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# A simplified analytical method for a phenotyping cocktail of major CYP450 biotransformation routes

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**Abstract**

An efficient, fast and reliable analytical method was developed for the simultaneous evaluation of the activities of five major human drug metabolising cytochrome P450 (1A2, 2C9, 2C19, 2D6 and 3A4) with a cocktail approach including five probe substances, namely caffeine, flurbiprofen, omeprazole, dextromethorphan and midazolam. All substances were administered simultaneously and a single plasma sample was obtained 2 h after the administration. Plasma samples were handled by liquid-liquid extraction and analysed by gradient high performance liquid chromatography (HPLC) coupled to UV and fluorescence detectors. The chromatographic separation was achieved using a Discovery semi-micro HS C18 HPLC column (5 μm particle size, 150 mm × 2.1 mm i.d.) protected by a guard column (5 μm particle size, 20 mm × 2.1 mm i.d.) The mobile phase was constituted of a methanol, acetonitrile and 20 mM ammonium acetate (pH 4.5) with 0.1% triethylamine mixture and was delivered at a flow rate of 0.3 mL $min^{-1}$. All substances were separated simultaneously in a single run lasting less than 22 min. The HPLC method was formally validated and showed good performances in terms of linearity, sensitivity, precision and accuracy. Finally, the method was found suitable for the screening of these compounds in plasma samples.


*Keywords:* Cytochrome P450; Cocktail; HPLC; Validation

## 1. Introduction

The cytochrome P450 (CYP) represents the major drug metabolising enzyme system in humans accounting for the metabolism of most medications. Genetics, dietary components, other environmental factors and drugs influence CYP activity resulting in inter-individual variability of plasmatic drug concentrations. The knowledge of the phenotypic profiles of the major CYP at the individual level will help to tailor efficient and safe drug prescriptions. Therefore, phenotyping cocktail can be useful since it allows a simultaneous determination of several CYP activities using a mixture of probes in a single test, with minimised intra-subject variability over time. However, this strategy has two major difficulties: the choice

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of the probes to avoid the occurrence of metabolic interactions when they are administered simultaneously and the availability of an appropriate analytical method. The aim of this study was to develop a phenotyping cocktail and a specific analytical method for the simultaneous evaluation of the in vivo activities of the most clinically relevant CYP for drug biotransformation (1A2, 2C9, 2C19, 2D6 and 3A4). The probe drugs selected for the present study were caffeine, flurbiprofen, omeprazole, dextromethorphan and midazolam. For each drug, a selective metabolite is generated by each cytochrome. The chemical structures of the drugs as well as their metabolites are illustrated in Fig. 1. Although previous cocktails have used combinations of several drugs [1], to the best of our knowledge, this is the first work combining these five

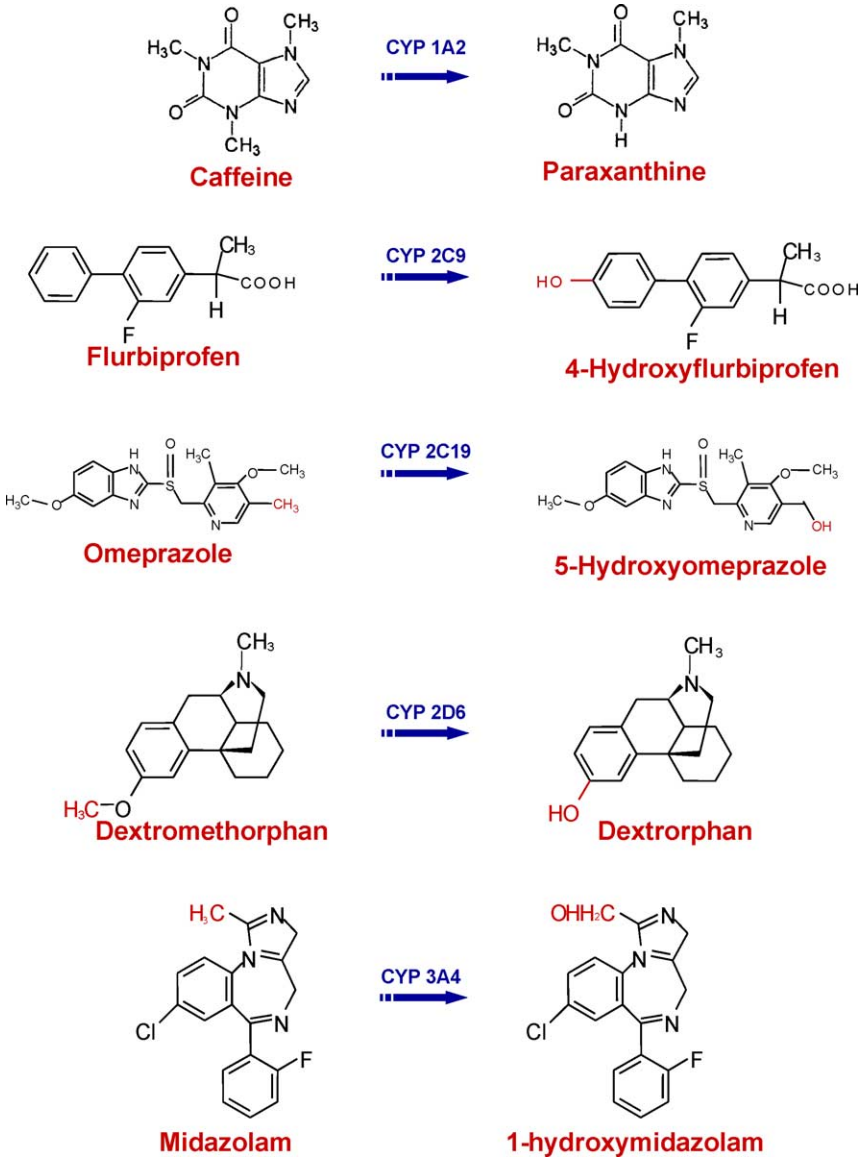


Fig. 1. Probe drugs and their selective metabolites.

probe drugs for these five CYPs. In the previous cocktails, a specific analytical method was applied for each probe test, thus making analyses long and tedious.

Additionally, other authors have described the usefulness of LC coupled to mass spectrometry (LC–MS, LC–MS-MS) to enhance selectivity and sensitivity of such complex separation [2–4]. The analytical method presented has been optimised to permit the separation of all substances in a single run. The chosen substances have been suggested as superior (in terms of safety, availability and specificity of enzyme metabolism) to the probes included in previous studies of phenotyping cocktails [5]. In vitro studies have showed which human CYP enzymes were involved in each drug's metabolism and in the generation of specific metabolites [6–11]. Then, in vivo, all drugs have been evaluated for use individually as enzyme-specific probes in phenotyping studies [12–17].

The present study focuses on the development and the optimisation of a rapid, selective and sensitive HPLC method allowing simultaneous analysis of five probes and their respective metabolites in a single run. This method was evaluated in terms of recovery, linearity, precision and accuracy, and applied to the analyse of plasma samples.

## 2. Experimental

### 2.1. Chemicals and reagents

Caffeine (caf), paraxanthine (para) were supplied by Fluka (Buchs, Switzerland). Flurbiprofen (flu), omeprazole (opz), naproxen, phenacetin and chloramphenicol were obtained from Sigma (Buchs, Switzerland). Dextromethorphan (dem), dextrorphan (dor), levallorphan, midazolam (mida), 1-hydroxymidazolam (1OH-mida) and diazepam were kindly donated by Hoffman-La-Roche (Basel, Switzerland). 4-Hydroxyflurbiprofen (4OH-flu) and 5-hydroxyomeprazole (5OH-opz) were gifts of Prof TS Tracy (School of Pharmacy, West Virginia University, Morgantown) and Astra Zeneca, (Mölndal, Sweden), respectively. Blank human plasma were obtained from the blood transfusion facilities (University Hospitals, Geneva, Switzerland).

HPLC-grade methanol and acetonitrile were supplied by Merck (Darmstadt, Germany). All other reagents (potassium phosphate, sodium carbonate, ammonium acetate, ammonium sulphate) and solvents were analytical grade reagents from Fluka (Buchs, Switzerland) and were used without any further purification. Ultra-pure water was supplied by a Milli-Q RG purification unit from Millipore (Bedford, MA, USA).

### 2.2. Instrumentation

LC data was generated in an Agilent 1100 Series LC system (Agilent, Palo Alto, USA) constituted of a quaternary pump, a vacuum degaser, an automatic injector, an autosampler, a thermostated column compartment, a diode-array and fluorescence detectors. An Agilent Chemstation software package was used for instrument control, data acquisition and data handling. LC analyses were carried out on a Discovery HS C18 column (150 mm × 2.1 mm i.d., particle size 5 μm) from Supelco preceded by a guard column with the same stationary phase (5 μm particle size, 20 mm × 2.1 mm i.d). The flow rate was set at 0,3 mL $min^{-1}$. Unless otherwise stated, the mobile phase consisted of a mixture of methanol, acetonitrile and 20 mM ammonium acetate (pH 4.5) in the presence of 0.1% triethylamine. Solvents and gradients are reported in Table 1. The column effluent was monitored either by UV or fluorescence detection. UV detection wavelengths were 280 for caffeine and paraxanthine, 302 for omeprazole and 5-hydroxyomeprazole and 254 nm for midazolam and 1-hydroxymidazolam. Emission and excitation wavelengths of the fluorescence detector were set at 271 and 315 nm (0–35 min) for dextrorphan and dextromethorphan, 271 and 353 nm (0–20 min) for OH-flurbiprofen and 260 and 320 nm

Table 1
Chromatographic conditions

| Time (min) | % Methanol | % Acetonitrile | % Ammonium acetate 20 mM pH 4.5 with 0.1% triethylamine |
|---|---|---|---|
| 0–2 | 15 | 0 | 85 |
| 2–12 | 10 | 40 | 50 |
| 2–18 | 10 | 40 | 50 |
| 18–19 | 10 | 70 | 20 |
| 19–25 | 10 | 70 | 20 |
| 25–25.1 | 15 | 0 | 85 |
| 25.1–35 | 15 | 0 | 85 |

(20–35 min) for flurbiprofen. The fluorescence detector gain was fixed at 10. The injection volume and column temperature were set at 20 μL and 25 °C, respectively.

### 2.3. Standard and sample preparation

#### 2.3.1. Standard solutions

Initial stock solutions of caffeine, paraxanthine, phenacetin, dextromethorphan, dextrorphan, levallorphan, naproxen, midazolam, diazepam, omeprazole, 5OH-omeprazole and chloramphenicol were prepared by dissolving 1 mg in 1 mL methanol. Stock solutions of flurbiprofen, 4OH-flurbiprofen were prepared by dissolving 1 mg in 1 mL $H_2O$/ACN 50:50. Stock solution of 1OH-midazolam was prepared by dissolving 1 mg in 1 mL ethanol. Intermediate standard solutions (1 and 10 μg mL$^{-1}$) were prepared by dilution of stock solutions in $H_2O$ except for omeprazole and 5OH-omeprazole where a 50 mM phosphate buffer (pH 8.5) was used. These stock and intermediate solutions were kept frozen at −20 °C. The standard mixture was prepared by dissolution of individual compounds in water to give required final concentrations.

#### 2.3.2. Plasma standard and quality control samples

Plasma standard solutions were prepared at the desired concentration by dilution of the intermediate standard solutions (10 or 1 μg mL$^{-1}$) with drug free human plasma. The plasma concentrations for dextromethorphan, midazolam and their respective metabolites were 1.25, 2.5, 6.25, 12.5, 25, 50 and 125 ng mL$^{-1}$. For omeprazole and 5OH-omeprazole, the plasma concentrations were 5, 10, 25, 50, 100, 200 and 500 ng mL$^{-1}$. For caffeine and paraxanthine, the plasma concentrations were 10, 20, 50, 100, 200, 400 and 1000 ng mL$^{-1}$, and for flurbiprofen and its metabolite, the concentrations were 170, 340, 850, 1700, 3400, 6800 and 17,000 ng mL$^{-1}$.

Quality control samples were prepared by the same manner. The plasma concentrations were 2.5, 12.5 and 50 ng mL$^{-1}$ for dextromethorphan, midazolam and their metabolites, 10, 50 and 200 ng mL$^{-1}$ for omeprazole and its metabolite, 20, 100 and 400 ng mL$^{-1}$ for caffeine and paraxanthine, and 340, 1700 and 6800 ng mL$^{-1}$ for flurbiprofen and 4OH-flurbiprofen.

After the extraction and evaporation procedures, residues were reconstituted in the appropriate volume to obtain the same final concentration for all substances (50, 100, 250, 500, 1000, 2000 and 5000 ng mL$^{-1}$) for plasma standards and (100, 500 and 2000 ng mL$^{-1}$) for quality controls.

### 2.4. Extraction procedures

Depending on the physico-chemical properties of the selected drugs, different sample preparations were applied. In particular, to improve the extraction recovery for basic drugs, two different extraction procedures were used on the one hand for omeprazole and its metabolite, and on the other hand for midazolam, dextromethorphan and their respective metabolites. Each extract was injected separately but using the same analytical method.

#### 2.4.1. Neutrals: caffeine and paraxanthine

Plasma (0.5 mL) containing caffeine and paraxanthine was extracted with 5 mL ethylacetate after the addition of 20 μL internal standard (phenacetin, 10 μg mL$^{-1}$) and 120 mg ammonium sulfate in a PE tube. After mixing for 15 min and centrifuging at 3500 rpm for 10 min, the organic phase was removed and evaporated under a gentle stream of nitrogen at 40 °C. The residue obtained was dissolved in 100 μL of the initial mobile phase and 20 μL was injected in the HPLC system.

#### 2.4.2. Acids: flurbiprofen and 4OH-flurbiprofen

To 100 μL plasma sample containing flurbiprofen and its metabolite were added 200 μL acetonitrile, 10 μL IS (10 μg mL$^{-1}$ of naproxen) and 40 μL of half-strength $H_3PO_4$ ($H_3PO_4$/$H_2O$, 50:50). The sample was vortexed for 30 sec and centrifuged at 10,000 rpm for 10 min. Hundred μL were placed into HPLC vials and 20 μL were subjected to HPLC [11].

#### 2.4.3. Basics

*2.4.3.1. Omeprazole and 5OH-omeprazole.* Five hundreds μL of plasma were mixed with 20 μL of IS (10 μg mL$^{-1}$ of chloramphenicol) and 500 μL of 500 mM phosphate buffer pH 8. Then, 5 mL of dichloromethane with 1% butanol were added and the tubes were shaken for 15 min before centrifuging at 3500 rpm for 10 min. The organic layer was

transferred and evaporated to dryness at 40 °C under a gentle stream of nitrogen gas. The residue was reconstituted with 50 μL of 50 mM ammonium acetate pH 8/MeOH (85:15, v/v) and 20 μL were submitted to HPLC analysis.

*2.4.3.2. Midazolam, 1OH-midazolam, dextromethorphan and dextrorphan.* After the addition of 20 μL each of diazepam (10 μg mL$^{-1}$) and levallorphan (10 μg mL$^{-1}$) as internal standards and 500 μL of 500 mM $Na_2CO_3$, 2 mL of plasma were extracted twice in glass tubes with 5 mL of hexane: ethylacetate 80:20 with 1% butanol. After mixing for 15 min and centrifuging at 3500 rpm for 10 min, the organic phase was removed and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was dissolved in 50 μL of initial mobile phase, and 20 μL were injected onto the HPLC system.

### 2.5. Validation

The following parameters were determined to validate the analytical method: selectivity, linearity, range, precision, accuracy, limit of quantification (LOQ) and recovery. To each sample, the appropriate internal standard was systematically added as described in the experimental part. Calibration curves were constructed by considering the peak area ratios of each compound to the internal standard versus the nominal concentration in the sample. The equations were calculated by least square linear regression analysis for dextromethorphan, omeprazole and their metabolites. For midazolam, 1OH-midazolam and flurbiprofen, the regression parameters were calculated by a weighted ($1/x^2$) least-squares linear-regression analysis. For caffeine, paraxanthine and 4OH-flurbiprofen, the best results were obtained by a weighted ($1/x$) least-squares linear-regression analysis.

The limit of quantitation (LOQ) of the overall analytical process was determined as the lowest plasma concentration that can be measured routinely with acceptable precision and accuracy (10-fold signal-to-noise). LOQ was determined after extraction followed by injection of spiked plasma with substances in decreasing concentrations.

Method precision (repeatability and intermediate precision) was expressed as the relative standard deviation (R.S.D.) and was determined by replicate analysis of control samples containing the drugs and their respective metabolites at three different concentrations as described in Section 2.3. Successive injections of the same sample were tested the same day to determine the intra-day (repeatability) precision, and aliquots of the same sample were injected on three different days to determine inter-day or intermediate precision. Accuracy was expressed as percent recovery after analysing drug-spiked plasma and comparing to the added amount.

The extraction recoveries of the analytes from the plasma samples were evaluated using blank plasma spiked with compounds. The concentrations of the spiked samples were calculated from the calibration curves and compared with those obtained by direct injection of an aqueous solution of the drugs.

## 3. Results and discussion

### 3.1. Optimisation of chromatographic conditions

For phenotyping purposes with a cocktail, a rapid, sensitive, selective and cost-effective analytical method was required for the simultaneous separation of the selected drugs and their metabolites. In addition, the method should allow the separation of the examined drugs and the internal standards, crucial to perform good quantitative data. In our first investigations, a mobile phase consisting of acetonitrile and 20 mM ammonium acetate pH 6 mixture was selected and the organic solvent percentage was varied between 20 and 70%. Under these conditions, caffeine and its metabolites eluted in the injection peak. Therefore, acetonitrile was replaced by methanol in the first part of the elution gradient. With 15% methanol during two minutes, caffeine metabolites were eluted without any interference, and subsequently by increasing acetonitrile percentage, a good separation of the other substances was obtained. Furthermore, in order to reduce the analysis time and improve the peak symmetry, the ammonium acetate pH was varied between 4 and 6. Unfortunately, at pH 4.5, the DEM peak tailed significantly due to interactions with silanol groups present on the stationary phase packing and therefore the addition of triethylamine as a competing base at various percentages was investigated. Adding 0.1% TEA in the mobile phase allowed a significant

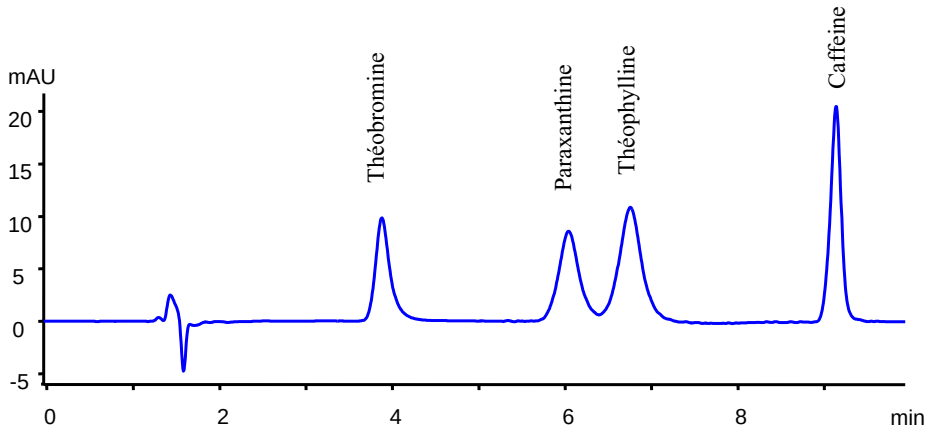


Fig. 2. Separation of caffeine metabolites.

improvement of DEM peak symmetry (tailing). Under these conditions (Table 1), a complete separation of the studied drugs, their metabolites as well as selected internal standards was achieved in less than 22 min. Furthermore, with these elution conditions, no interference was observed between caffeine and its three metabolites, namely theobromine, paraxanthine and theophylline likely to be present in plasma two hours after caffeine ingestion (Fig. 2).

### 3.2. Extraction procedures

Regarding the physico-chemical properties of all substances, three extraction procedures were used: firstly, a liquid–liquid extraction for the neutrals (caffeine and metabolites), secondly a protein precipitation for the acids (flurbiprofen and metabolite) and finally a liquid–liquid extraction for the bases (dextromethorphan, midazolam, omeprazole and their respective metabolites). For caffeine and paraxanthine extraction, a liquid–liquid extraction procedure was carried out. As described in the experimental part, ammonium sulfate was added to give a neutral pH, and ethylacetate was chosen because it is less toxic than chloroform, a solvent often used to extract caffeine and its metabolites [18–21]. As reported in Table 2, this method allowed an acceptable recovery for the two substances.

For flurbiprofen and its metabolite extraction, a sample preparation procedure based on protein precipitation was selected. Thus, plasma was treated with acidified (phosphoric acid) acetonitrile to precipitate proteins [22]. This procedure was found easy, rapid and efficient to achieve a high percentage recovery (Table 2). In fact, for these acidic compounds, recoveries higher than 75% were obtained.

For the basic substances, different solvents in various combinations were tested. Unfortunately, no combination allowed a good extraction in a single step and thus two extraction procedures were necessary. Dextromethorphan, midazolam and their respective metabolites were well extracted with a mixture of hexane-ethylacetate, while omeprazole was better extracted with dichloromethane. In order to improve recovery of OPZ and its metabolite, the effect of the extraction medium pH as well as the presence of butanol were investigated. A phosphate buffer 500 mM at pH 8 and the presence of 1% butanol in

Table 2
Percentage recovery of all drugs and their metabolites by final optimised extraction procedure

| | |
|---|---|
| Para (50 ng ml$^{-1}$) | 60 |
| 4OH-Flu (500 ng ml$^{-1}$) | 88 |
| 5OH-opz (25 ng ml$^{-1}$) | 70 |
| Dor (10 ng ml$^{-1}$) | 93 |
| 1OH-Mida (10 ng ml$^{-1}$) | 95 |
| Caf (50 ng ml$^{-1}$) | 60 |
| Flu (500 ng ml$^{-1}$) | 75 |
| Opz (200 ng ml$^{-1}$) | 88 |
| Dem (10 ng ml$^{-1}$) | 96 |
| Mida (40 ng ml$^{-1}$) | 81 |

Table 3
Linearity data for dem, opz and their metabolites ($y = ax + b$)

| Calibration | Dor | Dem | 5OH-opz | Opz |
|---|---|---|---|---|
| Intercept | 0.001 | −0.001 | 0.008 | 0.009 |
| Slope | 0.0002 | 0.0003 | 0.0007 | 0.0008 |
| Correlation coeff. | 0.997 | 0.998 | 0.999 | 0.999 |

dichloromethane permitted a good extraction of OPZ and its metabolite. For dextromethorphan, midazolam and their respective metabolites, higher pH (11) was selected and the effect of butanol percentage (0, 1 and 5%) was checked resulting in best recoveries of all analytes. Consequently, sodium carbonate 500 mM at pH 11 and a mixture of hexane:ethylacetate (80:20) with 1% butanol were selected to achieve an efficient extraction of these basic compounds with recoveries higher than 80%. It has to be noted that for further extraction recovery improvement, particularly in the case of midazolam, plasma samples were extracted twice.

### 3.3. Method evaluation

Method selectivity for measuring the investigated probes specifically without interferences was assessed throughout the injection of six blank plasma samples which have been previously subjected to the extraction procedures described in this study (data not shown).

Detector response linearity was performed by preparing seven duplicate calibration samples covering the range of expected concentrations. Calibration curves were obtained from spiked plasma samples as described previously. They were linear with equation $y = ax + b$ for dextromethorphan and omeprazole. A weighted least-squares linear regression analysis ($1/X$ and $1/X^2$) was used for the other probes as described previously. The linearity of the calibration graph was demonstrated by the good determination coefficient obtained ($r^2$) (Tables 3 and 4).

LOQ values were determined as 1.25 ng mL$^{-1}$ for dextromethorphan, midazolam and their metabolites, 5 ng mL$^{-1}$ for omeprazole and its metabolites, and 10 ng mL$^{-1}$ for caffeine and its metabolite. For flurbiprofen, LOQ was determined as 2.5 ng mL$^{-1}$. However, the lowest calibration level was fixed at 170 ng mL$^{-1}$ in accordance with the clinical samples values. The expected concentrations in clinical samples are above the obtained LOQ values for all substances [13,23–26].

Precision and accuracy were evaluated by replicate analysis ($n = 4$) of pooled plasma at three different concentrations and on three different days. As reported in Table 5, all accuracy and repeatability values proved to be satisfactory. In fact, the intra R.S.D.s ranged from 2.2 to 14.3% while inter-assay R.S.D.s were between 3 and 17%. Finally, method accuracy remained between 90.5 and 115%, which is in accordance with regulatory requirements [27].

### 3.4. Clinical application

In order to demonstrate the reliability of this method for the evaluation of the five selected CYP450 activities, it was used to determine all probe drugs and their respective metabolites in plasma samples 2 h after the administration of the cocktail to 10 healthy volunteers. The cocktail was constituted of 100 mg caffeine, 50 mg flurbiprofen, 40 omeprazole, 25 mg dextromethorphan and 7.5 mg midazolam. This study was approved by the Ethic Committee of Geneva University Hospitals. All volunteers have given written informed consent to participate in the study. Venous blood samples were collected and plasma was separated by centrifugation at 3000 × $g$ for 10 min and stored frozen at −20 °C until analysis.

A chromatogram obtained from one volunteer is shown in Fig. 3. The phenotype results for all volunteers will be published elsewhere. No interference was observed with endogenous compounds and all

Table 4
Linearity data for caffeine, mida, flu and their metabolites with a weighted least-squares linear regression analysis ($1/X$ or $1/X^2$)

| Calibration | Para | Caf | 1OH-Mida | Mida | 4OH-Flu | Flu |
|---|---|---|---|---|---|---|
| Intercept | 0.0055 | −0.0262 | 0.0032 | −0.0054 | 0.0065 | −0.0098 |
| Slope | 0.0009 | 0.0015 | 0.0006 | 0.0008 | 0.0005 | 0.0016 |
| Correlation coeff. | 0.999 | 0.999 | 0.992 | 0.994 | 0.999 | 0.997 |

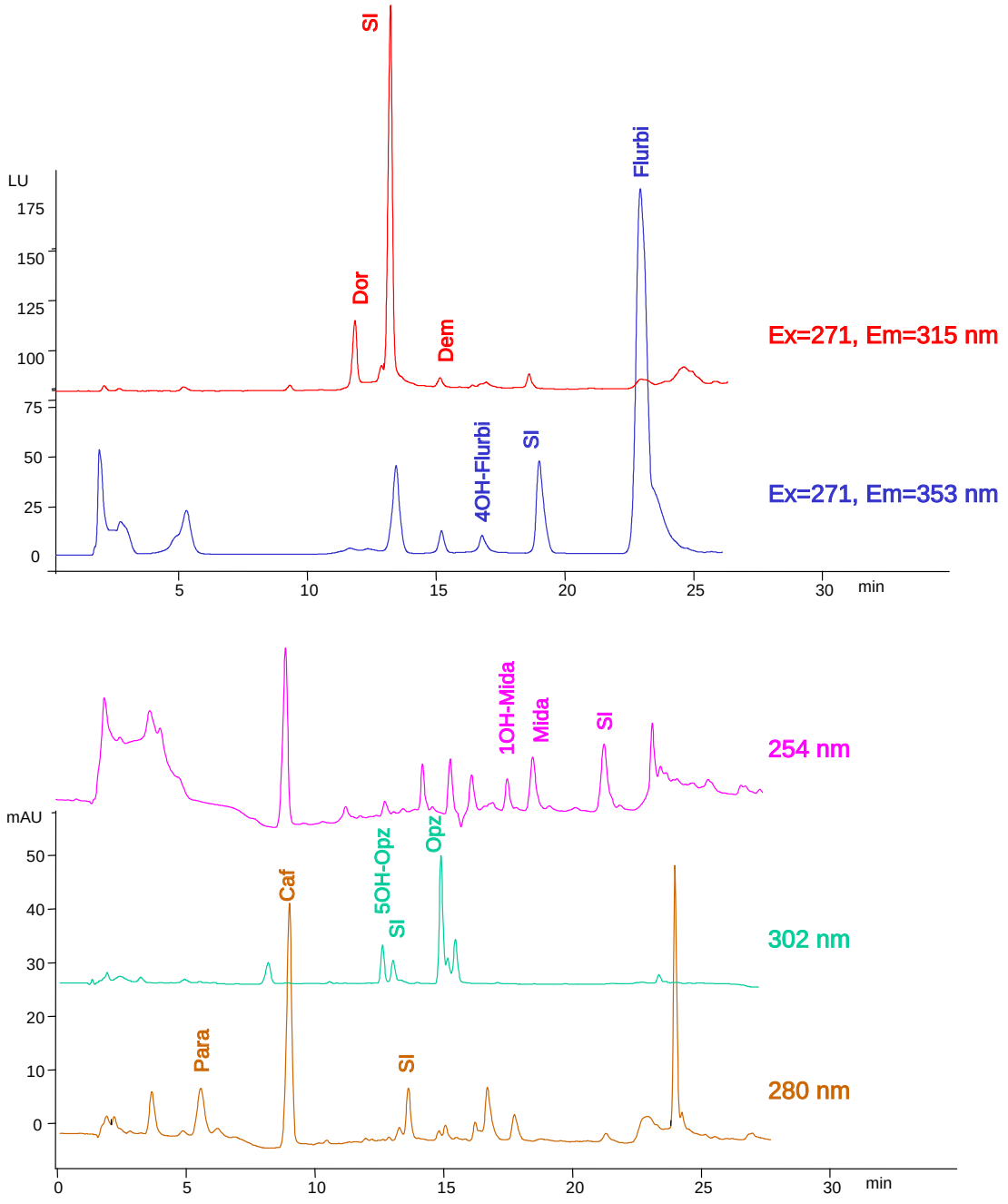


Fig. 3. Typical chromatograms of extracted plasma from a healthy volunteer 2 hours after ingestion of the cocktail.

Table 5
Precision and accuracy of the method for the determination of all drugs and their metabolites in human plasma

| | Intraday precision CV% | Interday precision CV% | Accuracy (%) |
|---|---|---|---|
| Concentration para ($ng\,mL^{-1}$) | | | |
| 20 | 10.7 | 10.7 | 101.8 |
| 100 | 8.9 | 10.4 | 105 |
| 400 | 5.5 | 6.3 | 101.9 |
| Concentration 4OH-Flu ($ng\,mL^{-1}$) | | | |
| 340 | 3.3 | 5.8 | 109.2 |
| 1700 | 2.2 | 3.1 | 99.4 |
| 6800 | 3.7 | 4.1 | 101.4 |
| Concentration 5OH-opz ($ng\,mL^{-1}$) | | | |
| 10 | 11 | 14.8 | 99.5 |
| 50 | 5.5 | 4 | 98 |
| 200 | 4.8 | 3.5 | 100.6 |
| Concentration dor ($ng\,mL^{-1}$) | | | |
| 2.5 | 9.5 | 10 | 90.5 |
| 12.5 | 7.9 | 6.1 | 103.5 |
| 50 | 11.2 | 8.5 | 100.3 |
| Concentration 1OH-Mida ($ng\,mL^{-1}$) | | | |
| 2.5 | 14.0 | 14.6 | 96.6 |
| 12.5 | 9.6 | 13.7 | 98 |
| 50 | 9.5 | 13.2 | 109.7 |
| Concentration caf ($ng\,mL^{-1}$) | | | |
| 20 | 9.1 | 9.1 | 99.7 |
| 100 | 11.5 | 11.5 | 109.2 |
| 400 | 9.4 | 10.8 | 107.9 |
| Concentration flu ($ng\,mL^{-1}$) | | | |
| 340 | 3.5 | 13.7 | 101.9 |
| 1700 | 5.7 | 6.6 | 103.8 |
| 6800 | 5.3 | 5.6 | 106.1 |
| Concentration opz ($ng\,mL^{-1}$) | | | |
| 10 | 10.2 | 15 | 96.9 |
| 50 | 6.1 | 5.3 | 97.2 |
| 200 | 5.6 | 4.4 | 102 |
| Concentration dem ($ng\,mL^{-1}$) | | | |
| 2.5 | 10.2 | 11 | 97.9 |
| 12.5 | 6.5 | 5 | 99.7 |
| 50 | 11.2 | 7.4 | 102.9 |
| Concentration mida ($ng\,mL^{-1}$) | | | |
| 2.5 | 14.3 | 15.8 | 104.1 |
| 12.5 | 8.8 | 14.2 | 93.3 |
| 50 | 9.1 | 17.1 | 115.2 |

probes and their metabolites were included in the calibration range. Hence, the developed analytical method is suitable for application to the clinical part of phenotyping.

## 4. Conclusion

Although the cocktail approach to characterise the activity of multiple metabolising enzymes offers several advantages, the analytical method to quantify each drug and its respective metabolite remains a key problem. In fact, the potential for interference increases with the addition of more parent drugs and metabolites to the sample matrix. After optimisation of the extraction procedure, gradient elution and detection specificity, the analytical method described in the present study allows a simultaneous separation of all drugs and their metabolites within a single run. Additionally, this new method showed satisfactory validation data in terms of specificity, linearity, sensitivity, accuracy and precision over the concentration range examined. Finally, the method was found appropriate for the dosage of the studied drugs and their metabolites in plasma samples, allowing the simultaneous phenotyping of five cytochromes. However, regarding the complexity of the extraction procedure as well as the sample mixture, the use of MS-MS detector will certainly simplify the overall procedure by enhancing both the selectivity and sensitivity. It has to be noted that the mobile phase used for this application was selected to be MS compatible. Future investigations will be devoted to the development of LC-MS method for this cocktail.

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