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Simultaneous analysis of cytochrome P450 probes—dextromethorphan, flurbiprofen and midazolam and their major metabolites by HPLC-mass-spectrometry/fluorescence after single-step extraction from plasma

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Abstract

Cytochrome P450 enzymes catalyze oxidative metabolism of most pharmaceutical compounds. Consequently dextromethorphan, flurbiprofen, midazolam and other compounds are commonly used as probe substrates to evaluate cytochrome P450 function in humans. A "cocktail" approach employing simultaneous administration of two or more of the probe substrates has been used by various investigators in recent years. An analytical strategy to simultaneously extract and analyze dextromethorphan, flurbiprofen and midazolam and their major metabolites (dextrorphan, 4′-hydroxy-flurbiprofen and 1′-hydroxy-midazolam) by HPLC-MS/fluorescence was developed and is described here. The three probe substrates and their major metabolites were extracted simultaneously by means of a solid-phase (Bond Elut Certify ® cartridges) extraction procedure from 200 μl of pig plasma. The extraction efficiency was more than 79.5% for each of the six analytes. The extracted compounds were chromatographically separated on a Luna C8(II) column (50 mm L × 3 mm ID) in a single run of 20 min and analyzed by either fluorescence (flurbiprofen and 4′-hydroxy-flurbiprofen) or selective ion monitoring (dextromethorphan, dextrorphan, midazolam and 1′-hydroxy-midazolam) with positive electrospray ionization. The limit of quantification was 2.5 ng/ml for midazolam and 5 ng/ml for the other five analytes. The assay was precise and accurate (error: −9.1 to 12.1) with total CVs of 13.9% or better for each of the 6 analytes. This method was used to analyze concentrations of the three probes and their metabolites in plasma after intravenous administration to a healthy pig.
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Keywords: Cytochrome P450; Dextromethorphan; Flurbiprofen; Midazolam; LC/MS; Phenotyping; Pharmacokinetics; Pig

1. Introduction

Cytochrome P450 (CYP) enzymes are responsible for phase I (oxidative) metabolism of many xenobiotics and are also involved in formation and catabolism of endogenous compounds [1,2]. Human CYP genes are categorized in 18 families and 57 subfamilies [1]. Of these, CYP1, 2 and 3 families are responsible for the metabolism of a majority of pharmaceutical compounds in humans [1–3]. There is variability in expression and activity of CYP enzymes across species, individuals and disease states. Consequently it is important to understand alterations in CYP expression and function in different groups and populations. Administration of probe substrates specific for a certain CYP isozyme followed by measurement of metabolism of that particular substrate is a commonly used technique for assessing the functionality of CYP isozymes. The probe substrates can be administered to assess CYP activity either alone or in combination [4–9]. The later approach is attractive as it allows for determination of activity of various isozymes in each subject and provides a more complete picture of metabolic characteristics of that particular individual. This combination or "cocktail" strategy also obviates the need for a large number

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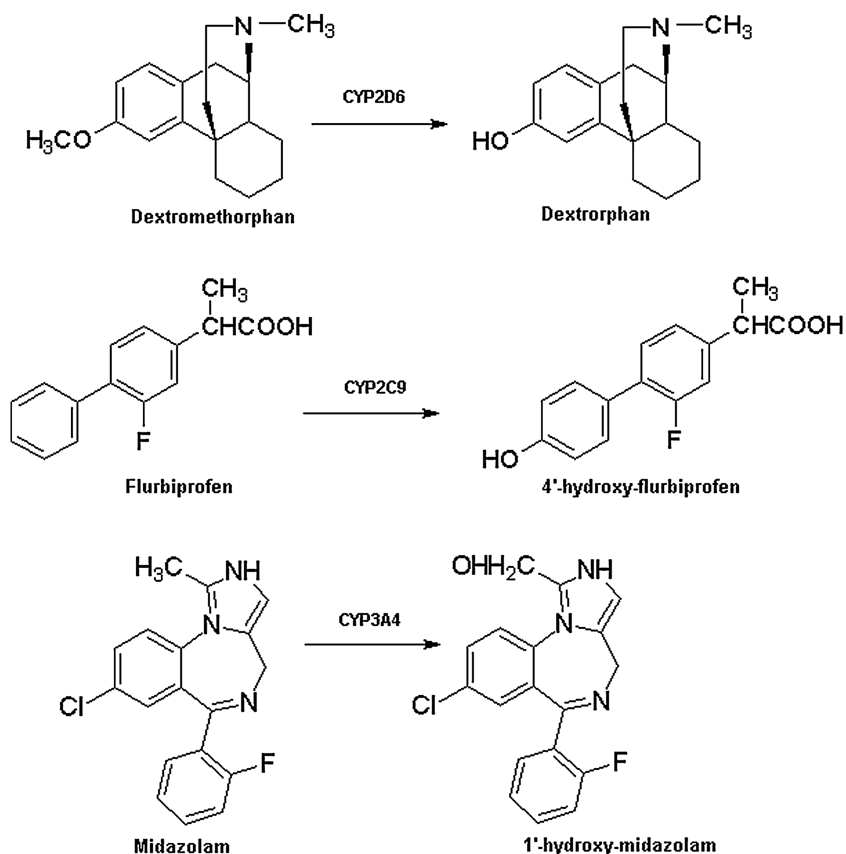

Fig. 1. Chemical structures of DEX, DXO, FLB, OHF, MDZ and OHM.

of subjects to ascertain a population's metabolic characteristics. In such studies, the metabolic profile of probe substrates is observed by analysis of blood or other biological matrix for the parent compounds and their metabolites. Most of the multiple-probe studies have employed separate methods for extraction and analysis of these compounds and their metabolites [4–9]. Therefore, these analytical strategies require a larger amount of the sample matrix and are labor-intensive due to multiple extraction and analysis steps. There has been one reported method to simultaneously analyze dextromethorphan (DEX), flurbiprofen (FLB), midazolam (MDZ), caffeine, omeprazole and their major metabolites in a single run by HPLC [10]. This method used four different extraction procedures to extract the analytes from separate samples. A simpler assay procedure for extracting multiple-probes and their major metabolites from a single plasma sample appears attractive. DEX, FLB and MDZ are metabolized to dextrorphan (DXO), 4′-hydroxy-flurbiprofen (OHF) and 1′-hydroxy-midazolam (OHM) by CYP2D6, CYP2C9, and CYP3A4, respectively (Fig. 1). Therefore, DEX, FLB and MDZ are used as probe substrates to assess CYP2D, CYP2C, and CYP3A activity in both humans and animals. Since about 80% of currently marketed drugs are metabolized by these three isozymes [2], administration of this probe-substrate cocktail can provide comprehensive information about CYP functionality in an individual. We report a method to simultaneously extract DEX, FLB and MDZ and their three main metabolites from a single 200 μl plasma sample. The extracted analytes were separated in a single run of HPLC and simultaneously analyzed by fluorescence and mass-specific detection.

2. Experimental

2.1. Materials and chemicals

Dextromethorphan hydrobromide, flurazepam dihydrochloride, flurbiprofen, midazolam hydrochloride, 1′-hydroxy-midazolam, levallorphan and β-glucuronidase (400,000 U/g lyophilized powder form; type H; derived from *Helix pomatia*) were purchased from Sigma–Aldrich (St. Louis, MO). Dextrorphan was obtained from Research Biochemical International (Natick, MA). 4′-hydroxy-flurbiprofen and 2-fluoro-4-biphenyl acetic acid were kindly provided by Dr Timothy Tracy at the University of Minnesota. Acetic acid (HPLC grade), ammonium acetate (ACS grade), ammonium hydroxide (20%; ACS grade), methanol (HPLC grade), phosphoric acid (85%; HPLC grade), sodium acetate (ACS grade) and sodium hydroxide (ACS grade) were obtained from Fisher Chemical (Fair Lawn, NJ). Formic acid (85%; HPLC grade) and HPLC-grade water were obtained from Fluka (Buchs, Switzerland) and EMD Chemicals (Gibbstown, NJ), respectively. A monosodium salt of flurbiprofen (for use in the *in-vivo* study) was synthesized by addition of an equimolar amount of a 1 mol/l sodium hydroxide solution to flurbiprofen followed by filtration and evaporation to dryness. Heparinized plasma from a pig not exposed to any drugs was used as blank matrix.

2.2. Chromatographic apparatus and operating conditions

The chromatographic system (Shimadzu, Japan) included two LC-10ADVP pumps, a DGU-14A degasser and a SIL-10ADVP autosampler. Chromatographic separation was achieved with a 50 mm long, 3 mm diameter Luna C8(II) column (Phenomenex, Torrance, CA) with a 3 μm particle size. The column effluent passed first through a RF10-XL fluorescence detector (Shimadzu, Japan) before reaching a mass selective detector (LCMS-2010A; Shimadzu, Japan). A biphasic mobile phase consisting of methanol (solvent A) and 20 mM ammonium acetate buffer (pH adjusted to 3.9 with formic acid) (solvent B) was delivered in a gradient manner through the column at a flow rate of 0.2 ml per minute at ambient temperature. The gradient started with 40% methanol and 60% acetate buffer and was raised to 90% methanol in 10 min and was held at that level for 10 min leading to a total run time of 20 min. The column was equilibrated with initial mobile phase mixture (40% methanol and 60% acetate buffer) for 5 min after each injection. The excitation and emission wavelengths of the fluorescence detector were set at 260 and 320 nm, respectively, for flurbiprofen and 4′-hydroxy-flurbiprofen [11]. The MS detector was equipped with an electrospray ionizer and was operated with the following conditions: ionization voltage (+ 4.5 kV); nebulizing gas (nitrogen) flow (1.5 l/min); ion source heating block temperature (200 °C); orifice voltage (−20 V); desolvation line temperature (250 °C); and detector voltage (1.5 kV). The MS detector was run in the selective positive-ion detection mode with the target ions being 258 (dextrorphan); 284 (levallorphan); 272 (dextromethorphan); 388 (flurazepam); 325.7 (midazolam); and 341.7 (1′-hydroxy-midazolam).

2.3. Calibration curve preparation

The three probe substrates and their metabolites were dissolved in methanol to produce six separate 1 mg/ml (as free base or acid) stock solutions. Appropriate volumes of these stock solutions were added to methanol to prepare four working solutions so that the addition of 5 or 10 μl of these four working solutions to 200 μl of plasma produced effective standard curve concentrations. The standard curve for DEX, DXO, FLB and OHF included concentrations of 10, 20, 50, 100, 250 and 500 ng/ml and 1 and 2 μg/ml. For MDZ and OHM, the standard curve comprised concentrations of 5, 10, 25, 50, 125, 250 and 500 ng/ml and 1 μg/ml.

Three compounds – levallorphan (for DEX and DXO), 2-fluoro-4-biphenyl acetic acid [11] (for FLB and OHF) and flurazepam (for MDZ and OHM) were used as internal standards. A stock solution containing 1 μg/ml each of levallorphan and 2-fluoro-4-biphenyl acetic acid and 0.5 μg/ml of flurazepam was prepared in methanol. Twenty microliters of this solution was added to each plasma sample prior to the extraction procedure.

2.4. Preparation of quality control standards

Separate 1 mg/ml stocks of the six analytes were prepared in methanol and the same were further diluted with methanol to prepare working solutions. Addition of 10 μl of the working solutions to 200 μl of plasma yielded effective plasma concentrations of 15 ng/ml (low QC), 200 ng/ml (medium QC) and 1500 ng/ml (high QC) for DEX, DXO, FLB and OHF and 7.5 ng/ml (low QC), 100 ng/ml (medium QC) and 750 ng/ml (high QC) for MDZ and OHM. The quality control plasma samples were extracted after addition of 20 μl of the internal standard solution as described below.

2.5. Determination of lower limit of quantification (LOQ)

Two separate working solutions containing the six analytes were prepared in methanol. Addition of 10 μl of these solutions to 200 μl of plasma yielded effective plasma concentrations of 10 and 5 ng/ml, respectively, for DEX, DXO, FLB and OHF and 5 and 2.5 ng/ml, respectively, for MDZ and OHM. Five replicates were employed at each of the two tested concentration levels. A 10-point standard curve (including eight points as described above in Section 2.3) was prepared. The two additional concentration points in the standard curve were 5 and 2.5 ng/ml for DEX, DXO, FLB and OHF and 2.5 and 1.25 ng/ml for MDZ and OHM. Twenty microliters of the internal standard solution was added to the each of the standard curve and LOQ plasma samples and the same were then extracted and analyzed as described below. The LOQ was defined as the lowest concentration measurable with an error of 20% or less for accuracy and precision.

2.6. Extraction from plasma samples

Extraction of analytes from plasma samples was accomplished with Bond Elut Certify® cartridges (Varian, Harbor city, CA). The cartridges were attached to a vacuum manifold and were prepared by sequential elution of 1 ml of methanol, 1 ml of water and 1 ml of 0.2 M phosphate buffer (pH 3) through them. Each plasma sample (200 μl) was acidified with 10 μl of 85% phosphoric acid and 250 μl of 0.2 M acetate buffer (pH 5). Each acidified plasma sample was passed through one prepared cartridge by centrifuging the cartridges at 75 × *g*. Each cartridge was subsequently washed with 1 ml of a 95:5 mixture of water and methanol (pH 2.7 with formic acid). The deposited compounds were then eluted with 1 ml of methanol containing 1% ammonium hydroxide. The eluent was dried under a gentle stream of nitrogen gas at 30 °C in a TurboVap® evaporator (Zymark, Hopkinton, MA). The residue was reconstituted with 75 μl of methanol, transferred to 200 μl plastic centrifuge tubes and centrifuged at 768 × *g* for 5 min. The supernatant was transferred to injection vials and 4 μl of this extract was injected into the HPLC column.

2.7. Study design for method validation

Plasma samples for standard curve and validation were spiked and processed in the manner described above. An eight-point standard curve along with three sets (low, medium, and high QC; $n = 5$ for each set) of validation samples was run on three separate days to determine intra- and inter-day accuracy and

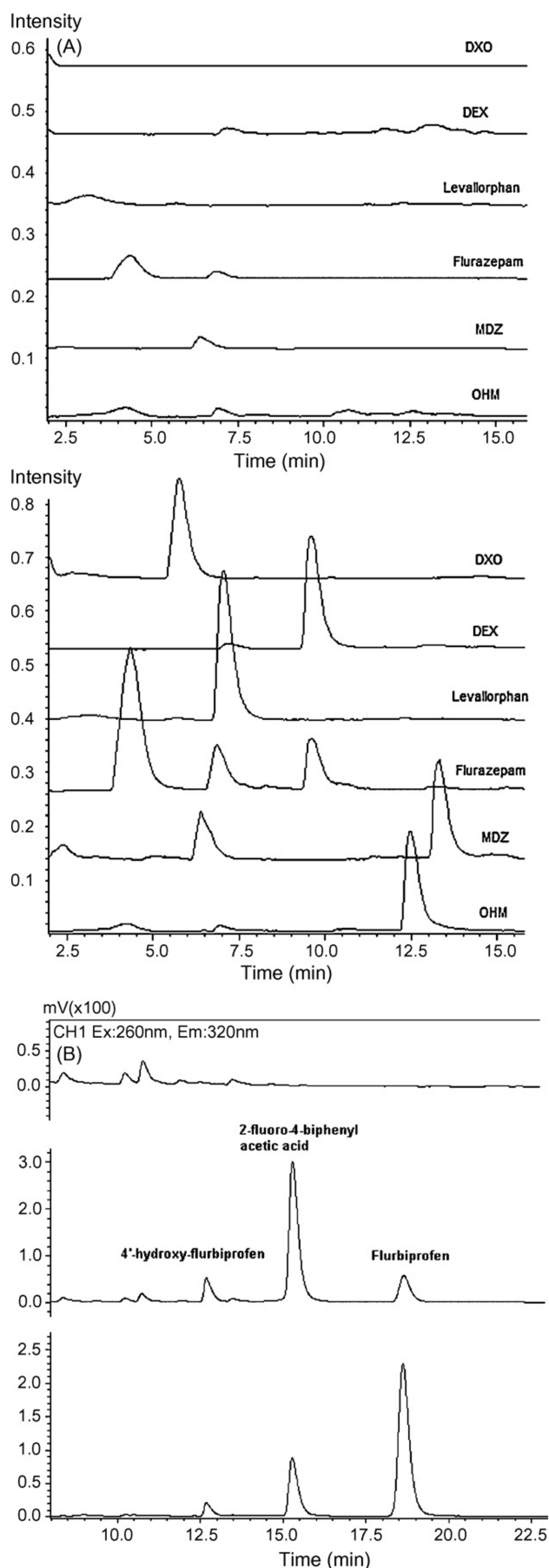

Fig. 2. (a) Selective-ion chromatogram showing DXO, levallorphan, DEX, flurazepam, OHM and MDZ (baselines shifted for clarity). The top and bottom panels represent a blank plasma sample and a spiked plasma sample (containing 50 ng/ml of DXO and DEX and 25 ng/ml of OHM and MDZ), respectively. (b) Fluorescence chromatogram showing OHF, 2-fluoro-4-biphenyl acetic acid and FLB. The top, middle and bottom panels represent a blank plasma sample, a spiked plasma sample (containing 50 ng/ml each of OHM and FLB) and sample from a pig, respectively.

precision. The stability of each analyte after two freeze–thaw cycles was determined at the three QC concentration levels ($n = 5$ for each analyte at each concentration level). Responses of QC samples from one set of the validation run were compared with unextracted standards containing the same amount of analytes to determine extraction recovery.

2.8. Analysis of plasma samples obtained from pig

The *in-vivo* experiment was approved by the institutional animal care and use committee (IACUC) of the University of Minnesota. A healthy Dorac pig weighing 22 kg was intravenously administered 0.25 mg/kg of flurbiprofen and 0.5 mg/kg each of dextromethorphan and midazolam. DEX hydrobromide and FLB sodium were dissolved in sterile 0.9% saline (Baxter healthcare, Deerfield, IL) and filtered through a 0.45 μm Acrodisc® syringe filter (Pall, East Hills, NY) under sterile conditions to prepare the IV dose just before the *in-vivo* experiment. A commercially available 5 mg/ml injection (Baxter healthcare, Deerfield, IL) was used to supply the dose of midazolam. Blood samples were collected at periodic intervals and centrifuged at 1200 × *g* to isolate plasma. Two hundred micoliters of each plasma sample was incubated with 250 μl of a 2000 U/ml β-glucuronidase solution (in 0.2 M acetate buffer; pH 5) for 12 h at 37 °C in a water bath. The plasma samples were then processed in the manner described above and analyzed with standard and QC samples of a validation run.

2.9. Data analysis

The fluorescence and MS detector signal (peak area) from standard curves for each analyte was plotted against corresponding concentrations with a 1/*x* weighting function for each analyte. The concentrations of the six analytes in the validation samples were determined from the corresponding standard curves. For each concentration data point, the calculated values were subjected to a one-way analysis of variance and the within-day component of variation (S^2_{wd}), the between-day component of variation (S^2_b), and total variance (S^2_t) were calculated as described elsewhere [12,13]. Accuracy (%) was determined by dividing the mean of calculated concentration by the true mean and multiplying it by 100.

3. Results

The representative selected ion and fluorescence chromatograms with retention times are shown in Fig. 2a and b. The retention times for DXO, levallorphan, DEX, flurazepam, OHM,

OHF, MDZ, 2-fluoro-4-biphenyl acetic acid and FLB were 5.6, 7, 9.8, 9.8, 12.5, 12.7, 13.3, 15.2 and 18.3 min, respectively. The standard curves obtained were linear ($r^2 > 0.993$) for each of the six analytes with the intercept values being of practically zero magnitude. The extraction recovery was more than 79.5% for each of the six analytes as shown in Table 1. The assay was sensitive for each of the six analytes with a LOQ of 5 ng/ml for DEX, DXO, FLB, OHF and OHM and 2.5 ng/ml for MDZ (Table 2). These LOQ values were obtained after extraction from 200 μl of plasma. The assay validation parameters are shown in Table 3. Results of the validation showed the assay methodology to possess good accuracy and precision for each analyte at each of the three tested levels. The six analytes also demonstrated good stability after two freeze–thaw cycles as shown in Table 4.

Table 1
Extraction recovery

	Level tested	Recovery (%)[a]	CV (%)
DEX	Low QC (15 ng/ml)	93.6	1.8
	Medium QC (200 ng/ml)	84.6	6.4
	High QC (1500 ng/ml)	92.2	3.3
DXO	Low QC (15 ng/ml)	96.7	1.8
	Medium QC (200 ng/ml)	91.3	3.6
	High QC (1500 ng/ml)	95.7	2.2
FLB	Low QC (15 ng/ml)	107.4	6.9
	Medium QC (200 ng/ml)	104.0	0.8
	High QC (1500 ng/ml)	101.4	1.2
OHF	Low QC (15 ng/ml)	111.4	4.2
	Medium QC (200 ng/ml)	105.0	2.4
	High QC (1500 ng/ml)	103.8	0.8
MDZ	Low QC (7.5 ng/ml)	96.1	4.1
	Medium QC (100 ng/ml)	97.8	2.7
	High QC (750 ng/ml)	98.5	2.2
OHM	Low QC (7.5 ng/ml)	80.0	4.8
	Medium QC (100 ng/ml)	86.3	3.4
	High QC (750 ng/ml)	79.5	4.7

[a] Average of five replicates.

Table 2
LOQ determination

	Level tested (ng/ml)	Accuracy (%)[a]	CV (%)
DEX	5	88.2	10.5
	10	107.2	5.4
DXO	5	108.6	15.2
	10	105.3	2.7
FLB	5	110.4	15.9
	10	98.0	7.3
OHF	5	112.2	6.0
	10	111.1	4.2
MDZ	2.5	120.0	14.2
	5	101.5	7.9
OHM	2.5	135.0	24.4
	5	118.9	12.2

[a] Average of five replicates.

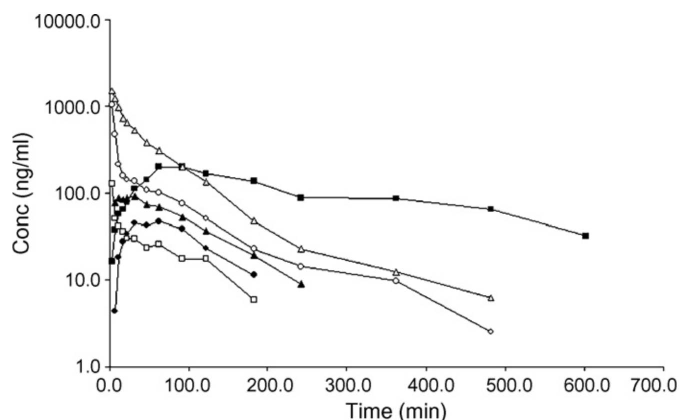

Fig. 3. Plasma concentration–time profiles of the six analytes after intravenous administration of 0.5 mg/kg of DEX and MDZ and 0.25 mg/kg of FLB to a healthy pig. Open squares, triangles and circles represent DEX, FLB and MDZ, respectively. Solid squares, triangles and circles represent DXO, OHF and OHM, respectively.

The plasma concentration–time profiles of the three parent drugs and their primary metabolites after intravenous administration of 0.5 mg/kg of DEX and MDZ and 0.25 mg/kg of FLB to a healthy pig are shown in Fig. 3. DEX was cleared rapidly from plasma and was below the limit of quantification in about 3 h. FLB and MDZ were below quantification limits by about 6 h. The metabolites DXO, OHF and OHM were detectable within 15 min after IV administration and DXO had a significantly longer $t_{1/2}$ than the parent compound.

4. Discussion

A number of methodologies to assay DEX, FLB and MDZ and their metabolites after extraction from plasma and other biological fluids have been reported till date. DEX and its metabolites have previously been analyzed by HPLC coupled with fluorescence and positive-ion mass spectrometry [14,15]. MDZ and OHM have been analyzed by HPLC linked with UV detection and positive-ion mass spectrometry [16,17]. FLB and OHF have been analyzed previously by fluorescence subsequent to their chromatographic separation [11]. FLB has also been assayed by mass spectrometry in negative-ion mode [18,19]. In recent years, phenotyping studies employing simultaneous administration of more than one probe substrate have been reported [4–9]. Each of these studies has used separate methods to extract and analyze various groups of analytes. Use of multiple extractions requires multiple aliquots of sample matrix and also is highly labor-intensive. Jerdi et al. have described a method to simultaneously analyze DEX, FLB, MDZ, caffeine (CYP1A2 probe) and omeprazole (CYP2C19 probe) and their major metabolites in a single HPLC run [10]. However, these authors used four different methods (due to differences in chemical natures of the compounds) and a total of 3.1 ml of plasma for analyte extraction. Also, while the authors demonstrated that their HPLC methodology was able to separate the analytes, the four eluted extracts were injected on four separate occasions. Our strategy, in contrast, allows for simultaneous extraction of the three most commonly used probe substrates and their major

Table 3
Results of assay validation done on three occasions

		n	Accuracy (%)	S_{wd}	S_b	S_t	CV_{wd} (%)	CV_b (%)	CV_t (%)
DEX	Low QC (15 ng/ml)	15	104.3	0.7	1.5	1.6	4.8	9.3	10.4
	Medium QC (200 ng/ml)	15	112.1	17.3	0.0	16.1	7.7	0.0	7.2
	High QC (1500 ng/ml)	15	101.6	70.5	199.4	211.5	4.6	13.1	13.9
DXO	Low QC (15 ng/ml)	15	90.9	0.5	0.1	0.6	3.9	1.0	4.1
	Medium QC (200 ng/ml)	15	110.7	8.0	5.0	9.5	3.6	2.3	4.3
	High QC (1500 ng/ml)	15	98.5	111.6	52.2	123.2	7.6	3.5	8.3
FLB	Low QC (15 ng/ml)	15	101.2	1.2	0.1	1.3	8.2	1.0	8.3
	Medium QC (200 ng/ml)	15	103.3	6.1	18.1	19.1	3.0	8.7	9.2
	High QC (1500 ng/ml)	15	96.3	25.3	154.8	156.9	1.8	10.7	10.9
OHF	Low QC (15 ng/ml)	15	112.1	0.8	0.0	0.6	4.6	0.0	3.9
	Medium QC (200 ng/ml)	15	102.1	6.8	22.8	23.8	3.3	11.1	11.6
	High QC (1500 ng/ml)	15	96.5	24.0	176.2	177.8	1.7	12.2	12.3
MDZ	Low QC (7.5 ng/ml)	15	91.5	0.6	0.2	0.6	8.8	3.3	9.4
	Medium QC (100 ng/ml)	15	111.2	5.2	0.0	5.0	4.7	0.0	4.5
	High QC (750 ng/ml)	15	97.4	44.4	0.0	38.6	6.1	0.0	5.3
OHM	Low QC (7.5 ng/ml)	15	92.1	0.5	0.2	0.6	7.8	3.3	8.5
	Medium QC (100 ng/ml)	15	110.9	8.6	0.0	8.4	7.8	0.0	7.6
	High QC (750 ng/ml)	15	96.7	48.9	19.0	52.5	6.7	2.6	7.2

S_{wd}, S_b, and S_t are within-day, between-day, and total standard deviation, respectively. CV_{wd}, CV_b and CV_t are within-day, between-day, and total coefficient of variation.

metabolites from a single 200 μl plasma sample. The isolated analytes are in a single extract obviating the need for multiple HPLC injections.

DEX and MDZ are weak bases with p*K*a values of 9.2 and 6.1, respectively. FLB is an organic acid with a pK_a of 4.2. The divergent chemical nature of these compounds precludes their simultaneous isolation from a plasma sample by traditional liquid–liquid extraction procedures. The Bond Elut Certify® extraction cartridges employ a mixed-phase comprised of a non-polar C8 sorbent and a cation-exchange phase. Acidification of 200 μl of plasma with 250 μl of 0.2 M acetate buffer and 10 μl of concentrated phosphoric acid leads to a sample pH below 2. This acidic treatment ionizes the basic moieties (DEX, DXO, MDZ and OHM) which then are retained on the cartridges. FLB and OHF are in a non-ionized state in the acidified plasma and are retained by the non-polar C8 groups of the cartridges. The

Table 4
Freeze–thaw stability

		Cycle 1		Cycle 2	
		Remaining[a] (%)	CV (%)	Remaining[a] (%)	CV (%)
DEX	Low QC	93.0	11.4	93.9	8.7
	Medium QC	100.5	12.1	100.5	8.1
	High QC	97.0	3.0	97.0	6.4
DXO	Low QC	88.1	6.2	89.5	5.3
	Medium QC	105.6	9.2	111.4	3.9
	High QC	96.0	1.9	96.2	3.7
FLB	Low QC	101.3	6.1	110.4	7.7
	Medium QC	106.8	1.2	110.4	10.0
	High QC	100.3	1.1	98.7	3.0
OHF	Low QC	108.0	5.8	113.5	8.1
	Medium QC	107.4	1.0	109.5	7.2
	High QC	99.6	1.1	97.4	2.6
MDZ	Low QC	97.1	7.8	96.0	5.4
	Medium QC	111.0	5.5	110.3	8.2
	High QC	104.2	8.8	92.5	3.9
OHM	Low QC	96.4	11.3	99.6	5.6
	Medium QC	115.0	9.3	104.8	7.8
	High QC	95.9	7.3	91.3	4.2

[a] Average of five replicates.

retained analytes are then eluted from the cartridges by a basic organic solvent (methanol with ammonium hydroxide). This strategy provides excellent sample cleanup for analysis by mass spectrometry and high extraction recoveries (>79.5%; Table 1)

Together, CYP 2C, 2D and 3A are responsible for metabolism of about 80% of currently marketed drugs [1–3]. Therefore, functional characterization of these three isozymes can provide an almost complete picture of the metabolic phenotype of an individual. The three probe substrates (DEX, FLB and MDZ) can be used for this purpose and the methodology described by us can be applied to facilitate rapid extraction and analysis of the plasma samples. The described assay is currently being used to determine the functional activity of the CYP enzymes in healthy pigs and in a porcine shock model.

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