored at −20 °C until analysis.

Analysis of DEX and the CYP2D6-dependent metabolite DOR was done according to a previously published method [13] with the following minor modifications: To 500 μL urine 5 μL internal standard levallorphan (2.3 pmol/μL) were added. The sample was centrifuged at 10,000 × *g* for 10 min and 10 μL of the supernatant were injected. Chromatography was performed isocratically with water/acetonitrile 70/30 (v/v), containing 0.1% formic acid (FA).

Individuals were classified on the basis of a metabolic ratio MR calculated by dividing the concentration of the parent drug DEX by the concentration of the CYP2D6-dependent metabolite DOR (MR = [DEX/DOR]) in urine [13–15]. Of the 152 persons phenotyped for CYP2D6 activity 12 were identified as PM and the remaining 140 as EM. For metabolite profiling, urine samples of all 12 PM plus 35 randomly chosen EM were used, which resulted in 47 samples.

2.3. Urine preparation (adjustment for dilution)

Creatinine concentrations were determined in the Central Laboratory of the University Hospital using a COBAS INTEGRA system (Roche Diagnostics) with enzymatic colour test. The blank urine samples contained 25–359 mg/dL creatinine. They were either used as such (=crude) or diluted with a solution containing 120 mM urea and 60 mM NaCl to give a final creatinine concentration of 25 mg/dL (=creatinine-adjusted). To 500 μL sample (crude or creatinine-adjusted) 2 μL solution of DORGlu standard (100 pmol/μL) were added. For quality control (QC) samples a 24-h blank urine sample from one individual was creatinine-adjusted to 25 mg/dL and treated as above. For glucuronide metabolite profiling after DEX ingestion all urine samples were creatinine-adjusted. Before LC–MS/MS analysis 2 μL FA 100% were added to all samples. After

centrifugation at 10,000 × *g* for 10 min 250 μL of the supernatant were injected.

2.4. Liquid chromatography/mass spectrometry; quality controls

The on-line extraction LC–MS/MS system had been described [16]. In brief, the autosampler (Agilent Series 1100, Waldbronn, Germany) introduced the sample into the system and pump 1 (Agilent Series 1100) carried the mobile phase (0.1% FA) at 750 μL/min to load the sample on the trap column (ReproSil-Pur C18-AQ, 33 mm × 3 mm, 5 μm, Maisch, Ammerbuch, Germany). After 1.0 min the valve switched to the elution position. Pump 2 supplied a gradient to back flush the trapped analytes from the trap column and to transfer them onto the analytical column (ReproSil-Pur C18-AQ, 150 mm × 2 mm, 3 μm, Maisch). The mobile phase was 0.1% FA (A) and acetonitrile containing 0.1% FA (B). The gradient started with 3% B for 1 min, increased to 60% B within 57 min, then to 90% B within 0.5 min, was held for 2 min at 90% B, and decreased to 3% B in 1 min. After an equilibration time of 9.5 min the next sample was injected. The slow gradient of 60 min was used to limit the number of molecules in the ion source at any given time in order to avoid ion suppression.

The column eluant was introduced into the MS/MS system consisting of a TURBO-Ionspray source operated in the negative ion mode and a hybrid quadrupole linear ion trap (QTRAP, Applied Biosystems/MDS Sciex, Concord, Ont.). The instrument parameters were source voltage (IS) −4.2 kV, vaporizer temperature 400 °C, curtain gas 30 psi, nebulizer gas 45 psi, turbogas 50 psi, CAD gas 10 psi. The compound specific parameters for glucuronides were obtained by infusion of DORGlu standard using the quantitative optimization function of Analyst software 1.4.1. They were as follows: declustering potential (DP) −50 V, entrance potential (EP) −9 V, collision energy (CE) −30 V, collision cell exit potential (CXP) −1 V. Glucuronides were recorded by monitoring theoretical transitions specific for glucuronides in the mass range from *m*/*z* 217 to *m*/*z* 466 with a dwell time of 5 ms each. Q1 selected the deprotonated molecular ion $[M-H]^-$ and Q3 the specific fragment of glucuronic acid conjugates *m*/*z* 113. In this way 250 multiple reaction monitoring (MRM) transitions (217 → 113, 218 → 113. . .465 → 113, 466 → 113) were acquired simultaneously.

Samples were analyzed in random order. Before the first sample three QC samples were measured to equilibrate the system. Then after every five samples one QC sample was inserted.

2.5. Acquisition of product ion spectra

Enhanced product ion spectra were recorded using information-dependent acquisition. In the negative ion mode the following MRM transitions were used as a survey scan (418 → 113, 432 → 113, 446 → 113, 448 → 113, dwell time 200 ms each). When the intensity of the MRM signal exceeded the threshold (500 counts), Q1 filtered the parent ion that was then fragmented in Q2 with a collision energy of −30 V. Fragment ions were trapped in Q3 before they were scanned from *m*/*z* 50 to 500 at a scan rate of 4000 amu/s. The parent ion was then excluded from fragmentation for 40 s. The other instrument and LC parameters were the same as stated above. In the positive ion mode the MRM transitions used as a survey scan were (420 → 244, 434 → 258, 448 → 272, 450 → 274). The instrument parameters were: IS 5 kV, DP 31 V, EP 10 V, CE 35 V, CXP 6 V and the intensity threshold 2×10^4 counts. All data acquisition was done using the Analyst 1.4.1 software (Applied Biosystems, Darmstadt, Germany).

2.6. Peak extraction, data transformation and scaling

Peak areas were extracted from the MRM chromatograms using MarkerView™ software version 1.2.0.1 (Applied Biosystems/MDS Sciex, Canada). Peaks were named by mass and RT in the form "*m*/*z*_RT". Parameters were optimized by visual inspection of the transitions for the two known glucuronide metabolites DORGlu and HOMGlu in order to ascertain that the same peak in all chromatograms was attributed to the same variable in all samples. This resulted in the following selection: for peak finding: smoothing half width 1 point, baseline sub. window 1.0 min, noise percentage 50%, peak-splitting factor 2 points; for filtering: minimum required intensity 2000 cps, minimum peak width 3 points, minimum signal/noise 5.0, maximum number of peaks 250 or 500 or 5000; retention time tolerance for sample alignment: 1.0 min.

The data matrix was imported into the SIMCA-P™ software (version 11.5, Umetrics, Umea, Sweden) for multivariate analyses. Each variable was $\log_{10}$ transformed to achieve approximate normal distribution. As the values for some variables in some observations were zero, a constant of 30 was added, corresponding to half of the minimum value observed. The variables were mean-centred before the significant components were calculated using the autofit function and sevenfold cross-validation. As scaling procedures, no scaling at all, scaling by the square root of the standard deviation (also known as Pareto scaling) and scaling by the standard deviation (unit variance scaling) were performed in the search of the models with highest predictive ability. Data were analyzed by principal component analysis and by discriminant analysis based on projections to latent structures by means of partial least-squares (PLS-DA).

3. Results

3.1. Theoretical MS/MS transitions to monitor glucuronides

Glucuronides share a common characteristic fragment of *m*/*z* 113 [11] in the negative ion mode. In preliminary experiments, we therefore, tried a precursor ion scan to monitor this class of metabolites. However, the data extraction step did not give reproducible results, possibly because *m*/*z* does not show up as discrete values in this acquisition mode. Alternatively, each *m*/*z* of the precursor ion scan was replaced by a transition in the form of *m*/*z* → 113, i.e., we used the mode of theoretical MRM. The resulting two-dimensional data could now easily be inspected in the extracted ion chromatogram and gave reproducible results. Using the range of *m*/*z* 217 to *m*/*z* 476 (250 transitions) included the majority of peaks.

3.2. Sample enrichment

Using direct injection of 10 μL urine, as usually done in high-dose animal studies resulted only in a small number of peaks. Therefore, a sample enrichment step was required for our low-dose human study. Both solid phase extraction [17] and online sample cleanup by column switching [18] had successfully been applied for metabolite profiling in previous studies. With column switching the analytes are retained on a trap column while matrix components are flushed to waste. Then a valve is switched to backflush the analytes onto an analytical column connected to the mass spectrometer [16]. In order to optimize the experimental parameters, preliminary experiments with control human urine spiked with DORGlu, the primary metabolite of DEX, were performed. Injection of 250 μL urine adjusted to pH 3 and a washing time of 1 min resulted in

highest intensities and a maximum number of peaks. A larger urine volume did not increase the intensities significantly due to signal suppression. A longer washing time resulted in loss of early eluting peaks.

3.3. Quality control samples

When analyzing a batch of samples the first requirement is that the instrument conditions are stable over the entire period of analysis. This means that measuring the same sample several times should only give a small variation in RT and peak area. For this purpose QC samples are inserted between groups of study samples. When the variation in these QCs is within adequate limits (relative standard deviations (R.S.D.) less than 15–20% [19,20]), the measurement of the study samples is considered acceptable. Our QC samples were taken from a 24-h control urine sample from one individual. Alternatively, QCs can consist of a mixture of all study urines, thus, representing an "average" sample [21].

3.4. Adjustment for creatinine concentration

With the variability of the QC being acceptable, the next step was the evaluation of the variability due to matrix effects. In conventional quantitative LC–MS methods, internal standards, preferentially stable isotope-labelled analogues are used to account for differences. In metabolomics this approach is not possible because most of the analytes are unknown. Therefore, each peak area needs to be a quantitative measure for the amount of the respective analyte, as the peak area of each compound will later be used to create the data matrix. This requirement is difficult to meet in mass spectrometry, particularly with a matrix like urine. Depending on the liquid intake, human urine samples can vary greatly in volume voided over a given period of collection. This in turn affects the concentrations of excreted compounds. As the ionization process is known to be susceptible to concentration [22], matrix effects were evaluated.

Twelve human urine samples with creatinine concentrations from 25 mg/dL to 359 mg/dL were analyzed as such (=crude) as well as after dilution of the urines to 25 mg/dL creatinine. DORGlu was added as standard. Three QCs were measured in the beginning and one after every six samples. The first two QC showed abnormal RT and peak area. Thereafter the system was stable: the RTs of the residual QCs were within 0.2 min and the peak areas of the DORGlu standard showed a R.S.D. of 9.5%. While these small variations in the QCs were acceptable, the results of the crude samples were not satisfactory. The open circles in chart A of Fig. 2 show the RTs of the DORGlu peak as a function of the original creatinine concentration. Values ranged from 17.1 min to 19.5 min and showed an increase with creatinine. The most pronounced change occurred between 25 mg/dL and 100 mg/dL creatinine. RTs of three unknown peaks eluting in the same time window were also analyzed. Fig. 2C for peak 295_18 shows a situation almost superposable to the one observed for DORGlu. Under such conditions, RT correction could theoretically be performed by the software. However, chart B shows that the RT of peak 326_18 decreased with increasing creatinine, and the effect became apparent only at higher creatinine levels. Chart D shows an example of a peak (266_19) that was barely affected. Since correct peak alignment is crucial for multivariate analyses, measures to improve the situation were sought. The idea was to make the urine samples as similar as possible for all aspects that might affect the analysis. Diluting the samples to equal creatinine concentration improved the situation substantially. The plus signs in Fig. 2A show that RT of DORGlu plotted as a function of the concentration in the original urine now ranged from 17.1 min to 17.5 min. For the three unknown peaks (charts B–D) all RTs were within 0.4 min also. Also peak areas depended on the urine concentration. As shown in Fig. 3, the DORGlu peak area decreased with increasing creatinine concentration in the crude samples (o) with a

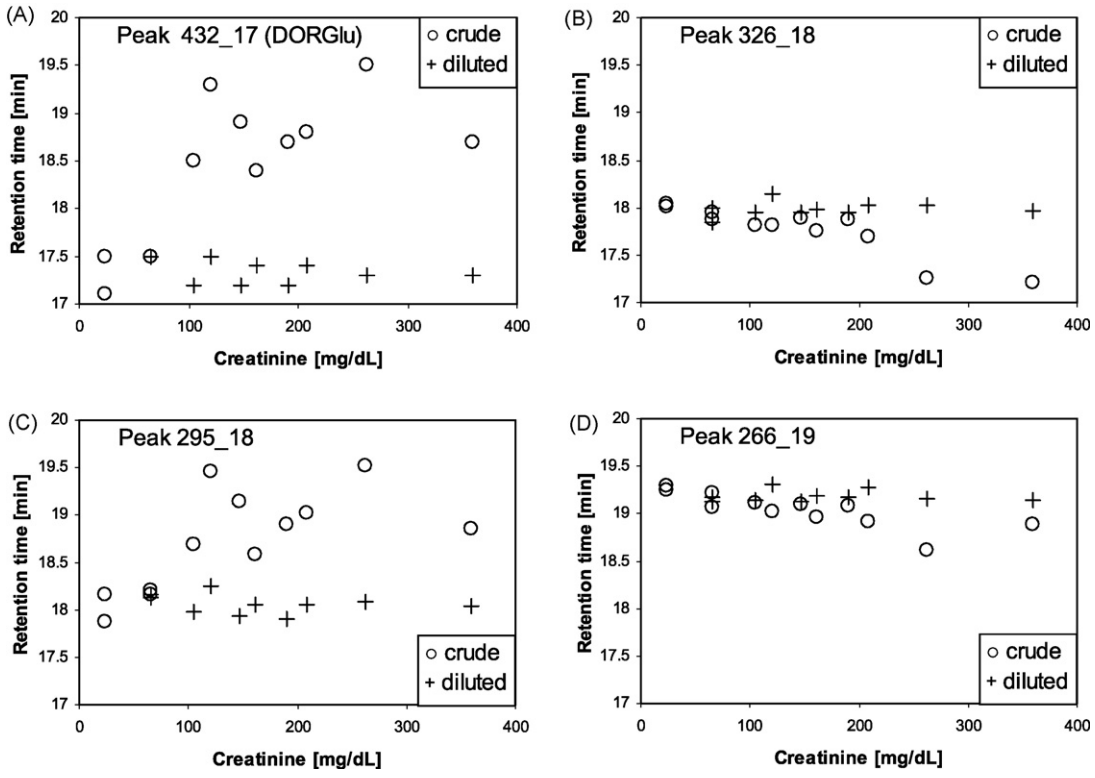

Fig. 2. Retention times of four different peaks eluting between 17 min and 20 min. Urine samples with different creatinine concentrations were analyzed as such (crude urine ○) or after adjustment to 25 mg creatinine/dL (+). The *x*-axis indicates the original creatinine concentration.

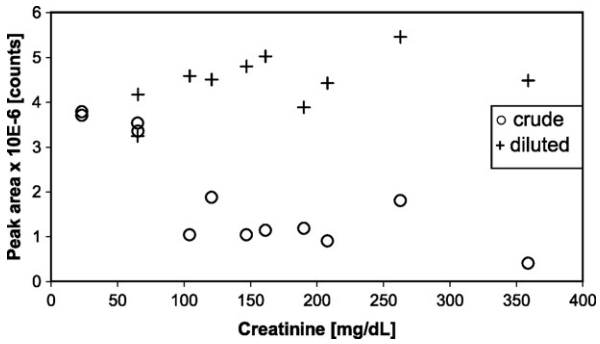

Fig. 3. Peak area of DORGlu standard added to crude urine (○) or creatinine-adjusted urine (+) in relation to the creatinine concentration. The *x*-axis indicates the original creatinine concentration.

R.S.D. of 63%, while the diluted samples (+) were within 13% R.S.D. Adjusting the samples to equal creatinine concentration resulted in an acceptable variation of both RT and peak area.

3.5. Profiling of glucuronides

The optimized method was applied to urine samples of 12 PM plus 35 EM after ingestion of 10 mg DEX. Fig. 4 shows TIC of the LC–MS/MS chromatograms of the 250 theoretical transitions acquired to monitor the glucuronides. Fig. 4A originates from an EM, whereas Fig. 4B was from a PM for CYP2D6. It was impossible by visual inspection to discern the difference in metabolizer phenotype or to pinpoint those peaks that could be metabolites of the administered drug. The major peaks originated from endogenous metabolites, constituents of food, or environmental exposures. The small dose of DEX did not result in metabolites of high intensity.

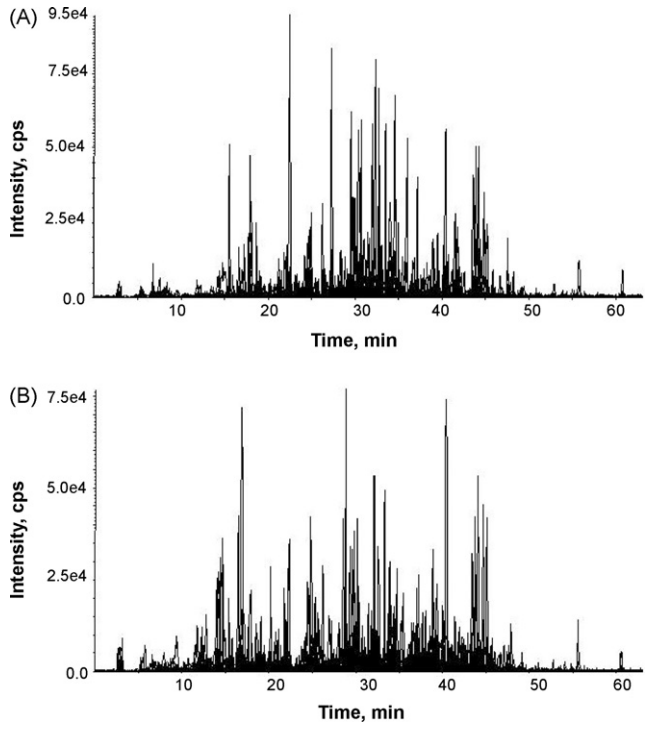

Fig. 4. Total ion chromatograms (TIC) of 250 MRM transitions specific for glucuronides in urine of an extensive metabolizer (EM; chart A) and a poor metabolizer (PM; chart B) for CYP2D6 after ingestion of 10 mg DEX.

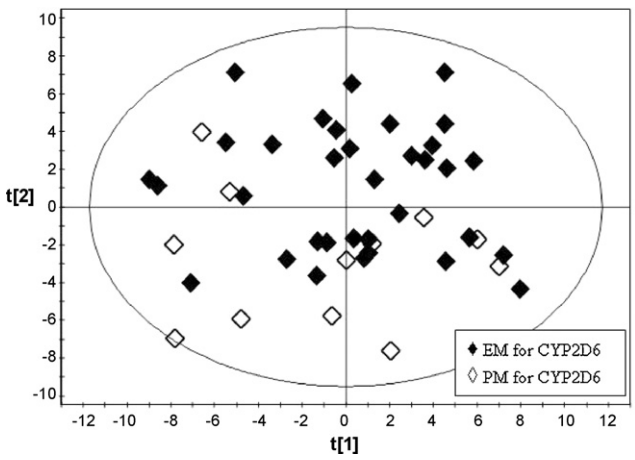

Fig. 5. Scores plot of a principal component analysis (PCA) of urinary glucuronides of extensive metabolizers and poor metabolizers for CYP2D6.

3.5.1. Data transformation and principal component analysis

The first principal component analysis was performed with all samples, i.e., QC samples were included. The latter group clustered with the exception of the first two samples run at the beginning of the series. This indicates that the analytical conditions were stable over the time of measurement after the initial two runs necessary to equilibrate the system. This was confirmed by the DORGlu standard that had been added to the QCs. RT variation was within 0.2 min and the R.S.D. of the peak area was 11.3% over the almost 71 h of measurement.

New data matrices with the 47 study samples only were then generated, in order to exclude any influence of the QC samples on the data extraction. The data were log-transformed and mean-centred. Principal component analysis showed that there was one outlier in the samples. When examining the data matrix it was evident that this sample contained peaks that were absent in all other samples. Therefore, this sample (an EM phenotype) was discarded for the subsequent analyses. Fig. 5 shows the scores plot of the first two principal components of principal component analysis of the remaining 46 samples. The explained variation was 14% and 9% for PC1 and PC2, respectively. While PM tended to locate at the lower left-hand side, neither PC1 nor PC2 separated them from the EM. As already seen in the chromatograms, the largest difference between samples resulted from interindividual factors other than differences in DEX metabolites. In animal studies, the homogeneous conditions for genetics and lifestyle often makes separation by the first PC possible [23–26].

3.5.2. Discriminant analysis, predictive ability, scaling

In order to more clearly separate EM from PM and find the responsible metabolites discriminant analysis was performed. In search for the best model three data matrices differing in the number of extracted peaks were generated after ranking for peak area: 250, 500 and 2016 (maximum number found). Three components were significant for all models. With 250 peaks (variables), the goodness of fit given by the explained variation (R^2Y cumulative) was 0.925. The predictive ability of the model determined by sevenfold cross-validation as given by the goodness of prediction Q^2 was 0.596. A model with $Q^2 > 0.5$ is generally considered good for the prediction of unknown samples [27]. Using 500 peaks for modelling resulted in slightly higher goodness of fit ($R^2Y = 0.930$) and clearly better predictive ability ($Q^2 = 0.641$). Inspection of the variables showed that the M + 1 isotopes of the two main glucuronide metabolites were not present in the data matrix with 250 peaks. The model generated with the maximum number of peaks found

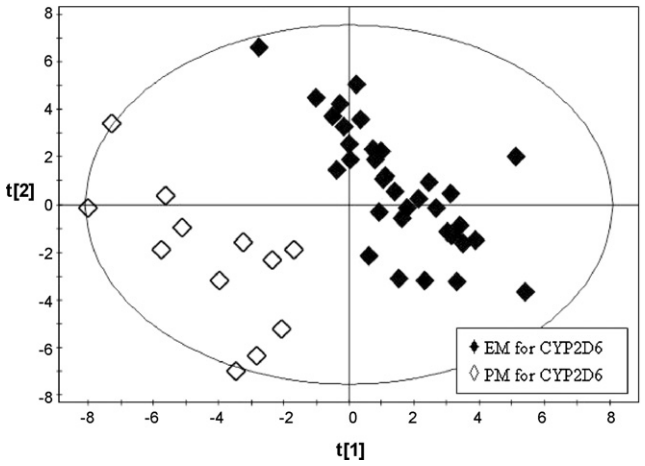

Fig. 6. Scores plot of a discriminant analysis (PLS-DA) of urinary glucuronides of extensive metabolizers and poor metabolizers for CYP2D6.

by MarkerView™ (2016) did not result in any significant further improvement, probably because of the statistical noise introduced by peaks that were not above background in all samples.

Fig. 6 shows the scores plot of the first two components resulting from the PLS-DA model with 500 variables that had been log-transformed and mean-centred. The explained variation in Y for t [1] and t [2] was 66% and 21%, respectively. A good separation between PM and EM was achieved, with the PM in the lower left-hand corner.

Using the data set with 500 variables we also tested the effect of different types of scaling of the data on the predictive ability. None of these actions improved the predictivity of $Q^2 = 0.641$ indicated above. Scaling by the square root of the standard deviation ("Pareto" scaling) resulted in $Q^2 = 0.608$, unit variance scaling in $Q^2 = 0.458$. Since scaling reduces the importance of large peaks these results indicate that the attribution of more statistical weight to small peaks deteriorated the models.

3.6. Detection of new CYP2D6-dependent metabolites

The loadings plot of the PLS-DA model represented in Fig. 6 is shown in Fig. 7. Variables that are important for the discrimination have high absolute values in PLS weights ($w \times c$) and the EM-specific variables show up in the upper right-hand corner of the two-component graph shown. The main known glucuronide metabolites of DEX, i.e., DORGlu (432_17.5 with the corresponding M + 1 isotope 433_17.6) and HOMGlu (418_17.3 with the isotope 419_17.3) showed up clearly. A number of additional variables contributed strongly. In order to investigate whether these compounds were additional glucuronides formed by DEX metabolism, product ion spectra in negative ($-H^+$) and positive ($+H^+$) ion modes were acquired (mass difference = 2 Da). The results are listed in Table 1. In the negative ion mode, all ions showed the neutral loss of 176 Da and the glucuronide fragments 175, 113, 95 and 85 Da. The DEX-specific fragments of the morphinan structure appeared in the positive ion mode. The spectra of m/z 434 (RT 17.5) and m/z 420 (RT 17.3) are in accordance with the product ion spectra of DORGlu and HOMGlu published before [13]. The other ions in Table 1 all show some of the characteristic fragments and most probably are also DEX metabolites. The two positive ions with m/z 448 (RT 29.5 and 30.6) could originate from the oxidation of a methylene group of DORGlu to a carbonyl group (oxo-DORGlu, +14 Da). The positive ion with m/z 434 (RT 22.0) could be the equivalent for HOMGlu (oxo-HOMGlu, +14 Da). The positive ion with m/z 450 (RT 19.2) could be a hydroxylation product of DORGlu (hydroxy-DORGlu, +16 Da).

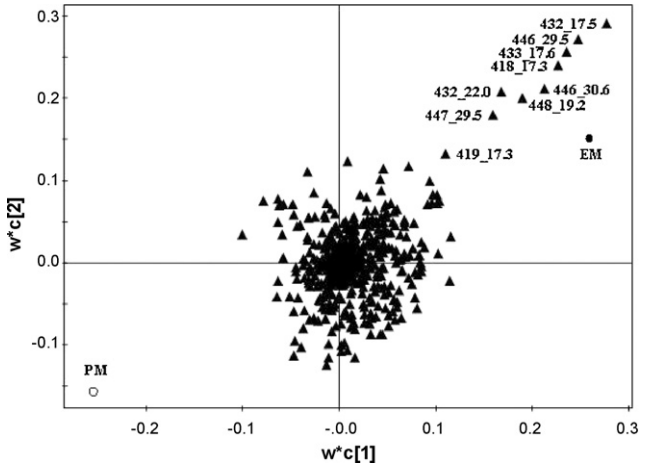

Fig. 7. Loadings plot of the PLS-DA model shown in Fig. 6. Variables (▲) close to the "dummy variable Y" (●: EM for extensive, ○: PM for poor metabolizers for CYP2D6) contribute strongly to the separation according to the metabolic phenotype. The numbers indicate mass and retention time of the respective peak (variable).

4. Discussion

4.1. Methods development

4.1.1. Creatinine adjustment

We have shown that the dilution of urine ("wateriness") can affect both retention time and ion yield of analytes. This is not tolerable if the data are to be used in a matrix for multivariate analysis, where correct attribution of peaks to variables is a prerequisite. Dilution of all urines to the same creatinine concentration markedly improved the situation. We did not use water but a solution containing 120 mM urea and 60 mM NaCl, because the concentration of these two urinary constituents is relatively constant, irrespective of the water consumption.

Normalization is often performed by applying a correction factor for creatinine after the measurements. This can be misleading if the creatinine concentration is determined by factors other than the water consumption. Since creatinine is an excretion product related to muscle activity males excrete more creatinine than females per kg body weight. This difference will be reflected in the principal component analysis by a separation of males and females, which may detract from the observation of other differences between subgroups. The confounding effect is lessened with a discriminant analysis where individuals are attributed to a class according to criteria that are independent of gender.

It has to be noted that normalizing the urine samples before analysis improves the quality of the data matrix but does not overcome the gender difference. In our analysis, differences between EM and PM were so dominant that minor confounding by gender did not render a classification impossible. Whether males and females tend to cluster within the two subgroups EM and PM could not be investigated because of the anonymity of the data.

4.1.2. Logarithmic data transformation and scaling for multivariate analysis

Principal component analysis and DA provide best results if the variables are normally distributed in the samples (=individuals). This criterion may be met by analytes that are present in all individuals and at a concentration of the same order of magnitude. In human urine many compounds are not present in some of the

Table 1
Mass, retention times and product ion spectra of the variables that contributed most to the separation of extensive from poor metabolizers for CYP2D6

Ionization	*m/z* of parent ion	Retention time	Product ions	Metabolite[a]
Neg	432	17.5	414, 256, 175, 113, 95, 85	DORGlu
Pos	434	17.5	258, 227, 201, 199, 185, 159, 157, 145, 133	
Neg	418	17.3	400, 242, 175, 113, 95, 85	HOMGlu
Pos	420	17.3	244, 227, 215, 201, 199, 185, 159, 157, 145, 133	
Neg	446	29.5	428, 270, 175, 113, 95, 85	Oxo-DORGlu
Pos	448	29.5	272, 227, 201, 199, 185, 159, 157, 145, 133	
Neg	446	30.6	428, 270, 175, 113, 95, 85	Oxo-DORGlu
Pos	448	30.6	272, 227, 201, 199, 185, 159, 157, 145, 133	
Neg	432	22.0	414, 256, 175, 113, 95, 85	Oxo-HOMGlu
Pos	434	22.0	258, 240, 199, 157, 145, 133	
Neg	448	19.2	430, 272, 175, 113, 95, 85	Hydroxy-DORGlu
Pos	450	19.2	432, 274, 256, 227, 201, 199, 159, 157, 150, 145, 133	

[a] Abbreviations: DORGlu, dextrorphan-*O*-glucuronide; HOMGlu, 3-hydroxymorphinan-*O*-glucuronide; Oxo-DORGlu, DORGlu with a methylene oxidized to a carbonyl group; Oxo-HOMGlu, HOMGlu with a methylene oxidized to a carbonyl group; Hydroxy-DORGlu, hydroxylated DORGlu.

samples due to differences in food or drug intake or not detectable because of differences in metabolism. These samples show a null entry in the matrix for the corresponding variable, while the entries of the other samples often extend over several orders of magnitude. For instance, the peak area of DORGlu ranged from undetectable to 19,000 and the distribution was skewed to the right (positive skewness). Using these original data, no significant model could be achieved, irrespective of the scaling procedure.

In such a situation, logarithmic transformation can reduce the degree of skewness as well as the minimum to maximum ratio, i.e., achieve approximate normality [27]. Since log(0) is undefined, a value >0 has to be added to the null entries; we used half the minimum value appearing in the data matrix (30). Using this procedure, the log-transformed values ranged from 1.48 [$\log_{10}(30)$] to 4.28 [$\log_{10}(30)$] for DORGlu and to 5.46 when including all variables. Significant models were now accomplished. Using 10 or 1 instead of 30 to be added to the null entries did not affect the model characteristics noticeably.

Measured values (or their logarithm) are not used as such for the multivariate analyses. The data are mean-centred or mean-centred and scaled. Mean centring uses the difference between the sample value and the mean of the respective variable over all samples. This mode gives highest importance to variables that show largest absolute differences between individuals or groups of individuals. In "unit variance scaling", the difference between an individual measure and the mean value of the given variable is divided by the standard deviation of the respective variable. In this case, all variables get the same weight. Low-level analytes have the same chance as have high-level analytes to be indicative of differences between individuals. On the other hand there is the danger that noise may influence the model inappropriately. The so-called Pareto scaling uses the square root of the standard deviation as the divisor and is a compromise between the fore-mentioned procedures. In our analysis, scaling did not improve the models. On the contrary it deteriorated the predictivity of the models. Logarithmic transformation had already reduced the undue impact of the high-level analytes.

4.2. Insight into dextromethorphan metabolism

Metabolites of DEX in human urine had been investigated by GC–MS [28]. In this study, glucuronides were hydrolyzed by incubation with glucuronidase. Metabolites that carried a hydroxyl group could therefore, have originated from glucuronides. One example is hydroxy-DOR from excreted hydroxy-DORGlu as detected in our analysis. Oxidized DEX and MOM that carry a carbonyl group instead of a methylene group (oxo-DEX and oxo-MOM) had also been found in the cited report, but the analogue oxo-DOR and oxo-HOM detected here in the form of glucuronides had not been described. Metabolite profiling and multivariate analysis therefore, allowed detection of new pathway-specific metabolites. Their impact on the metabolic ratio DEX/DOR remains to be investigated.

4.3. Urinary biomarkers: metabolomics and metabolite profiling

Analysis of compounds excreted in urine is an established method for biomonitoring exposure of humans to specific compounds. More recently, comprehensive analysis of a metabolic fingerprint in a biological fluid was introduced as the field of "metabolomics". The hypothesis is that urine may contain biomarkers of functional changes or disease, under the assumption that a given stimulus results in characteristic changes in the profile [29]. In the past, the metabolite spectrum was dominated by the high-level urinary constituents of biochemical pathways.

With the emergence of more refined analytical and statistical methods, additional types of investigations come into reach. Metabolite profiling, i.e., the analysis of a full metabolic profile of a substance is becoming a promising tool for preclinical drug development [30–32], drug toxicity [33–36], and safety assessment [37]. Exemplified in an animal model, pre-dose urinary metabolite profiles of rats had been used to predict the ratio of paracetamol glucuronide to paracetamol obtained post-dose from the individual rat. This approach shows promise in developing personalized drug therapy [38,39], but to our knowledge no respective studies in humans exist so far.

4.4. Applications of LC–MS/MS for class-specific metabolites

Mass spectrometry does not only allow for highly sensitive and selective analysis of known molecules but also lends itself to analyze classes with common structural elements. In this study, we showed the potential to do so for glucuronides that form an important group of phase II metabolites. Neutral loss of 176 Da combined with the characteristic glucuronic acid fragments 175, 113, 95, and 85 are highly indicative of a glucuronide conjugate.

Conjugation with sulfate results in another important class of phase II metabolites. The changes in sulfate conjugation have been investigated after gentamicin-induced nephrotoxicity in the rat by monitoring a neutral loss of 80 [40]. Another group has developed a method for profiling sulfoconjugates in human urine and compared

the neutral loss of 80 with precursor ion scanning of m/z 80 (SO_3^-) and m/z 97 (HSO_4^-). The method was applied to the characterization of urinary biomarkers for heavy metal toxicity in rats [41]. The same authors used a quadrupole ion trap for metabolite profiling of rat urine for various chemical families, including carboxylic acids, amines, sulfated compounds, glucuronides and glycosides, based on information-dependent acquisition of product ion spectra [42].

The same analytical principle can be applied to investigate the reaction products of electrophilic intermediates originating from epoxides, quinones or aldehydes. They react with nucleophilic groups such as thiols or amino groups to form conjugates for instance with glutathione or adducts with cysteine or lysine. Excretion of the resulting mercapturic acids or cysteine/lysine adducts can be analyzed by MS in a class-specific manner on the basis of their characteristic neutral losses and fragmentation. Neutral loss of 129 Da is characteristic for *N*-acetyl-cysteine, 171 Da for *N*-acetyl-lysine [18,26,43].

Formation of reactive intermediates in the metabolism of drugs is a major problem in drug development because adduct formation with protein can result in cell death. Analysis of mercapturic acids in urine of animals treated with the respective compound could provide an early warning against putative reactive metabolites. Similarly, reaction with nucleophilic sites of DNA nucleotides would be indicative for a mutagenic and carcinogenic potential and could be discovered on the basis of a neutral loss of 116 (2′-deoxyribose) from carcinogen nucleoside adducts excreted in urine [16].

In addition to these applications for toxicity testing of compounds in development, the investigation of individual differences in metabolism could even be more refined than for the phenotyping of polymorphically expressed enzymes as presented here. Modulation of metabolism by co-medication with drugs with inhibiting or inducing activity, smoking, nutrition, or occupational and environmental exposures could be studied. This would protect individuals from potentially toxic exposures and result in a big step forward towards personalized drug therapy.

Acknowledgments

The authors thank Drs. R. Bonner and L. Burton, Applied Biosystems/MDS Sciex, Canada, for providing a time-limited demo version of MarkerView™ software and for support.

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