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Development and validation of a chemical hydrolysis method for dextromethorphan and dextrophan determination in urine samples: Application to the assessment of CYP2D6 activity in fibromyalgia patients

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Abstract

Dextromethorphan (DEM) is a widely used probe drug for human cytochrome P450 2D6 isozyme activity assessment by measuring the ratio between DEM and its *N*-demethylated metabolite dextrorphan (DOR). DOR is excreted in urine mainly conjugated to glucuronic acid. Prior to quantification, DOR must be deconjugated to avoid variability caused by the polymorphic glucuronosyltransferase enzyme. A chemical hydrolysis method was optimized using a chemometric approach. Three factors (acid concentration, hydrolysis time and temperature) were selected and simultaneously varied to study their effect on conjugated DOR hydrolysis. Hydrolysis conditions that maximize DOR release without significant degradation of both DEM and DOR were chosen and results were compared to those obtained by enzymatic method using β-glucuronidase. An HPLC method with fluorescence detection was developed for the simultaneous quantitation of DEM, DOR and levallorphan, used as an internal standard. Separation was performed on a phenyl analytical column (150 mm × 4.6 mm i.d., 5 μm) with a mobile phase consisting of 18% acetonitrile and 50 mM phosphoric acid (pH 3). The overall analytical procedure was validated and showed good performances in terms of selectivity, linearity, sensitivity, precision and accuracy. Finally, this assay was used to determine DEM/DOR molar ratios in fibromyalgia patients for the purpose of determining phenotype status for the CYP2D6.

Keywords: Chemical hydrolysis; Experimental design; CYP2D6; Phenotype; Dextromethorphan

1. Introduction

Drug pharmacokinetics may vary considerably between individuals, and may be caused by differences in expression and activity of cytochrome P450 enzymes [1]. For CYP2D6, more than 70 different allelic variants have been described [2]. Different phenotypes can be distinguished: poor metabolizers (PM) lack the functional enzyme; intermediate metabolizers (IM) carry two different alleles, leading to partial activity; efficient metabolizers (EM) have two normal alleles; and ultra-rapid metabolizers (UM) have multiple gene copies [3]. DEM is a widely used and validated probe for assessing CYP2D6 activity [4–7]. DEM is metabolized to DOR by CYP2D6, and to 3-methoxymorphinan (3-MM) by CYP3A4 (Fig. 1). Moreover, 3-hydroxymorphinan (3-OH) is obtained through *N*,*O*-didemethylation by CYP3A4 and CYP2D6, respectively [8].

DOR is excreted in urine mainly conjugated to glucuronic acid [9]. Prior to quantification, urine must be deconjugated to avoid variability caused by the polymorphic glucuronosyltransferase enzyme. Hydrolysis of conjugated DOR is generally achieved by enzymatic method using β-glucuronidase [10]. However, this method is expensive, tedious and time consuming (18–24 h). A new chemical hydrolysis method using hydrochloric acid is described in this study. The most important parameters are hydrolysis time, temperature and acid concentration. Reaction optimization is achieved by using a chemometric approach which represents a valuable statistical tool for the development

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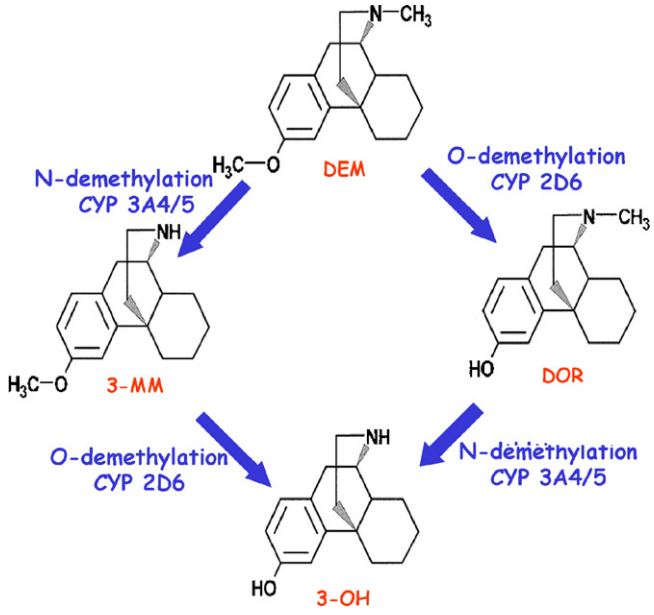

Fig. 1. Dextromethorphan demethylation pathways catalyzed by CYP2D6 and CYP3A4/5.

and optimization of analytical processes [11]. In fact, in contrast to the most popular strategy based on the one-variable-at-a-time approach, the use of an experimental design methodology is extremely efficient in locating the true optimum when interaction effects between the experimental factors are present. Therefore, an experimental design was applied to study the effect of the selected experimental parameters as well as their interactions. Previous studies described the use of similar approaches to optimize chemical hydrolysis methods using mineral acids [12].

Several analytical techniques including gas chromatography (GC) [13], capillary electrophoresis (CE) [14] and high-performance liquid chromatography (HPLC) with fluorescence [15] or mass spectrometry detection [16] were described for DEM and its metabolites analysis in biological fluids. Nevertheless, gas chromatography requires a tedious derivatization step for a polar metabolites determination and the principal drawback of CE-UV method consists of its poor sensitivity due to low loading capacity and short optical pathlength because of the small capillary dimensions. Moreover, since DEM and its metabolites are predominantly eliminated in urine, very high concentrations are recovered in this matrix. Hence, very sensitive and cost-effective methods such as LC–MS–MS are not needed and thanks to the fluorescent functional groups in the investigated molecules, fluorescence detection is sensitive enough to allow DEM and its metabolites quantification with high precision and accuracy. However, in the case of plasma or saliva samples, more sensitive methods such as LC–MS–MS are mandatory since concentrations are in pg to ng level. Recently, several papers devoted to the determination of DEM and metabolites in plasma [17,18] or saliva [19] using this technology were published.

The present paper reports on the use of experimental design approach to optimize the chemical hydrolysis of conjugated DOR. As a part of validation, results were compared to those obtained by the well-established enzymatic method using β-glucuronidase. Once the optimized conditions determined, the performances of the overall analytical method for the simultaneous assay of DEM and its metabolite DOR in urine samples were evaluated in terms of linearity, sensitivity, precision and accuracy. Finally, the validated method was applied to determine CYP2D6 phenotypes in 169 subjects constituted of fibromyalgia and control patients in a clinical trial.

2. Experimental

2.1. Chemicals and reagents

DEM hydrobromide, DOR *d*-tatrate, 3-MM hydrochloride, 3-OH hydrobromide and levallorphan tartrate (IS) were kindly provided by Hoffmann-La Roche (Basel, Switzerland). The β-glucuronidase (Helix pomatia, type H-1) was purchased from Sigma–Aldrich (Buchs, Switzerland). All solvents and chemicals for mobile phase preparation, standards and chemical hydrolysis were analytical or HPLC grade and purchased from Sigma–Aldrich (Buchs, Switzerland). To prepare buffers and solutions, ultra-pure water was supplied by a Milli-Q purification unit from Millipore (Bedford, MA, USA).

2.2. Instrumentation

The chromatographic equipment consisted of an Agilent 1100 Series LC system (Agilent, Paolo Alto, USA) with a quaternary pump, a vacuum degaser, an autosampler, a thermostated column compartment and a fluorescence detector. An Agilent Chemstation software package was used for instrument control, data acquisition and data handling. DEM and DOR separation was carried out on a phenyl column (150 mm × 4.6 mm i.d., particle size 5 μm) from Macherey-Nagel (Oensingen, Switzerland) coupled with a guard column with the same stationary phase. Mobile phase was prepared by mixing acetonitrile with a buffer solution consisting of a 50 mM orthophosphoric acid adjusted to pH 3.0 with sodium hydroxide 4N, in a volume ratio 18:82. The mobile phase was delivered at 0.8 ml/min flow rate and the column temperature was set at 25 °C. Fluorescence was measured with excitation and emission wavelengths set at 280 and 310 nm, respectively.

2.3. Standard solutions

Initial stock solutions of DEM (2.7 mmol/l), DOR (2.35 mmol/l) and levallorphan (2.3 mmol/l) were prepared by dissolving 10 mg of each compound in 10 ml of methanol. Intermediate standard solution containing a mixture of DOR at 600 μmol/l and DEM at 60 μmol/l and one intermediate levallorphan standard solution at 23 μmol/l were prepared, in duplicate, from the primary stock solutions. Stock and intermediate solutions were kept frozen at −20 °C and remained stable for at least 12 months.

Working standard solutions (1, 3, 10, 20 and 60 μmol/l for DEM and 10, 30, 100, 200 and 600 μmol/l for DOR) were prepared in duplicate by appropriate dilution of the intermediate solution.

2.4. Standards and quality controls (QC) preparation

Standards and QC samples were prepared by spiking 200 μl of blank urine with 50 μl of each working standard solution of a mixture of DEM and DOR and 50 μl of IS solution in order to obtain calibration standard samples of (0.1, 0.3, 1, 2 and 6 μmol/l for DEM and 1, 3, 10, 20 and 60 μmol/l for DOR) and QC of (0.2, 1.5 and 3 μmol/l for DEM and 2, 15 and 30 μmol/l for DOR).

2.5. Urine samples extraction

To 250 μl of urine, 85 μl of HCl 10N were added and the vials were screw capped. Chemical hydrolysis was performed at different temperatures and times (see experimental model for exact values). Once the hydrolysis is finished, 50 μl of IS (levallorphan: 23 μmol/l) were added and After being alkalinized by adding 90 μl KOH 10N and 500 μl Na_2CO_3 1 M, samples were extracted with 5 ml of hexane-ethyl acetate (50–50) for 15 min. The extracted solution was subjected to centrifugation at 5000 × *g* and the upper organic phase was transferred to a second tube and re-extracted for 15 min with 0.5 ml of orthophosphoric acid (50 mM). The upper organic layer was discarded while the aqueous phase was kept in the dark for 20 min to ensure complete elimination of the organic solvents. Fifty microliters were injected automatically onto the HPLC system.

2.6. Enzymatic hydrolysis

Enzymatic hydrolysis was performed as previously reported [20]. Briefly, 250 μl of urine was hydrolyzed overnight at 37 °C with β-glucuronidase (2000 units) in 1 ml of 0.1 M sodium acetate buffer (pH 5.0). The extraction procedure was as described in Section 2.5.

2.7. Computation

Experimental design and data processing were generated with Modde 7.0 (Umetrics AB, Umea, Sweden). Coefficients for the regression model and optimized hydrolysis conditions were calculated with Modde software. Response surfaces were drawn using the same software.

2.8. Method validation

The strategy applied for the validation of DEM and DOR in urine samples was based on the approach proposed by the "Société française des Sciences et Techniques Pharmaceutiques" (SFSTP) [21]. This procedure allows to confirm the linearity over the tested concentration range and to assess the selectivity, precision and accuracy of the bioanalytical method. These different criteria were validated using two kinds of samples prepared in an independent way, namely calibration and validation standards. The DEM and DOR calibration standards are samples prepared by adding specified aliquots of the intermediate solutions. These samples ($n = 5$) are only used for calibration. The validation standards, corresponding to QC samples used in the routine analysis, are reconstituted samples with the urine matrix containing known concentrations of the analytes of interest, which are considered as true values by consensus. In the present study, the validation standards were prepared at three concentration levels. The validation range was selected on the basis of preliminary experiments and covered the range of DEM and DOR concentrations expected in patients. These concentrations were 0.1–6 μmol/l for DEM and 1–60 μmol/l for DOR. Moreover, to better take into account the between-day variability, a relatively high number of days were considered for method validation (5 days) as previously suggested by Hartmann et al. [22].

2.8.1. Selectivity

The selectivity was studied by analysing six sources of urine and several drugs structurally related to dextromethorphan (tramadol, buprenorphine, venlafaxine, morphine, etc.) and the two other metabolites of dextromethorphan (3-methoxymorphinan and 3-hyroxymorphinan).

2.8.2. Response function/linearity

The response function of an analytical procedure is, within the range selected, the existing relationship between the response (signal) and the concentration (quantity) of the analyte in the sample. The linearity criteria must only be applied to the results [calculated concentration = *f*(introduced concentration)]. Thus, a regression line was fitted on the back-calculated concentrations by applying the selected regression model. As described in Section 2.4, five concentration levels were analysed in duplicate.

2.8.3. Accuracy

Accuracy was expressed as percentage recovery of the target value and assessed by means of validation standards in urine at three independent concentration levels (0.2, 1.5 and 3 μmol/l for DEM and 2, 15 and 30 μmol/l for DOR).

2.8.4. Precision

In the present study, precision was estimated by measuring repeatability and intermediate precision at the same concentration levels mentioned in Section 2.8.3. Variance of repeatability and intermediate precision were computed from estimated concentrations and precision was expressed by relative standard deviation (RSD) at each concentration level.

2.9. Assay application

As a part of a clinical trial, approved by the Ethic Committee of Geneva University Hospitals, the method developed was applied to assess CYP2D6 activity in 169 subjects constituted of fibromyalgia patients and controls. According to a standardized protocol, a single 25 mg oral dose of dextromethorphan HBr was administrated to the patients and phenotypes were evaluated using metabolic ratio between deconjugated DEM/DOR in urine (overnight collection).

Table 1
Experimental design: coded and real values of three variables for face-centred design model as well as DOR and DEM responses

Trial	Hydrolysis temperature (X_1)		Hydrolysis time (X_2)		Acid concentration (X_3)		Responses	
	Coded value	Real value (°C)	Coded value	Real value (min)	Coded value	Real value (μl of HCl 10 M)	DOR/IS	DEM/IS
1	−1	60	−1	60	−1	50	1.276	1.179
2	1	140	−1	60	−1	50	9.937	0.961
3	−1	60	1	120	−1	50	1.517	1.093
4	1	140	1	120	−1	50	11.430	1.138
5	−1	60	−1	60	1	250	2.128	1.227
6	1	140	−1	60	1	250	12.436	0.373
7	−1	60	1	120	1	250	2.141	1.026
8	1	140	1	120	1	250	12.677	0.122
9	−1	60	0	90	0	150	1.602	1.070
10	1	140	0	90	0	150	12.650	0.585
11	0	100	−1	60	0	150	10.221	1.223
12	0	100	1	120	0	150	9.863	1.203
13	0	100	0	90	−1	50	9.465	1.184
14	0	100	0	90	1	250	10.071	1.232
15	0	100	0	90	0	150	10.266	1.132
16	0	100	0	90	0	150	10.347	1.240
17	0	100	0	90	0	150	9.742	1.175
18	0	100	0	90	0	150	9.742	1.204
19	0	100	0	90	0	150	10.401	1.276

3. Results and discussion

3.1. Optimization of the chemical hydrolysis conditions

For chemical hydrolysis optimization, a central composite face-centred design (CCF) was selected. This type of experimental design allows the response surface to be built and the localization of factor settings or operating conditions that maximize the dextromethorphan and particularly the dextrorphan responses.

The CCF consisted of a two level full factorial 2^3 design (eight hypercube points) that has been augmented with six extra star points (centred on the faces of the cube) and five central points in the cube. The replicates in the centre of the square are used to estimate the variability of the experimental measurements. Modde software was used to design the experiments and to model and analyse the results. All the 19 ($2^3 + 2 \times 3 + 5$) set point combinations that were performed for the CCF design are described in Table 1 together with the obtained values of the experimental responses studied in each experiment. The runs were randomized to provide protection against the effect of hidden variables that could have introduced bias into the measurements. Each factor was evaluated at three levels. The minimum, central and maximum values used in the CCF design were: hydrolysis temperature (60–100–140 °C), hydrolysis time (60–90–120 min) and acid concentration, expressed as the volume of HCl 10 M (50–150–250 μl). Urine sample used for the hydrolysis optimization was selected from the group of patients described in Section 4.2. Before liquid-liquid extraction, levallorphan (IS) was added to the hydrolysed urines to reduce the variability in the responses caused by the extraction step. Hence, DEM and DOR responses are expressed as DEM/IS and DOR/IS.

The CCF design provides data to fit a second-degree expression as given below:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{1}^{3} b_{ii} X_i^2 + \sum_{\substack{i,\ j \\ i<j}}^{3} b_{ij} X_i X_j$$

where Y is the experimental response, X_i the studied factors, b_0 the intercept term, b_i the main effect coefficients for each variable, b_{ii} the quadratic terms coefficients responsible for the curvature effects and $b_{ij(i\neq j)}$ are the interaction effects.

The estimates of the coefficients for the second-order models of each response were calculated (Table 2) by least squares linear regression and these models were statistically analysed and validated by analysis of variance (ANOVA). In particular, the coefficient of determination (R^2), which represents the percent of the variation explained by the model and the adjusted coefficient of determination (R^2_{adj}) which is related to the number of parametric coefficients in the model, were calculated. Table 3

Table 2
Regression coefficients estimated for each response

Coefficient	DOR/IS	DEM/IS
b_0	10.11	1.20
b_1	5.05	−0.24
b_2	0.16	−0.04
b_3	0.58	−0.16
b_{11}	−2.99	−0.36
b_{22}	−0.07	0.03
b_{33}	−0.35	0.02
b_{12}	0.19	0.03
b_{13}	0.28	−0.20
b_{23}	−0.19	−0.07

Table 3
Statistical data obtained for the selected model

Response	R^2	R^2_{adj}	n	ν
DOR	0.995	0.99	19	9
DEM	0.94	0.88	19	9

R^2 = regression coefficient; R^2_{adj} = adjusted regression coefficient; n = number of experiments; ν = degree of freedom.

shows that R^2 and R^2_{adj} were higher than 0.94 and 0.88, respectively, indicating the good fitting of these models. Therefore, the models were accepted and were used to predict any response within the experimental domain.

For each response, the effects estimates of each variable and all their possible linear and quadratic interactions on the response variables with their confidence intervals are reported in Fig. 2. According to the ANOVA, the hydrolysis temperature has the most prominent effect on the response variables. In fact, when the hydrolysis temperature was varied from its low to its high level, an increase was observed for DOR response and a decrease in DEM response. No important interactions were found in the case of DOR response and only the temperature quadratic effect was found to be significant. Moreover, the acid concentration had a negative effect on the DEM response while the hydrolysis time was not significant within the experimental domain. This behaviour means that a high hydrolysis temperature is beneficial for the chemical hydrolysis of conjugated DOR but a careful attention should be carried out to avoid possible degradation of DEM. For DEM response, the interaction of hydrolysis temperature and acid concentration was also significant meaning that both factors had to be studied together.

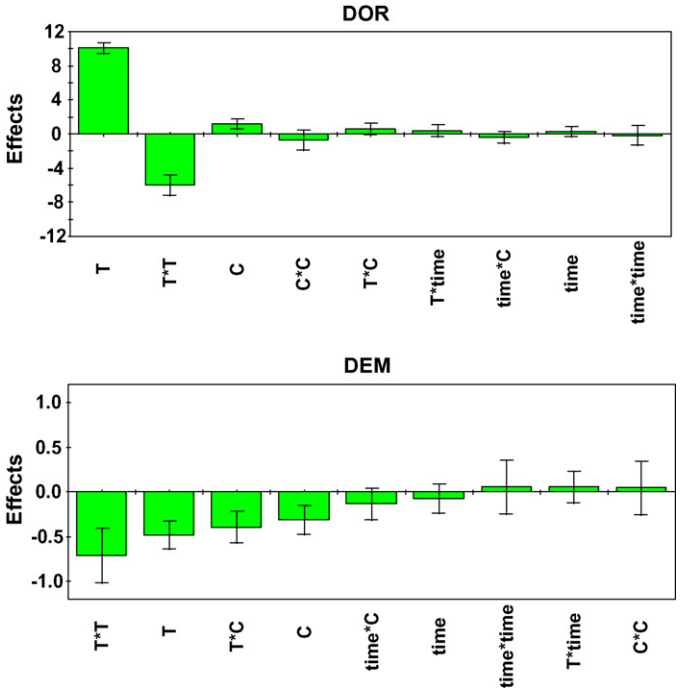

Fig. 2. Scaled and centred coefficients with confidence intervals for DEM and DOR responses.

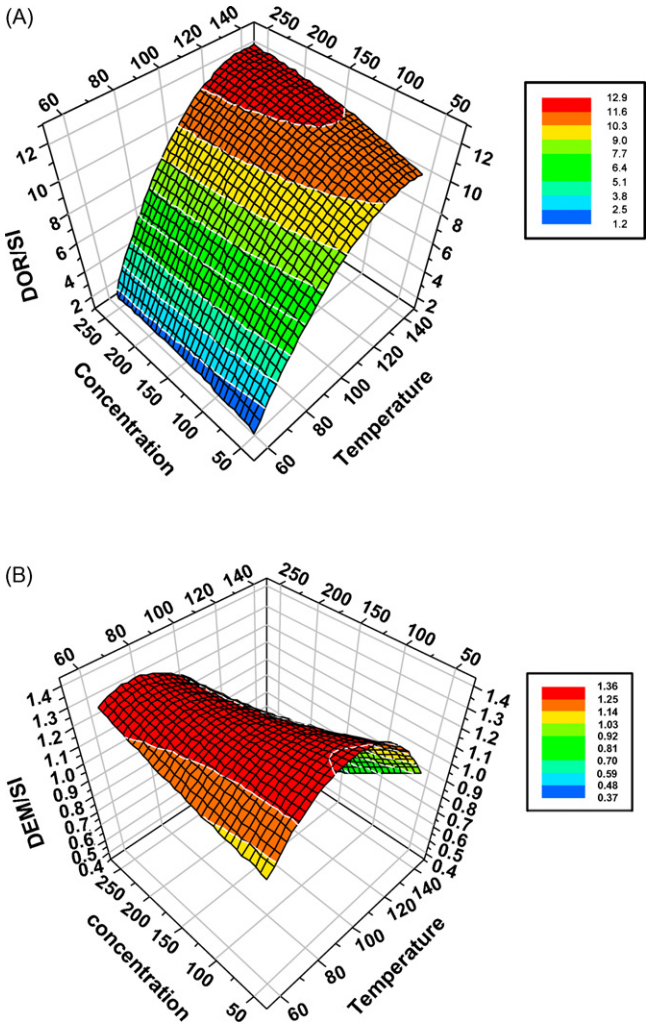

Fig. 3. Response surface plots for the DOR (A) and DEM (B) as a function of hydrolysis temperature and acid concentration, hydrolysis time being fixed at its low value.

Based on the regression models obtained, response surfaces can be illustrated as three-dimensional plots by presenting the DEM and DOR responses in function of two factors while keeping the other factors constant (Fig. 3). According to the ANOVA performed, the hydrolysis temperature and hydrochloric acid concentration have a significant effect on both responses. Hence, these were the variables chosen to plot the response surfaces while maintaining the hydrolysis time at its low value in order to speed up the chemical hydrolysis and to avoid any possible dextromethorphan degradation. As previously seen, the DOR response was mainly affected by temperature variation while the HCl concentration increase did not significantly modify the DOR response. In the case of DEM, at a low acid concentration, intermediate temperature conditions are favourable while at a high acid concentration higher hydrolysis temperatures have to be avoided.

The quadratic regressions allowed determining the optimal conditions by maximizing both DOR and DEM responses. The hydrolysis time was set at its low value in order to perform the chemical hydrolysis in a reasonable time. As a result, the chem-

ical hydrolysis conditions chosen were: 85 °C as a hydrolysis temperature, 110 μl of HCl 10N and 60 min for the hydrolysis time.

3.2. Extraction and chromatography

Extraction of DEM and DOR is challenging due to their opposite physico-chemical properties. In fact, DOR (log $P = 3.7$) [23] is more polar than DEM (log $P = 4.1$) [23]. A mixture of hexane-ethyl acetate (50:50, v/v) was previously described to permit a good recovery of DEM without loss of DOR [20]. Unfortunately, using this simple extraction procedure, interfering substances were observed. Hence, a double extraction was tested by back-extracting the solvent in phosphoric acid 50 mM. As a result, interfering peaks were avoided and recoveries were higher than 80% (see Section 3.3.5). In order to improve throughput and automation of the extraction procedure, new developments are ongoing in our laboratory to use 96-well plates.

Different columns were reported for the separation of DEM and metabolites (i.e., Cyano, C-18, C-8, phenyl) [20,24–27]. However, phenyl column was by far the most cited to provide optimal resolution between DEM and its three metabolites. Different conditions were tested (solvent nature and percentage, buffer pH and molar concentration) and the optimal conditions consisted of a mixture of acetonitrile and orthophosphoric acid 50 mM (18/82) adjusted to pH 3.0 with sodium hydroxide 4N. Under these conditions, baseline separation of the investigated compounds was achieved in less than 14 min. The retention times for DOR, levallorphan (IS) and DEM were 4.2, 7.2 and 11.8 min, respectively (Fig. 4).

3.3. Method validation

3.3.1. Selectivity

Six blank urines from different sources were analyzed and no interference was observed. Furthermore, several drugs structurally related to dextromethorphan (tramadol, buprenorphine, venlafaxine, morphine, etc.) and the two other metabolites of dextromethorphan (3-methoxymorphinan and 3-hyroxymorphinan) were tested and no interference was detected. As a consequence, the absence of interfering endogenous components at the retention time of the compounds of interest clearly demonstrates the good selectivity of the method.

3.3.2. Calibration and linearity

The linearity of the method was carried out on fresh series of standards over the different days of validation to assess intra and inter-day performances. Each day, five calibration standards ($n = 5$), ranging from 0.1 to 6 μmol/l for DEM and from 1 to 60 μmol/l for DOR, were prepared as detailed in Section 2.4 and analysed by LC-Fluo. On the basis of the calibration standard data, different commonly used response functions were considered to express the Fluorescence signal (peak area) and the analyte of interest concentration. As a result, a weighting factor of $1/X$ (where X represents the introduced concentration) was chosen taking into account the relationship between natural variance logarithms and concentration as described elsewhere

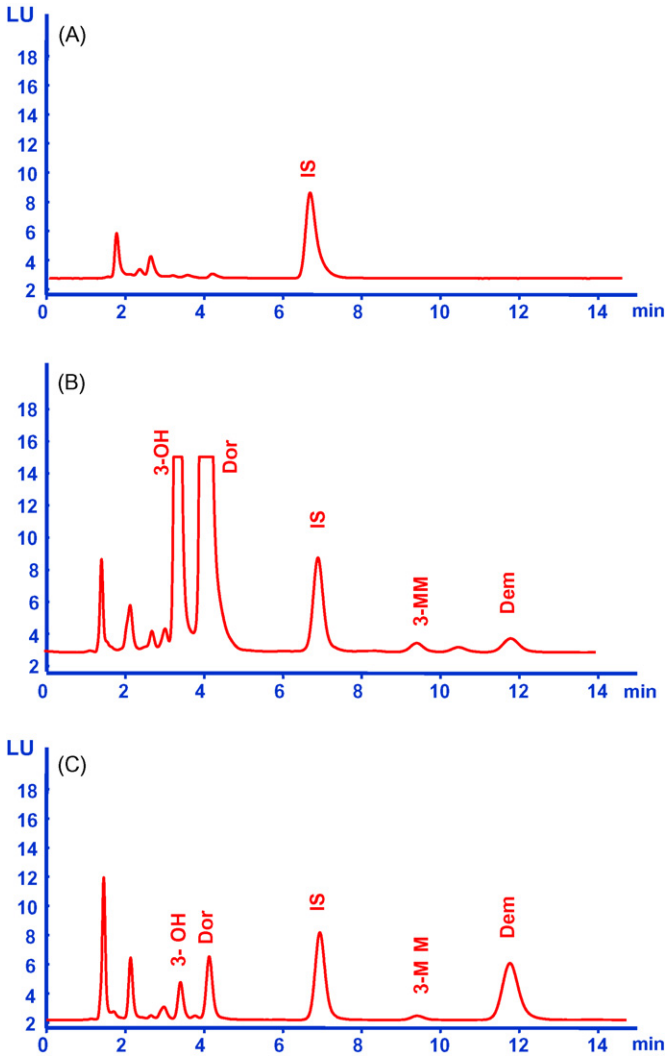

Fig. 4. Typical chromatogram of blank plasma (A), an extensive metabolizer (B) and a poor metabolizer (C). Chromatographic conditions are described in Section 2.2.

[28]. The regression analysis over the validation days ($k = 5$) showed that the determination coefficients (R^2) were always higher than 0.99 and for each point on the calibration curve, the concentration back-calculated from the regression equation was constantly below 15% of the nominal value (data not shown). Finally, to demonstrate the good method linearity, the recovered concentrations were reported versus the introduced ones showing very satisfactory correlation as witnessed by the good correlation coefficients (data not shown).

3.3.3. Accuracy and precision

For validation samples, three independent concentrations (0.2, 1.5 and 3 μmol/l for DEM and 2.0, 15 and 30 for DOR) were analysed. Precision and accuracy results are presented in Table 4 and demonstrated the method effectiveness for quantitative determination of DEM and DOR in urine samples. Accuracy, expressed as percentage recovery of the target value, exhibited excellent results with bias inferior to 10% throughout the tested range.

Table 4
Accuracy, repeatability and intermediate precision for validation samples ($k = 5$ days, $n = 2$)

Concentration (μmol/l)	Accuracy (%)	Repeatability CVR (%)	Intermediate precision CVR (%)
DEM			
0.2	108.9	4.9	5.7
1.5	92.7	5.1	5.1
3	99.3	9.6	9.6
DOR			
2	100.5	11.3	11.3
15	94.4	9.0	11.0
30	99.0	9.8	9.8

The bioanalytical method precision was determined by calculating the relative standard deviation (RSD) values of repeatability and intermediate precision at three concentration levels (Table 4). As recommended by regulatory guidelines [29], in all cases, method precision was inferior to 15%.

3.3.4. Sensitivity

The LOQ and LOD were estimated with a signal-to-noise ratio (S/N) of 10 and 3, respectively. For both DEM and DOR, LOQ was 0.05 μmol/l and LOD was 0.02 μmol/l.

3.3.5. Extraction efficiency

The extraction efficiency was determined by comparing the peak area from the extracted samples versus aqueous standard solutions at the same concentration. The extraction efficiency was determined using 6 replicates of each QC sample. The mean extraction efficiencies (SD) were 91.6% (5.0), 82.6% (8.4) and 88.1 (6.1) for dextromethorphan, dextrorphan and levallorphan (IS), respectively. Thus, high extraction efficiency was obtained for the investigated compounds.

3.3.6. Stability

Stability was tested for low, medium and high QCs urine samples stored at −20 °C for 1 month, at ambient temperature for 24 h and during three freeze-thaw cycles. In all cases, mean calculated concentrations were within 15% of the nominal value (data not shown). Consequently, no significant degradation was observed. Moreover, comparison was made between hydrolyzed and non-hydrolyzed QC samples in order to test the stability of DEM, DOR and IS under the optimized chemical hydrolysis conditions. Calculated concentrations were comprised between 90 and 110% indicating a good stability of the studied substances under the selected conditions.

4. Application

4.1. Chemical and enzymatic hydrolysis methods

In order to demonstrate the reliability of the described method for the evaluation of CYP2D6 activity in humans, results obtained with the chemical hydrolysis method were compared to those achieved by the widely used enzymatic method using β-glucuronidase in 20 urine samples from patients who received 25 mg of DEM. As shown in Fig. 5, good correlation was observed ($r^2 = 0.98$ for all subjects and $r^2 = 0.76$ when only EM were selected) indicating that both methods can be used for DEM and DOR hydrolysis in urine. However, the chemical hydrolysis method developed is simple, rapid and cost-effective.

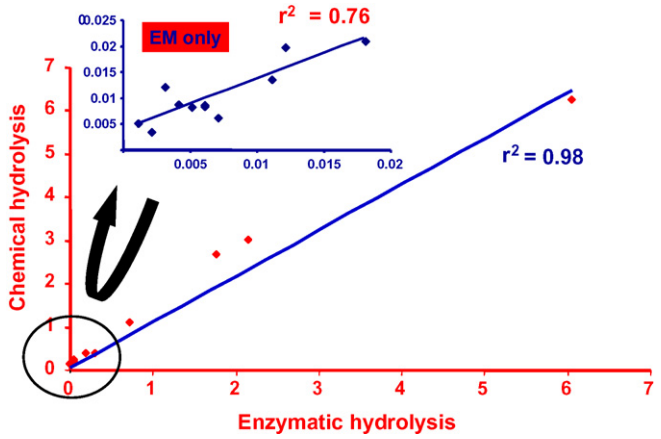

Fig. 5. Correlation between chemical and enzymatic hydrolysis methods.

4.2. Clinical application

As a part of a clinical trial, where the investigators studied the impact of CYP2D6 polymorphism on pain intensity in fibromyalgia patients, 169 subjects where phenotyped for CYP2D6 as described in Section 2.9. As shown in Fig. 6, the distribution of the measured CYP2D6 phenotypes exhibits a high variability (MR varies by 4–5 orders of magnitude) since CYP2D6 is well known as a highly polymorphic enzyme. The antimode (separation between extensive and intermediate metabolizers) was fixed at 0.3 based on published literature [6,20]. As previously reported for CYP2D6 activity assessment using debrisoquine, a logarithm scale can be used to classify the different CYP2D6 metabolizers [30–32]. Accordingly, UM's have a DEM/DOR ratio below 0.003, EM's have a ratio between 0.003 and 0.03, IM have a ratio between 0.03 and 0.3 while PM have a ratio above 0.3. Moreover, CYP2D6 genotype was systematically performed in all individuals using AmpliChip™

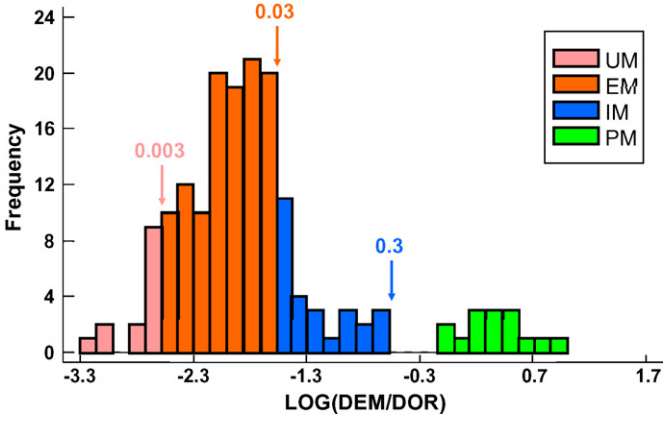

Fig. 6. Frequency distribution of the CYP2D6 metabolic ratio in fibromyalgia and control volunteers ($n = 169$).

(Roche) and correlation between predicted and measured phenotypes will be published elsewhere.

5. Conclusion

A cost-effective, reliable and rapid chemical hydrolysis method was developed and optimized using a chemometric approach. Three relevant factors were selected and simultaneously varied to study their effect on conjugated DOR hydrolysis. After the optimization step, optimal conditions allowing efficient DOR hydrolysis without sacrificing the DEM content were found at 60 min for the hydrolysis time, 110 °C for the hydrolysis temperature and 85 μl for the acid volume. This method was validated by comparison with the well-established enzymatic method showing a very good correlation. Liquid chromatography coupled to a fluorescence detector was sensitive enough for DEM, DOR determination in 250 μl of urine when 25 mg of dextromethorphan were administrated to the patients. Good recoveries and high selectivity were obtained with liquid–liquid extraction using hexane-ethyl acetate (50/50, v/v) followed by back-extraction of the solvent with phosphoric acid 50 mM. The method was validated and showed good performances in terms of selectivity, linearity, sensitivity, precision and accuracy over the concentration range examined. Finally, this method was successfully applied to several clinical studies when dextromethorphan was used as a probe for CYP2D6 activity assessment.

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