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Development of a chromatographic bioreactor based on immobilized β-glucuronidase on monolithic support for the determination of dextromethorphan and dextrorphan in human urine

E. Calleri [a,*], G. Marrubini [b], G. Massolini [a], D. Lubda [c], S.S. de Fazio [a], S. Furlanetto [d], I.W. Wainer [e], L. Manzo [b], G. Caccialanza [a]

[a] *Department of Pharmaceutical Chemistry, University of Pavia, Via Taramelli 12, Pavia I-27100, Italy*
[b] *Clinical Toxicology Division, Department of Internal Medicine, University of Pavia, Via Brodolini 7, I-27028 San Martino Siccomario, Pavia, Italy*
[c] *Merck KGaA, LSP R&D MDA, Frankfurter Str 250, Darmstadt D-64293, Germany*
[d] *Department of Pharmaceutical Sciences, University of Florence, Polo Scientifico, Via Ugo Schiff 6, Sesto Fiorentino 50019 (FI), Italy*
[e] *Bioanalytical and Drug Discovery Unit, Gerontology Research Center, National Institute on Aging, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, MD 21224-6825, USA*

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Abstract

We here reported the development and application of an immobilized enzyme reactor (IMER) based on β-glucuronidase to the *on-line* determination of urinary molar ratios of dextromethorphan (DOMe)/dextrorphan (DOH) for the assessment of the metabolic activity of CYP2D6, a genetically variable isoform of cytochrome P-450 (CYP).

β-Glucuronidase was immobilized on an HPLC monolithic aminopropyl silica support. Catalytic activity and stability of the chromatographic reactor were evaluated using 8-hydroxyquinoline glucuronide (8-HQG) as substrate. The IMER was coupled through a switching valve to a reversed-phase column (C8) for the simultaneous determination of dextromethorphan and its main metabolite dextrorphan. On purpose a selective reversed-phase ion pair HPLC method coupled with fluorescence detection was developed. Urine samples were first centrifuged to remove insoluble materials and then aliquots of the supernatants were injected into the coupled-column analyser.

Linearity, precision and accuracy of the method were established. The method reliability was verified by comparing our data with previous data of a phenotyping study carried out by the Poison Control Centre of Pavia-Clinical Toxicology Division.

Keywords: β-Glucuronidase; Immobilized enzyme reactor; Dextromethorphan; Dextrorphan; Cytochrome P-450 2D6

* Corresponding author. Tel.: +39-382-507788; fax: +39-382-422975.
E-mail address: enrica.calleri@unipv.it (E. Calleri).

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1. Introduction

The response of an individual to a drug therapy may be highly variable. Genetically variable drug metabolism can result in therapeutic failure and unanticipated toxicity in individuals who have variant alleles for drug-metabolizing enzymes [1]. This poses a major clinical problem because this inter-individual variability is until now only partly predictable.

Members of the human cytochrome P-450 (CYP) superfamily play a role in the metabolism of many drugs and several of them have been shown to be polymorphic. In particular cytochrome P-450 2D6 (CYP2D6) is polymorphically distributed and is responsible for the metabolism of several clinically important drugs [2,3]. For this reason the determination of this genetic polymorphism is important and could provide the basis for a rational approach to drug prescription.

The individual activity of CYP2D6 enzyme can be assessed by means of genotyping or phenotyping. Genotyping involves identification of defined genetic mutation that give rise to the specific drug metabolism phenotype. Phenotyping requires intake of a probe drug whose metabolism is known to be solely dependent on CYP2D6 enzyme. The excretion of parent compound and/or metabolite in urine allows to calculate the metabolic ratio, which is a measure of individual CYP2D6 activity. Phenotyping methods using drug probes have several drawbacks, but currently represent the only approach to evaluate enzyme function. Moreover, phenotyping is useful in revealing drug–drug interactions or defect in overall process of drug metabolism.

Different probe drugs have been proposed for CYP2D6 phenotyping but dextromethorphan (DOMe) represents the only probe drug readily available as OTC drug in most of the countries.

Dextromethorphan is a mild cough suppressant drug extensively metabolized by *O*-demethylation to the major active metabolite, dextrorphan (DOH) and by *N*-demethylation to 3-methoxymorphinan. These metabolites are further demethylated to 3-hydroxymorphinan. The 3-hydroxyl group position of dextrorphan and 3-hydroxymorphinan is rapidly conjugated via glucuronidation. *O*-Demethylations are catalyzed by cytochrome P-450 2D6 while *N*-demethylations are primarily catalyzed by the cytochrome P-450 CYP3A. The metabolic pathways of dextromethorphan are reported in Fig. 1. In vivo, the urinary molar ratios of DOMe/DOH are used to assess

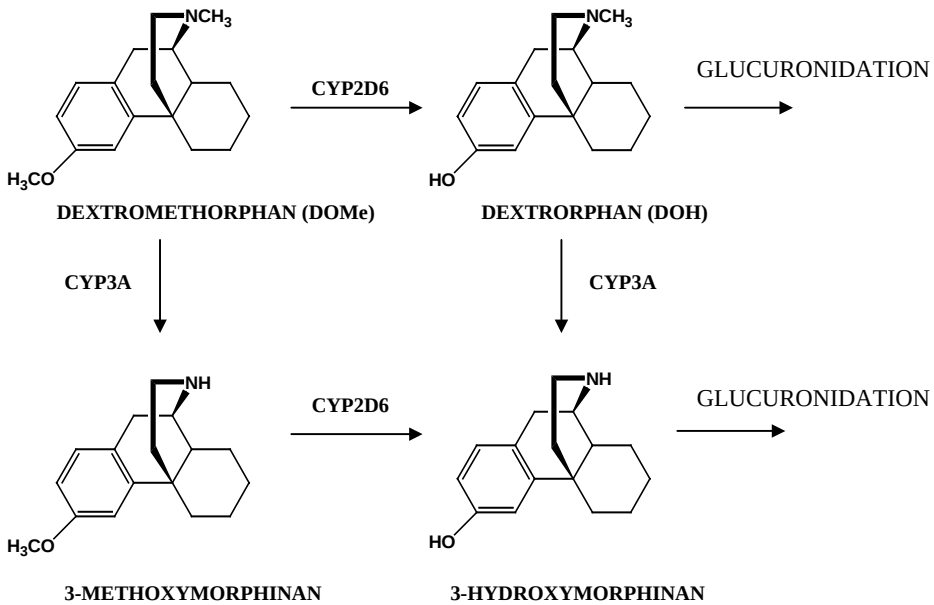

Fig. 1. Chemical structures and metabolic pathways of dextromethorphan.

the metabolic activity of CYP2D6. Dextromethorphan and its metabolites can be measured in biological matrices by several methods including high-performance liquid chromatography (HPLC) with UV, MS or fluorescence detection, gas chromatography and capillary electrophoresis [4–11].

Because of lack of commercially available standard of DOH-glucuronide, hydrolysis procedures during sample preparation are unavoidable. The conjugate may be cleaved by acid-, base- or β-glucuronidase-catalysed hydrolysis with the latter being the method of choice in most investigations addressed to determine the total concentration of dextrorphan. The *off-line* deconjugation of the glucuronide is often followed by an extraction procedure of the aglycone from the biological matrix and analysis by a suitable analytical method. These procedures can be time consuming, require extensive sample clean-up and may yield unreproducible results because of the instability of the enzymes in the assays. One approach to overcome the problems involved in the *off-line* deconjugation, is the development of a HPLC-compatible bioreactor based on immobilized β-glucuronidase for the *on-line* conversion of the conjugate. The feasibility of this approach was previously demonstrated in the *on-line* deconjugation of some standard glucuronides [12], but up to now only the analysis of urinary chloramphenicol-β-D-glucuronide has been reported [13].

Among the glucuronides considered in reference [12], the hydrolysis of morphine-glucuronide with β-glucuronidase immobilized on epoxide silica support was very low. The authors inferred that the immobilization process could have sterically hindered the active site of the enzyme. In view of the structural similarity between morphine and dextrorphan, a new chromatographic support was considered for β-glucuronidase immobilization to overcome the problem faced for morphine-glucuronide.

In recent years the monolithic stationary phases have attracted attention in liquid chromatography due to their excellent properties concerning fast mass transfer. Monolithic stationary phases differ from conventional columns because of their hydrodynamic properties. Monoliths have mass transfer between the eluent and the active sites of the silica support as rapid as that of 3 μm particulate materials while offering a pressure drop comparable to that of columns packed with 15 μm particles. All these features are ideal for enzyme immobilization and fast conversion of substrates.

Considering altogether these favorable properties for chromatographic performance and speed, β-glucuronidase has been covalently immobilized on a silica-based monolithic type HPLC-column [14,15]. The potentiality of the developed immobilized enzyme reactor (IMER) for the *on-line* deconjugation of dextrorphan-β-D-glucuronide to its aglycone in human urine samples was investigated in a coupled-column HPLC system.

2. Experimental

2.1. Reagents and materials

β-Glucuronidase (EC 3.2.1.31, type H-1, from *Helix pomatia*, 374.000 U/g solid), 8-hydroxyquinoline (8-HQ), 8-hydroxyquinoline glucuronide (8-HQG), *N*,*N*′-disuccinimidyl carbonate (DSC) and USP-grade dextromethorphan hydrobromide monohydrate were purchased from Sigma–Aldrich (Milan, Italy). Dextrorphan D-tartrate (98% purity) was obtained from ICN Phrmaceuticals s.r.l. (Milan, Italy). USP-grade Levallorphan D-tartrate was provided by Sigma-RBI (Milan, Italy).

Concentrated solution of β-glucuronidase from *Escherichia coli* in 0.2 M sodium acetate buffer at pH 5 (cat. no. 127060) was purchased from Boehringer-Roche (Milan, Italy). Sodium azide, citric acid and Chromolith® Performance NH_2 (2 μm macropores, mesopore size 13 nm) (4.6 mm × 150 mm i.d.) research sample were from Merck (Darmstadt, Germany). HPLC-grade acetonitrile and sodium dodecyl sulfate (SDS) were from Carlo Erba (Milan, Italy). Water was deionized by passing through a Direct-Q™ system (Millipore, Bedford, MA, USA). Zorbax Eclipse® XDB-C8-column (4.6 mm × 150 mm i.d., 5 μm) was purchased from Agilent Technologies (Germany).

2.2. Apparatus

A schematic drawing of the column-switching system is given in Fig. 2. Chromatographic experiments were performed with two Series 1100 Hewlett-Packard (HP, Palo Alto, CA, USA) HPLC modular sys-

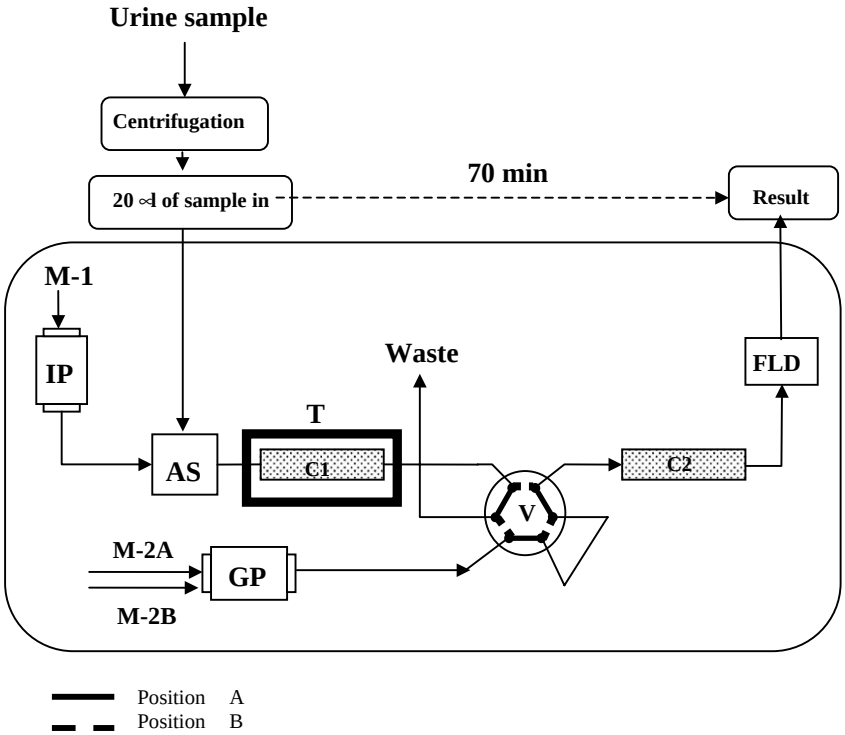

Fig. 2. Chromatographic system coupling the enzyme column with the reversed-phase analytical column. The substrate is loaded onto the enzyme column using Position A; the analytes are switched to the analytical column using Position B; the analyte separation is carried out on the analytical column using Position A.

tems connected to a Vectra VE Hewlett-Packard personal computer operating the HPLCs by the Hewlett-Packard ChemStation software (Revision A.04.01). System 1 consisted of an isocratic pump (IP), a thermostated column oven ($40.0 \pm 0.5\,^{\circ}C$), an autosampler (AS), and the enzyme column (C1). System 2 consisted of a quaternary gradient pump (GP) equipped with a HP Model 1046 fluorimetric detector (FLD), a vacuum degasser and a Zorbax Eclipse® XDB-C8-column (C2) (4.6 mm × 150 mm i.d., 5 μm).

Systems 1 and 2 could be used independently or the eluent from System 1 could be directed onto System 2 through a Rheodyne six-port switching valve (V) as shown in Fig. 2.

2.3. Preparation and characterisation of the enzymatic stationary phase

β-Glucuronidase was covalently immobilized on a Chromolith-NH_2 column according to a previously described procedure following the in situ method [16,17]. Briefly, a total of 6 g of DSC were dissolved in 200 ml acetonitrile and the resulting solution was re-circulated for 18 h at 0.5 ml/min through the column previously equilibrated with the same solvent. The column was washed at the same flow-rate, first with 30 ml acetonitrile, then with 30 ml water and finally with 30 ml of 1 mM phosphate buffer (pH 7.0). Five hundred and seventy-six milligrams enzyme were dissolved in 200 ml of 50 mM phosphate buffer (pH 7.0) and the solution continuously circulated at 0.5 ml/min for 24 h. The flow was inverted every 15 min in the first hour and then every 30 min in the following 4 h. After 24 h the column was washed with 200 ml water, 200 ml of a 0.5 M NaCl solution and finally with 100 ml of 0.2 M glycine solution to block any residual activated group. When not in use the column was stored at 4 °C in a sodium azide solution (0.01%, w/v). The hydrolysis of 8-HQG was used as standard assay for determining the activity of the im-

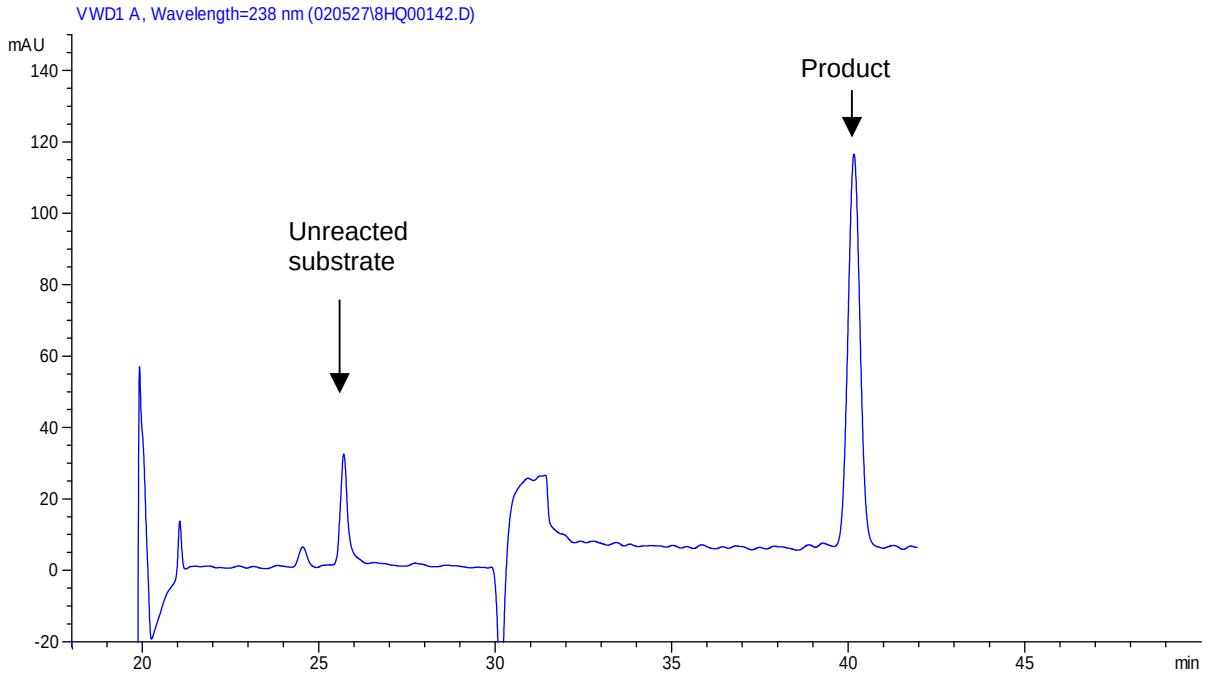

Fig. 3. Chromatogram showing the deconjugation of 8-hydroxyquinoline glucuronide (0.3 mM); see text for experimental conditions.

mobilized enzyme using the system reported in Fig. 2 by an on-column procedure (16). Stock standard solutions of 8-HQ (10 mM in methanol) and 8-HQG (3 mM in water) were prepared. Test samples of 8-HQG at concentrations ranging between 0.025 and 30 mM were prepared by diluting the stock solution in a 20 mM citrate buffer (pH 5.6). The experiments were carried out at 40 °C, measuring the activity of the immobilized enzyme (U, μmol of 8-HQG hydrolysed per minute). The enzyme column was first equilibrated for 30 min with 20 mM citrate buffer (pH 5.6), and then 20 μl of 8-HQG test samples were injected onto System 1 at a flow-rate of 0.5 ml/min (switching valve in position A). After 5 min, C1 was connected on-line to C2 (switching valve in position B) and 8-HQ and unreacted 8-HQG were flushed for 13 min. The valve was then switched back to its original position (Position A) for the separation of the analytes with the eluent delivered by System 2. The analyses on System 2 were carried out at 1 ml/min applying on C2 a 20 mM citrate buffer (pH 3.0)–acetonitrile (70:30, v/v) containing 10 mM SDS for the first 10 min. At 10.01 min a step gradient to 20 mM citrate buffer (pH 3.0)–acetonitrile (60:40, v/v) was applied. A representative chromatogram with UV detection at 238 nm is given in Fig. 3. The chromatographic retention factors were approximately 14.82 for 8-HQ and 4.39 for 8-HQG. The amount of 8-HQ recovered after hydrolysis was dependent on the concentration of 8-HQG injected. The Michaelis–Menten trend was found plotting the rate of reaction against the substrate concentration [*S*].

The rate of the enzymatic reaction (*V*) expressed as (Δarea 8-HQ/min) was calculated by:

$$V\left(\frac{\Delta\text{area (8}-\text{HQ)}}{\text{min}}\right)=\frac{\text{area (8}-\text{HQ)}}{\text{time (min)}}=\frac{\text{area (8}-\text{HQ)}}{18\,\text{min}} \quad (1)$$

V_{max} was estimated by Lineweaver and Burk reciprocal plots of $1/V$ versus $1/[S]$.

To calculate the units (U) of immobilized enzyme the following equation was used:

$$U\left(\frac{\mu\text{mol}}{\text{min}}\right)=\left[\frac{(\Delta\text{area/min})_{max}}{\text{extintion coefficient}}\right]\times\text{column void volume}(1.76\,\text{ml}) \quad (2)$$

In order to determine the 8-HQ extinction coefficient for concentration assessment (area of a 1 mM solution of 8-HQ), increasing concentrations of 8-HQ were injected onto the enzyme column. Four 8-HQ calibration solutions in the range between 0.03 and 15 mM were prepared and each solution was injected twice. A linear correlation ($r^2 = 0.9866$) was found providing the extinction coefficient value. The amount of active immobilized units was found to be 0.123.

The hydrolysis of a 0.1 mM standard solution of 8-HQG was used as a standard assay to test the stability of the immobilized enzyme. The conversion degree (C%) was calculated by comparing the area of produced 8-HQ to the area of a 8-HQ standard solution (0.1 mM) in the same chromatographic conditions according to following equation:

$$C\% = \frac{A_{8-\mathrm{HQpr}}}{A_{8-\mathrm{HQst}}} \times 100 \quad (3)$$

The column activity was constantly checked during the research work (4 months). The mean conversion degree of 8-HQG was 70.86 ± 6.57% showing that the column activity was stable after more than 250 injections.

2.4. Standard solutions

Stock solutions of DOMe (1 mg/ml in methanol), DOH (2 mg/ml in water), and Levallorphan (1 mg/ml in water) were prepared and immediately stored at −20 °C in the dark. Prior to every analytical session dilutions of the stock solutions were prepared at the working solutions concentrations. DOMe working solution was at the concentration of 10 μg/ml in 0.1 M sodium acetate buffer (pH 5), and further diluted in the same buffer to the calibrators concentrations at 1.0, 0.5, 0.2, 0.02, and 0.01 μg/ml. DOH working solution was at the concentration of 100 μg/ml in 0.1 M sodium acetate buffer (pH 5), and further diluted in the same buffer to the calibrators concentrations of 5.0, 2.5, 1.0, 0.1, and 0.05 μg/ml. Levallorphan (internal standard, IS) working solution for the off-line method was prepared at 50 μg/ml in 0.1 M sodium acetate buffer (pH 5). In the off-line procedure 20 μl aliquots of IS solution were added to all samples, i.e. calibrators, aqueous controls, and urines.

2.5. Chromatographic method

The following experimental steps were followed:

Step 1 (0–15 min, valve in position A): the sample was loaded on the enzymatic column (C1); citrate buffer 20 mM (pH 5.6) (M1) was used as eluent delivered by IP using a flow-rate of 0.1 ml/min.

Step 2 (15.01–55 min, valve in position B): the valve was switched to position B and the analytes were flushed and focused directly onto the reversed-phase analytical column (C2) using a flow-rate of 0.1 ml/min for the first 25 min and increasing the flow-rate to 1 ml/min during the following 15 min. DOH and DOMe were contained in 17.5 ml of eluate and focused onto the head of the analytical column.

Step 3 (55.01–70 min, valve in position A): the valve was switched back to its original position for the analytical separation. The mobile phase used consisted of M-2A: M-2B (40:60, v/v), with M-2A being a 20 mM citrate buffer (pH 3.0) containing 10 mM SDS, and M-2B being a mixture of M-2A-acetonitrile (20:80, v/v) containing 10 mM SDS.

The flow-rate was 1.0 ml/min. Fluorimetric detection at 210 nm (excitation) and 312 nm (emission) wavelengths was performed.

2.6. Method validation

Quantitation of DOMe and DOH was accomplished by external calibration on standard additions to pooled blank urine. The use of internal standard procedure was not considered necessary for the straight-forward on-line procedure. At the beginning of each analytical session, five separate samples were prepared by spiking urine with known amounts of stock solutions of DOH and DOMe to final concentrations of 0.05, 0.1, 1.0, 2.5, and 5.0 μg/ml and 0.01, 0.02, 0.2, 0.5, and 1.0 μg/ml, respectively (Table 1). Aqueous controls were also prepared in 0.1 M sodium acetate buffer (pH 5) at the same concentrations. The urine calibrators were randomly inserted into the injections sequence together with real samples, urine blanks, and aqueous controls. Correlation coefficients and the equations describing the calibration curves were determined by linear regression analysis. Limits of detection and quantitation (LOD, LOQ) were calculated according to [18] based on three replicate analyses of blank pooled urine.

Table 1
Results of the calibration plots[a]

DOH				DOMe			
Nominal concentration (μg/ml)	Area[b]	Measured mean concentration (μg/ml)	Recovery (%)	Nominal concentration (μg/ml)	Area[b]	Measured mean concentration (μg/ml)	Recovery (%)
5.00	1660.1	4.99	99.8	1.00	470.6	1.01	101
2.50	837.0	2.51	100	0.50	218.6	0.47	94
1.00	335.4	1.00	100	0.20	94.5	0.20	100
0.10	35.0	0.10	100	0.020	13.1	0.027	137
0.05	15.1	0.04	80	0.010	7.4	0.015	151

[a] Samples were prepared by spiking urine at different DOH and DOMe concentrations. Each sample was analysed once. Linear regression data equation: $y = 332x + 1.9$, $r^2 > 0.999$ for DOH and: $y = 464x + 0.38$, $r^2 = 0.998$ for DOMe.
[b] Raw areas were corrected by subtracting the reading in the blank at the same retention time of the analytes. See text for more details.

Precision was tested by intra-day and inter-day repeatabilities together with inter-laboratory transferability and assessed by multiple analysis of real samples, as summarised by the data presented in Tables 2 and 3.

Accuracy was tested thoroughly during all sessions by comparing the experimental concentration measured for each calibrator of the calibration plot with their corresponding nominal concentration (Table 1), and with aqueous controls of equal concentration.

Accuracy on real samples was verified by comparison with data obtained 2 years before from a pilot phenotyping study carried out by the Poison Center of Pavia-Clinical Toxicology Division. At the time, determinations were conducted with a method (Method 2) which was essentially that described by Francis Lam and Rodriguez [19] with slight modifications. Briefly, the 8 h total urine production of healthy volunteers and of selected patients were collected after administration of one tablet of Bronchenolo Tosse® (7.5 mg destromethorphan) under controlled conditions. From each sample, 10 ml urine aliquots were kept stored for external cross-validation. After addition of IS (1 μg/ml), enzymatic hydrolysis was carried out in glass tubes on 1 ml aliquots of urine by adding 0.980 ml of 0.2 M sodium acetate buffer (pH 5) and 0.02 ml of β-glucuronidase solution in sodium acetate buffer at pH 5. The tubes were kept closed in a thermostated bath at 38–40 °C for 18 h. The hydrolyzed urine was then brought to pH 11–12 by adding 0.02 ml of concentrated NH_4OH. Liquid–liquid extraction followed by adding 5 ml of *n*-hexane: ethylacetate (3:1, v/v) and Vortex mixing for 2 min. After centrifugation (20 min, 2500 rpm), the organic layer was transferred to a clean tube and re-extracted with 0.2 ml of 0.01N HCl (Vortex mixing, 2 min). After centrifugation (5 min, 2500 rpm), the organic layer was discarded, the residual traces of *n*-hexane and ethylacetate were evaporated under a gentle nitrogen stream for few minutes, and 50 μl of the hydrochloric extract were injected into the HPLC system. Quantitation was performed on the concentrations of 0.01, 0.02, 0.2, 0.5, and 1.0 μg/ml for DOMe and of 0.05, 0.1, 1.0, 2.5, and 5.0 μg/ml for DOH. Fluorimetric detection

Table 2
Results of *intra-day* repeatability and *inter-laboratory* transferability[a]

Sample 10	DOMe (μg/ml)	DOH (μg/ml)
Laboratory 1 ($n = 3$)	0.16	2.35
	0.18	2.43
	0.17	2.52
Mean ± S.D.	0.17 ± 0.01	2.43 ± 0.09
R.S.D. (%)	6	4
Laboratory 2 ($n = 4$)	0.26	2.41
	0.27	2.51
	0.28	2.20
	0.24	2.50
Mean ± S.D.	0.26 ± 0.02	2.40 ± 0.1
R.S.D. (%)	7	6

[a] Sample 10 has been processed in replicate analysis in two different laboratories.

Table 3
Results of *inter-day* repeatability[a]

Sample	DOMe		DOH		*N*
	Mean ± S.D. (μg/ml)	R.S.D. (%)	Mean ± S.D. (μg/ml)	R.S.D. (%)	
2	0.0223 ± 0.001	6	0.53 ± 0.04	7	4
3	0.045 ± 0.017	38	0.53 ± 0.01	1	3
4	0.029 ± 0.01	4	0.80 ± 0.01	1	3

[a] The samples were analysed at least for 3 days.

was used at 210 nm excitation and 312 nm emission wavelenghts for DOH and DOMe, and 400 nm emission for IS. Chromatographic separation was accomplished isocratically at 1 ml/min by applying a mobile phase of water–acetonitrile–H_3PO_4–triethylamine (84.88:15:0.06:0.06, v/v) at pH 2.7–3.0 on a Supelco LC-CN (150 mm × 4.6 mm, 5 μm) stainless steel column.

3. Results and discussion

3.1. Method development

Based on previous reports [20], describing the optimization of the hydrolysis of morphine-3-β-D-glucuronide by β-glucuronidase from *H. pomatia*, the mobile phase chosen was a 20 mM citrate buffer at pH 5.6 incubated at 40 °C. Such conditions were selected as better compromise between maximum activity and stability of the enzyme.

The flow-rate is an additional parameter affecting the hydrolysis yield. The relation between hydrolysis yield of DOH-β-glucuronide and flow-rate was therefore studied on one urine sample obtained from a subject enrolled in the phenotyping study. The flow-rates tested were 0.1, 0.2 and 0.5 ml/min. DOH concentration increased six-fold decreasing the flow-rate from 0.5 to 0.1 ml/min. The hydrolysis yield thus was higher at the lower flow-rate, at which the substrate–enzyme contact time is longer. However, the analysis of a standard mixture of DOH and DOMe on the enzyme column at 0.1 ml/min provides two broad peaks at approximately 48 and 93 min, respectively (chromatogram not shown). Given the higher polarity of the glucuronide compared to the aglycone, and supposing that the reaction takes place before the elution of the product, we decided to apply a flow gradient. The flow-rate was set at 0.1 ml/min for 40 min and increased to 1.0 ml/min for the last 15 min of analysis. No significant variation in the peak areas of DOH and DOMe was observed after the injection of the urine sample with and without the flow-rate gradient, confirming the hypothesis that the hydrolysis reaction took place during the first 40 min of run.

Furthermore, a selective reversed-phase ion pair high-performance liquid chromatography method coupled with fluorescence detection was developed in order to attain optimal conditions for resolution and selectivity between DOMe, DOH, and interferences in the urine samples.

The ion pair mode at mildly acidic pH (pH 3.0) using 0.01 M sodium dodecyl sulfate gave symmetrical and well resolved peaks for DOH and DOMe in a run time of 12 min. To overcome incompatibility problems due to different mobile phases of the two columns in the on-line coupling of the enzyme reactor, the 20 mM citrate buffer at pH 3.0 was adopted as mobile phase for the analytical column.

Ion pairing with the hydrophobic anion SDS enhanced retention allowing the separation of the target compounds from large hydrophilic peaks arising from the injection of urine (Fig. 4A–C). The conditions adopted, however, could not produce a base-to-base peak separation of the analytes from minor contaminants present in hydrolyzed urine. Given the goal of our study, i.e. the evaluation of the efficiency on real samples of a coupled-column analyser including a β-glucuronidase IMER, the matrix noise was considered acceptable for quantitative analysis of DOH and DOMe.

3.2. Validation

The linear calibration equations for urine showed acceptable *y*-axis intercepts and high correlation co-

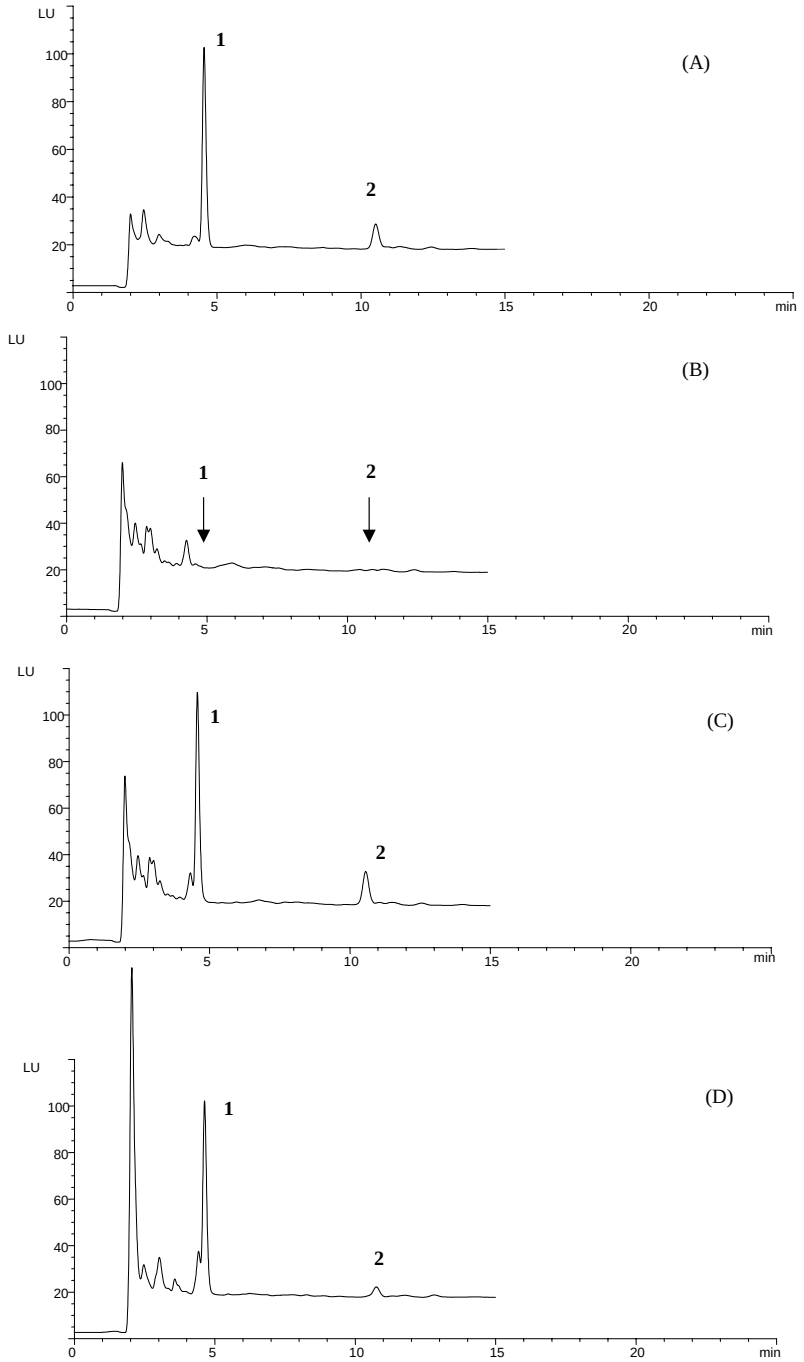

Fig. 4. Chromatograms showing: (A) aqueous control. A 2.5 μg/ml of DOH (peak 1) and 0.5 μg/ml of DOMe (peak 2). (B) Blank urine. The arrows correspond to the retention times were DOH (peak 1) and DOMe (peak 2) would appear. (C) Urine spiked with 2.5 μg/ml of DOH (peak 1) and 0.5 μg/ml of DOMe (peak 2). (D) Analysis of urine sample from a volunteer who took orally 7.5 mg dextromethorphan bromide (Brochenolo Tosse®). Urine was collected over a 0–8 h period.

Table 4
The determination of DOMe and DOH concentrations in urine samples from subjects dosed with DOMe using on-line (Method 1) and off-line (Method 2) assays

Sample	Method 2: DOMe (μg/ml)	Method 1: DOMe (μg/ml	Error (%)	Method 2: DOH (μg/ml)	Method 1: DOH (μg/ml)	Recovery (%)
1	0.65	0.70	8	13.2	4.00	30
2	0.03	0.02	33	1.33	0.53	40
3	0.01	0.05	400	1.53	0.53	35
4	0.25	0.29	16	1.77	0.80	45
5	0.03	0.02	33	2.38	0.59	25
6	<0.01	<0.01	[a]	2.80	0.72	26
7	0.02	0.02	0	4.00	1.99	50
8	<0.01	<0.01	[a]	0.87	0.49	56
9	0.01	<0.01	[a]	0.65	0.30	46
10	0.55	0.17	69	7.10	2.43	34
11	<0.01	0.02	[a]	1.43	0.41	29

[a] Not determinable.

efficients ($y = 332x + 1.9$, $r^2 > 0.999$ for DOH, and $y = 464x + 0.38$, $r^2 = 0.998$ for DOMe). The LOD and LOQ were respectively established at 0.005 and 0.05 μg/ml for DOH and at 0.001 and 0.01 μg/ml for DOMe. Intra-day and inter-day repeatabilities together with inter-laboratory transferability (see Tables 2 and 3) prove that method precision is good (CV% comprised between 1 and 7% over three–four replicates in different days) except in one case (sample 3, CV% 38 over three replicates in different days) where the chromatographic set-up failed to fully separate DOMe owing to a significant background matrix noise.

3.3. Method application

In order to assess if the β-glucuronidase IMER could be used in the determination of metabolic phenotyping, both the *on-line* (Method 1) and *off-line* (Method 2) methods were used to analyze 11 urine samples. The samples came from subjects involved in a pilot CYP2D6 phenotyping study carried out by the Poison Control Center of Pavia-Clinical Toxicology Division which utilized DOMe as the probe drug. The comparison of the data obtained with the two methods are reported in Table 4.

The on-line method failed to determine correctly DOMe in one sample (sample 3, Table 4), and produced significant errors assessing the concentrations of 4 samples out of 11 (samples 2, 4, 5, and 10, Table 4). The DOH total concentrations found were in all cases lower than the reference value. This observation points to an incomplete hydrolysis obtained through the β-glucuronidase IMER, which was expectable considering the morphine-like frame of DOH and previous literature [12].

Table 5
The determination of DOMe and DOH concentrations in urine samples from subjects dosed with DOMe using on-line (Method 1) and off-line (Method 2) assays and the determination of the metabolic ratio (log DOMe/DOH)

Sample	Method 2			Method 1		
	DOMe (μM)	DOH (μM)	Log DOMe/DOH	DOMe (μM)	DOH (μM)	Log DOMe/DOH
1	2.39	51.29	−1.33	2.58	15.54	−0.78
6	<0.04	10.88	−2.43	<0.04	2.80	−1.84
7	0.07	15.54	−2.35	0.07	7.73	−2.04
8	<0.04	3.38	−1.93	<0.04	1.90	−1.68
9	0.04	2.52	−1.80	<0.04	1.16	−1.46
11	<0.04	5.55	−2.14	0.07	1.59	−1.36

When the urine concentration of DOMe was below the lower limit of quantitation (i.e. <0.04 μM) the values of 0.04 was used to calculate the metabolic ratio.

In earlier studies using DOMe as the probe drug, metabolic ratios of 0.3 to 0.6 (expressed as log values of −0.52 and −0.22) were used as antimode to divide extensive from poor CYP2D6 metabolizers [21–23]. The ratio values used to discriminate between poor and extensive metabolizer was based on standard analytical methods in which there is more complete hydrolysis of dextrorphan-glucuronide. It would be expected that the "true" antimode for our method would be shifted. The metabolic ratios determined by the two methods with the exclusion of samples 2, 3, 4, 5 and 10 are presented in Table 5. It was interesting to observe that the log of the metabolic ratios determined by the two methods were correlated, $r^2 = 0.7878$ ($P = 0.017$) and in each case, the *on-line* method assigned the extensive metabolic phenotype to the subject which was consistent with the results from the *off-line* study.

4. Conclusions

The immobilization of β-glucuronidase on monolithic support was described and the developed reactor was coupled with an analytical column for the separation and quantitation of total DOMe and DOH. The use of monolithic material lead to a better performance of immobilized β-glucuronidase and to a very stable bioreactor. Nevertheless the glucuronide hydrolysis of DOH is still a rate-limiting step as the hydrolysis of the glucuronate was not complete. It was interesting to observe a linear correlation ($r^2 = 0.944$) between the measured levels of DOH with the two methods indicating that in the explored substrate concentration range the saturation of the enzyme was not reached. Further developments will include the optimization of the experimental conditions and of the immobilization reaction in order to increase the hydrolysis yield.

The proposed method is of interest as it could potentially simplify the determination of phenotype by using a direct urine injection. Unfortunately, the experimental cohort did not contain a poor metabolizer and therefore the full validation of the application for clinical purpose will require a more extensive data set.

References

[1] E. Spina, G.M. Campo, A. Avenoso, A.P. Caputi, P. Zuccaro, R. Pacifici, G. Gatti, G. Strada, A. Bartoli, E. Perucca, Pharmacol. Res. 29 (1994) 281–289.
[2] G. Schmitz, C. Aslanidis, K.J. Lackner, Clin. Chim. Acta 308 (2001) 43–53.
[3] E. Michalets Landrum, Pharmacotherapy 18 (1998) 84–112.
[4] E.-K. Bendriss, N. Markoglou, I.W. Wainer, J. Chromatogr. B 754 (2001) 209–215.
[5] D.R. Jones, J.C. Gorski, M.A. Hamman, S.D. Hall, J. Chromatogr. B 678 (1996) 105–111.
[6] J.M. Hoskins, G.M. Shenfield, A.S. Gross, J. Chromatogr. B 696 (1997) 81–87.
[7] J. Ducharme, S. Abdullah, I.W. Wainer, J. Chromatogr. B 678 (1996) 113–128.
[8] S. Hartter, D. Baier, J. Dingemanse, G. Ziegler, C. Hiemke, Ther. Drug Monit. 18 (1996) 297–303.
[9] H.T. Kristensen, J. Pharm. Biomed. Anal. 18 (1998) 827–838.
[10] S.S. Vergurlekar, J. Heitkamp, F. McCush, P.R. Velagaleti, J.H. Brisson, S.L. Bramer, J. Pharm. Biomed. Anal. 30 (2002) 113–124.
[11] R.A. Bartoletti, F.M. Belpaire, M.T. Rosseel, J. Pharm. Biomed. Anal. 14 (1996) 1281–1286.
[12] M. Pasternyk Di Marco, G. Felix, V. Descorps, M.P. Ducharme, I.W. Wainer, J. Chromatogr. B 715 (1998) 379–386.
[13] M. Pasternyk Di Marco, M.P. Ducharme, V. Descorps, G. Felix, I.W. Wainer, J. Chromatogr. A 828 (1998) 135–140.
[14] D. Lubda, K. Cabrera, W. Kraas, C. Schaefer, D. Cunningham, R.E. Major, LC/GC Europe 14 (12) (2001) 730–734.
[15] D. Lubda, K. Cabrera, H. Minakuchi, K. Nakanishi, J. Sol–Gel Sci. Technol. 23 (2002) 185–187.
[16] G. Massolini, E. Calleri, A. Lavecchia, F. Loiodice, C. Temporini, G. Fracchiolla, P. Tortorella, E. Novellino, G. Caccialanza, Anal Chem. 75 (2003) 535–542.
[17] E. Calleri, E. De Lorenzi, D. Siluk, M. Markuszewski, R. Kaliszan, G. Massolini, Chromatographia 55 (2002) 651–658.
[18] J.D. Winefordner, G.L. Long, Anal. Chem. 55 (1983) 712A–724A.
[19] Y.W. Francis Lam, S.Y. Rodriguez, Ther. Drug Monit. 15 (1993) 300–304.
[20] M.R. Taylor, S.A. Westwood, D. Perret, J. Chromatogr. A 768 (1997) 67–71.
[21] R.J. Straka, S.R. Hansen, P.F. Walker, Clin Pharmacol. Ther. 58 (1995) 29–34.
[22] L.B. Anthony, T.J. Boeve, K.R. Hande, Cancer Chemother. Pharmacol. 36 (1995) 125–128.
[23] W.M. O'Neil, B.M. Gilfix, N. Markoglou, A. Di Girolamo, C.M. Tsoukas, I.W. Wainer, Eur. J. Clin Pharmacol. 56 (2000) 231–240.