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An improved HPLC assay with fluorescence detection for the determination of domperidone and three major metabolites for application to *in vitro* drug metabolism studies

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Abstract

Domperidone is currently used in Canada and Europe for the treatment of intestinal motility disorders as well as for its antiemetic properties. Recent drug metabolism studies have indicated that domperidone is a substrate of different subtypes of CYP3A family and consequently, the drug requires complete characterization of its metabolism for the identification of major drug–drug interactions. Therefore, the purpose of our studies was to develop a simple, sensitive and rapid HPLC assay for the determination of domperidone and its major metabolites. This assay had to be suitable for the conduct of *in vitro* drug metabolism study with human liver microsomes. Baseline resolution of internal standard, domperidone and three of its major metabolites was achieved in a run time of less than 15 min using an Ultrasphere ODS column (250 mm × 4.6 mm × 5 μM) and a mobile phase consisting of disodium citrate buffer (10 mM, pH 3.4):methanol:acetonitrile:trietylamine, 54.6:34.7:9.9:0.8 at a flow rate of 1.0 mL/min. Chromatographic separation was executed at room temperature. Quantification was performed by tandem fluorescence (excitation λ = 282 nm and emission λ = 328 nm) and ultraviolet detectors (λ = 254 nm for the quantification of encainide, internal standard). Calibration curves were constructed and showed linearity in the range of 0.1–20 μmol/L and 10–250 μmol/L. Intra- and interday coefficients of variation were less than 8% and 11%, respectively. Mean accuracy was 100.5 ± 9.9% and limit of quantification was established at 0.06 μmol/L for domperidone and its metabolites. The assay allows estimation of enzymatic parameters (K_m and V_{max}) of domperidone for the formation of its various metabolites and sensitivity is sufficient for the conduct of inhibition studies with potent CYP3A inhibitors.
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Keywords: HPLC; Domperidone; CYP3A4; Cytochrome P450; Metabolites; Drug metabolism

1. Introduction

Domperidone, 5-chloro-1[1-[3-(2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)propyl]-4-piperidinyl]-1,3-dihydro-2H-benzimidazol-2-one, is a selective antagonist of the D2 receptor with a chemical structure related to butyrophenone. The drug is principally used in Canada and European countries (not USA) for the treatment of motility disorders as well as for its antiemetic properties [1,2]. Domperidone was for a long time a second choice to cisapride but the removal of the latter from the market has increased its clinical use.

Information on the metabolism of domperidone in humans has remained limited for years. At the beginning of the ‘80s, oral and intravenous administrations of the ^{14}C-labelled compound to rats, dogs and human have led to the identification of three major metabolites formed following oxidative N-dealkylation and hydroxylations at two different sites [3]. Further characterization of the metabolism of the drug by our group has allowed identification by LC–MS–MS of four isomers of ring hydroxylated metabolites [4]. Finally, Ward et al. demonstrated that 5-hydroxydomperidone (labelled M3) was the major hydroxylated metabolite [5]. They, as we did, identified M2 as the N-dealkylated major metabolite (Fig. 1) [4,5].

Recent drug metabolism studies performed have demonstrated that CYP3A4 is the most prominent member of the CYP3A-family which is involved in domperidone metabolism, with minor contribution of CYP1A2 and CYP2D6 [4,5]. The

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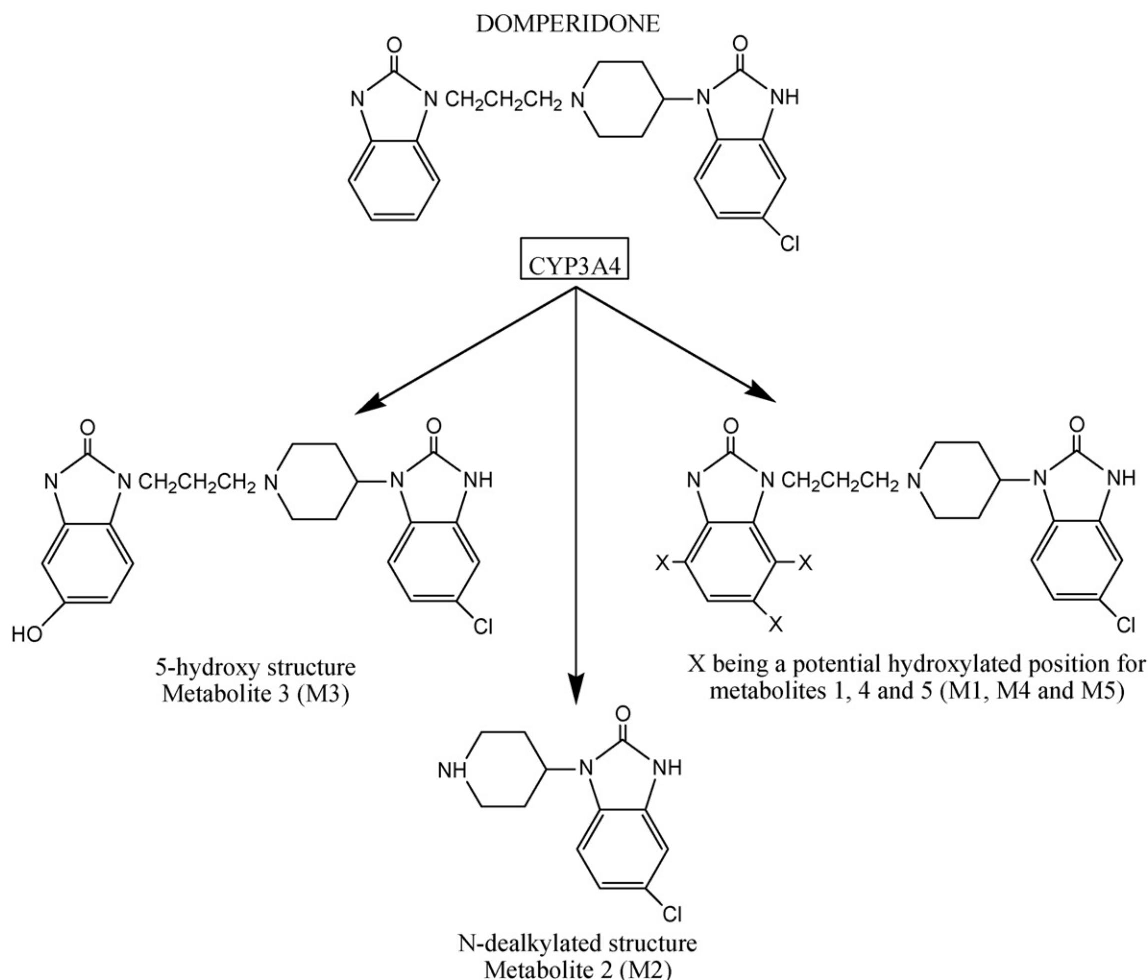

Fig. 1. Major metabolic pathways of domperidone.

CYP3A family, the most abundantly expressed in human liver, is highly variable and contributes greatly to variation in oral bioavailability and systemic clearance of CYP3A substrates [6,7]. It is the purpose of some studies conducted in our laboratory to characterize the metabolism of domperidone and evaluate the risk of drug–drug interactions with this drug. The overall objective of these studies is to further evaluate the risk of drug-induced Long QT Syndrome since electrophysiological studies have demonstrated that domperidone is a potent blocker of the rapid component of the delayed rectifier potassium current (I_{Kr}; IC_{50} 162 nM) [8].

Several methods have been reported for the determination of domperidone in various matrices such as plasma, urine and milk [3,9,10]. Some of these assays use ^{14}C-labelled drug and are very sensitive but they do not permit the determination of major metabolites of domperidone [3,11–13]. Other assays measure levels of domperidone by radio-immunoassay but antibodies raised in rabbits against domperidone are not commercially available and do not allow for the determination of domperidone metabolites [3,11,13,14]. The use of LC–MS and ESI-MS have been recently reported and could be adapted for the conduct of drug metabolism studies [5,10,15,16]. Unfortunately, the required equipments are not available in several laboratories. Finally, other previously reported HPLC assays with fluorescence/UV detection were either only partially described, required post-column derivatization or lacked resolution required for the quantification of all identified metabolites [5,9,17].

Therefore, the objective of our study was to develop a simple and sensitive HPLC assay, using non-radioactive materials, and allowing for the determination of domperidone and at least three of its major metabolites formed by human liver microsomes. The method is based on a previously described HPLC assay by the group of Yamamoto et al. and employs reversed-phase chromatography (HPLC) with fluorescence and ultraviolet detection [17].

2. Experimental

2.1. Chemicals and reagents

Domperidone (analytical grade) was purchased from Janssen Pharmaceutica N.V. (Beerse, Belgium). Encainide hydrochloride (analytical grade), used as the internal standard, was provided by Bristol-Myers Pharmaceutical research and development division (Wallingford, CT, USA). Acetonitrile and

methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Dimethylsulfoxamide (DMSO) and trietylamine were obtained from Aldrich Chemical (Milwaukee, WI). Other chemicals used were of highest qualities commercially available. Metabolites of domperidone were generated following the incubation of the parent drug with human liver microsomes. Reaction cofactors (NADP+, D-glucose 6-phosphate and glucose 6-phosphate dehydrogenase) were obtained from Sigma Chemicals (St. Louis, MO, USA). Human liver microsomes were purchased from Human Biologics International (Scottdale, AZ, USA).

2.2. Standard solutions

A stock solution of domperidone was prepared by dissolving 29.8 mg of the drug in 1 mL of DMSO (70 mM). Dilutions were performed by using various volumes of this stock solution added to DMSO to create secondary stock solutions with final concentrations varying from 0.01 to 50 mM. Stock solutions of internal standard, encainide hydrochloride, were prepared in distilled water to final concentrations of 129 μmol/L or 257 μmol/L. The stock and working solutions were stored at −20 °C and the incubations mixtures containing metabolites of domperidone were stored at −80 °C.

2.3. Apparatus

The chromatographic HPLC system (Thermo Separation products, Fremont, CA) consisted of a SpectraSystem P4000 pump, a SpectraSystem AS3000 autosampler, a model FL3000 fluorescence detector, a SpectraSystem UV3000 ultraviolet detector and a model PC1000 System Software. Chromatographic separation of domperidone and its major metabolites was performed at room temperature on an Ultrasphere ODS column (250 mm × 4.6 mm × 5 μM; Beckman Coulter, Fullerton, CA) using a mobile phase containing disodium citrate buffer (10 mM, pH 3.4):methanol:acetonitrile:trietylamine, 54.6:34.7:9.9:0.8 at a flow rate of 1.0 mL/min. Eluent was monitored with fluorescence detection (excitation $\lambda = 282$ nm and emission $\lambda = 328$ nm) and by ultraviolet absorbance at $\lambda = 254$ nm (quantification of encainide, internal standard).

2.4. Incubations

The incubation mixture (final volume, 500 μL) consisted of 20 μL human liver microsomes, 375 μL phosphate buffer 50 mM (pH 7.4), 100 μL NADPH-regenerating system solution and 5 μL substrate (domperidone dissolved in DMSO; 1% final reaction volume). Incubations containing microsomes, buffer and NADPH-regenerating system solution were pre-incubated at 37 °C for 8 min. Reactions were initiated by the addition of the substrate to the incubation mixture. After 45 min, enzymatic process was stopped by adding 500 μL of ice-cold acetonitrile. Encainide was added as the internal standard. The incubation mixture was centrifuged at 12,000 rpm for 5 min. The supernatant, 10–30 μL, was injected onto the HPLC system and major metabolites of domperidone were monitored.

2.5. Standard curve

Calibration curves based on the area under the peak of domperidone to internal standard were constructed using 8 different concentrations in duplicate. Aliquot (5 μL) of the secondary stock solutions were added to incubation mixtures (final volume 500 μL) to obtain a range of domperidone concentrations varying between 0.1 and 250 μmol/L. The internal standard, encainide hydrochloride was added (30 μL) to final concentrations of 7.3 or 14.5 μmol/L. To improve precision, two calibration curves were constructed: the first one covered the range of 0.1–20 μmol/L while the second covered the range of 10–250 μmol/L. In the lower range, final concentration of the internal standard was fixed at 7.3 μmol/L while a final concentration of 14.5 μmol/L was used for the curve in the highest range. The data were then subjected to linear regression analysis to give the appropriate calibration factor.

Frozen standards of secondary stock solutions (−20 °C), frozen samples from standard curves (−80 °C) and finally frozen incubation mixtures (−80 °C) were reanalyzed periodically to confirm stability of the compounds and reproducibility of the assay.

Pure drug substance for the metabolites of domperidone was not available. Hence, the standard curve constructed for the parent compound were used and applied to the peak area of the metabolites. The degree of fluorescence emission for these compounds relative to domperidone was assumed to be equal to 1.

2.6. Precision

Intraday variability in the analysis of domperidone and its metabolites was assessed by the repeated analysis ($n = 6$) of fixed volumes of incubations performed with domperidone (2.5, 5, 20, 50 and 100 μmol/L). The range of concentrations analyzed varied between 0.1 and 72 μmol/L. This range of concentrations corresponded to 42.6 ng/mL to 30.7 μg/mL for domperidone (molecular weigh of 425.9 g), 44.2 ng/mL to 31.8 μg/mL for hydroxylated metabolites (M1 and M3; molecular weigh of 441.9 g) and 25.2 ng/mL to 18.1 μg/mL for the N-dealkylated metabolite (M2, molecular weigh of 252 g). Interday precision was determined by repeated analysis of similar samples for domperidone and its metabolites (0.1–72 μmol/L) generated from incubation mixtures on 5 consecutive days. The precision was calculated as percent coefficient of variation (CV):

$$CV = \frac{\text{Standard Deviation} \times 100}{\text{mean of repeated measurements}}.$$

3. Results

3.1. Description of assay improvements

Yamamoto et al. described in 1998 a quantitative HPLC assay for the determination of domperidone in plasma [17]. This assay that uses fluorescence detection exhibited high sensitivity with

a detection limit of 1 ng/mL (2.37 nmol/L) for domperidone in plasma. However, this assay did not allow for the quantification of the metabolites of domperidone.

In order to do so, we decided to modify the mobile phase by adding acetonitrile and by increasing the proportion of the disodium citrate buffer. Various ranges of pH were also assessed: a decrease in the pH of the mobile phase was found to be associated with shorter retention times and an improvement in peak resolution. Finally, sharper peaks and baseline resolution were obtained by the addition of the ion-pairing agent triethylamine. We also had to change the internal standard to prevent interference with some of domperidone metabolites. Consequently, tandem fluorescence and UV detection were used for the determination of the new internal standard namely, encainide. The liquid–liquid extraction procedure was eliminated and proteins were precipitated by the addition of acetonitrile.

3.2. Chromatographic analysis

A typical chromatogram of domperidone and its major metabolites detected in incubations performed with human liver microsomes is shown in Fig. 2A. Retention times were 4.2, 4.7, 5.1, 5.7, 6.5 and 12.1 min for a hydroxylated metabolite (M1), a N-dealkylated metabolite (M2), a minor hydroxylated metabolite (M4), a major metabolite (5-hydroxydomperidone, M3), internal standard (encainide) and domperidone, respectively. Eluting fractions corresponding to these compounds were collected and reanalysed by LC–MS–MS [4]. Results obtained were compared to data published by Ward et al. and by Meulderman et al. and identification of the various metabolites was reported in Simard et al. [3–5]. As can be noted, our HPLC assay allowed for the detection and separation of four oxidative metabolites of domperidone. Quantification was however feasible only for the three major metabolites using a signal to noise ratio of 5. Even though levels of the minor metabolite (M4) were too low for quantification, complete separation was sough for in order to prevent overestimation of other nearby metabolites. Complete base peak resolution for all metabolites, internal standard and domperidone was achieved with conditions used. A typical chromatogram obtained from a blank and control incubation is shown in Fig. 2B.

3.3. Linearity and sensitivity

Equations determined by linear regression analyses used to determine concentrations of domperidone and its major metabolites were: $y = 0.383x + 0.018$ in the range of 0.1–20 μmol/L ($r^2 = 0.996$; $p < 0.05$), and $y = 0.195x + 0.104$ in the range of 10–250 μmol/L ($r^2 = 0.989$; $p < 0.05$). Mean accuracy values were $102.7 \pm 11.2\%$ and $98.1\% \pm 8.1\%$ for low and high standard curves, respectively (Table 1). Detection limit was 0.06 μmol/L for domperidone and its major metabolites. Intraday and interday coefficients of variation were less than 8% and 11%, respectively (Table 2).

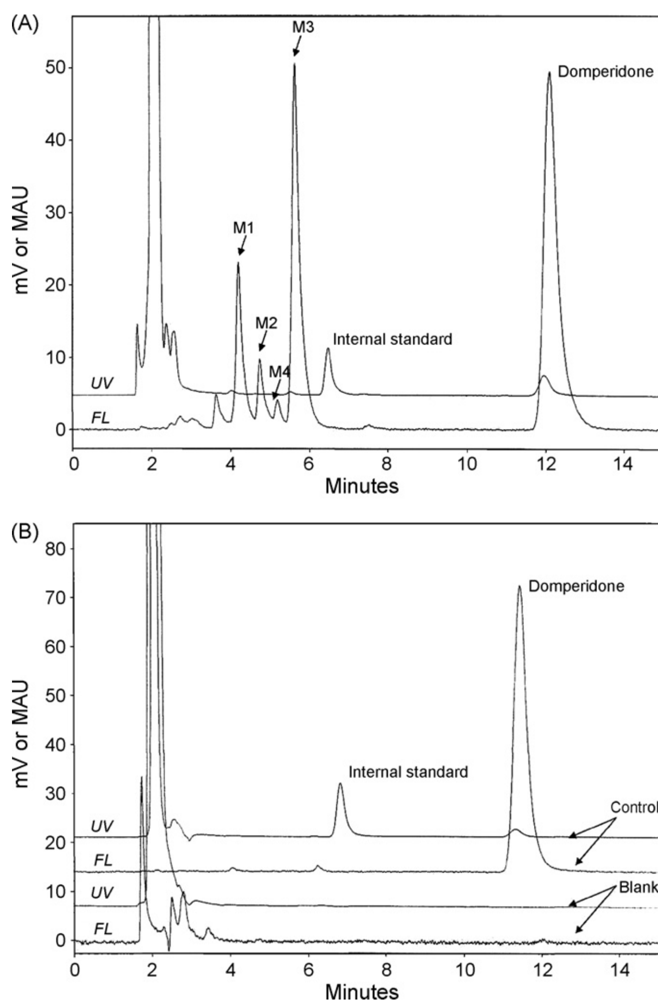

Fig. 2. (A) Typical chromatogram of domperidone and its four major metabolites. Retention time are 4.2, 4.7, 5.1, 5.7, 6.5 and 12.1 min for a hydroxylated metabolite (M1), a N-dealkylated metabolite (M2), a minor hydroxylated metabolite (M4), a major 5-hydroxylated metabolite (M3), internal standard (encainide) and domperidone, respectively. (B) Typical chromatogram obtained from a blank and control incubation (incubation performed without NADPH regenerating system).

3.4. Application to in vitro drug metabolism studies

Our improved HPLC method was applied to the study of the metabolism of domperidone in human liver microsomes. The sensitivity observed with our method using fluorescence detection was sufficient to allow detection of all major metabolites of domperidone. The sensitivity was also sufficient for the determination of enzymatic parameters related to the formation of these metabolites (K_m and V_{max}) and allowed the conduct of inhibition studies. For example, incubations conducted with human liver microsomes in the presence of inhibitors such as imidazole drugs indicated that formation rate of domperidone metabolites was decreased by ∼80% (Fig. 3).

4. Discussion

We have described herein an improved HPLC based on the previously reported assay by Yamamoto et al. for the deter-

Table 1
Accuracy and precision associated with the quantification of domperidone in standard curves

Domperidone (duplicate)		
Spiked concentration (nmol/mL)	Observed concentration (nmol/mL)	Accuracy (%)
Low concentration curve		
0.1	0.13	128.2
0.25	0.24	97.1
0.5	0.49	97.8
1	1.0	100.9
2.5	2.3	92.7
5	4.8	96.7
10	10.9	109.3
20	19.6	98.0
High concentration curve		
10	9.2	92.3
20	19.3	96.6
30	28.3	94.2
50	47.5	94.9
75	71.9	95.8
100	106.6	106.6
150	149.2	99.5
250	247.3	98.9

mination of domperidone in plasma (Table 3) [17]. Mobile phase was redefined to allow for the determination of domperidone and three of its major metabolites formed during *in vitro* drug metabolism studies. The resulting assay is rapid, simple and reproducible and allows separation of domperidone and four major metabolites with a run time of less than 15 min.

Several assays were considered prior to the conduct of our *in vitro* drug metabolism studies with domperidone. Sensitivity (studies performed with limited amounts of human liver microsomes) and specificity (determination of four major metabolites of domperidone) were required characteristics of the selected assay. LC–MS and ESI-MS could have been appropriate but the required equipments were not readily available to us [5,10,15]. We have also considered a radioimmunoassay procedure using antibodies raised against domperidone that was described for the quantification of domperidone in plasma. Although this assay would have provided enough sensitivity, it was known that hydroxylated metabolites of domperidone show cross-reactivity with the parent compound [3,11,13,14]. We also considered the HPLC assay by Meuldermans et al. for domperidone and its metabolites [3]. Unfortunately, the mobile phase also had to be modified because of interfering peaks with the metabolites and sensitivity was not sufficient enough due to UV detection [3]. Several studies have been developed using ^{14}C-labelled dom-

Table 2
Reproducibility of domperidone analysis

Metabolite 1		Metabolite 2		Metabolite 3		Domperidone	
Concentrations (μmol/L)	Coefficient of variation (%)	Concentrations (μmol/L)	Coefficient of variation (%)	Concentrations (μmol/L)	Coefficient of variation (%)	Concentrations (μmol/L)	Coefficient of variation (%)
(a) Intraday coefficients of variation ($n = 6$)							
0.3	6.6	0.1	7.4	1.5	2.1	0.6	2.0
0.5	6.1	0.3	5.1	2.5	1.6	1.7	1.0
1.7	1.4	1.0	4.9	7.9	1.3	9.4	1.3
3.5	2.6	2.3	1.4	12.5	2.4	31.7	0.7
6.4	1.7	4.8	2.9	16.4	2.6	71.5	0.4
(b) Interday coefficients of variation ($n = 3$)							
0.3	10.8	0.14	10.5	1.5	6.1	0.6	8.8
0.5	6.1	0.3	7.2	2.5	4.9	1.7	7.8
1.7	2.9	1.0	8.4	7.9	5.6	9.4	5.8
3.5	2.9	2.3	8.0	12.5	6.1	31.7	7.7
6.4	9.1	4.8	8.8	16.4	7.5	71.5	8.0

Table 3
Comparison of the herein described and previously reported assays for domperidone

	Yamamoto et al.	Kobylinska et al.	Ward et al.	Michaud et al.
Accuracy	<110%	94–105.6%	ND	100.5 ± 9.9%
Intra-day variability	5%	<10%	ND	<8%
Inter-day variability	ND	<10%	ND	<11%
LOQ	1 ng/mL	1 ng/mL	ND	0.06 μmol/L (25 ng/mL)
Sample preparation	Liquid–liquid extraction	Solid-phase extraction using nitrile SPE cartridges	No extraction required	No extraction required
Analysis time	Total analysis time of 8.6 min for domperidone only	Total analysis time of 7.62 min for domperidone only	Baseline resolution of domperidone and 3 metabolites within 15 min	Baseline resolution of domperidone and 4 metabolites within 13 min
Medium	Suitable for detection of domperidone in plasma	Suitable for detection of domperidone in plasma	Suitable for *in vitro* drug metabolism studies	Suitable for *in vitro* drug metabolism studies

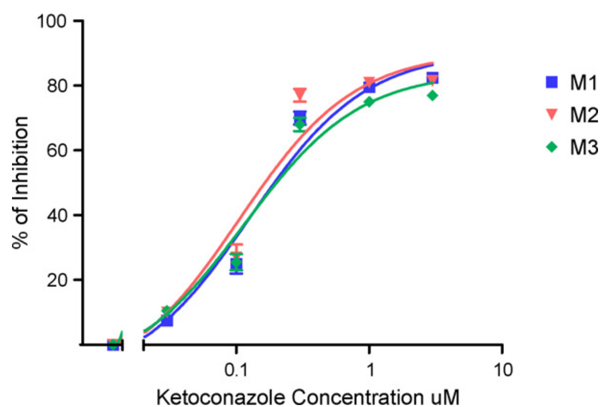

Fig. 3. Representative kinetic plots for the inhibition of the formation rate of major metabolites of domperidone. Each data point represents the mean of duplicate experiments. M1; hydroxylated metabolite, M2; N-dealkylated metabolite and M3; major 5-hydroxylated metabolite.

peridone [3,11–13]. HPLC chromatography coupled with liquid scintillation spectrometry does not offer great peak separation or requires very long time run. Some metabolites may also be missed depending on the labelling site of the parent drug.

Kobylinska et al. described a quantitative HPLC assay for the determination of domperidone in plasma using fluorescence detection [9]. Detection limit for domperidone was also 1 ng/mL (2.37 nmol/L) but required a post-column photoreactor. Post-column derivatization is generally associated with a decrease in peak resolution which would have prevented base peak resolution of the four metabolites of domperidone or would have required longer run time.

Our assay suffers from an important limitation. Indeed, none of the pure substances of the metabolites of domperidone were available for the conduct of our experiments. For quantitative purposes, we therefore had to use standard curves constructed for domperidone and applied them to metabolites. The risk for potential error was limited by the fact that elution times and characteristics of domperidone and its metabolites were very similar. Retention times varied only from 4.2 to 12.1 min for all 5 compounds. Peaks were all very symmetrical and no tailing was observed. The ratio of peak width at half peak height over retention time was 0.35 for M1 (retention time 4.2 min), 0.32 for N-dealkylated domperidone (M2, 4.7 min), 0.29 for a minor hydroxylated metabolite (M4, 5.1 min), 0.35 for 5-hydroxydomperidone (M3, 5.7 min) and 0.33 for domperidone. To estimate the potential error, we analysed several incubation mixtures with various concentrations of domperidone (as substrate) and its metabolites and we compared it to respective control incubations at the same concentrations of domperidone. We could demonstrate that the sum of the area under the curve of the peak of each metabolite added to that of domperidone (AUC of domperidone plus AUCs of its metabolites) in incubations mixtures was similar to AUC of corresponding control incubations. Different concentrations were analysed and various incubations were performed with different enzymatic sources (human liver microsomes, mouse liver microsomes, Supersomes) to evaluate relative peak intensities of metabolites. Results obtained converged and suggested that fluorescence coefficients of domperidone and its metabolites are similar.

5. Conclusion

In summary, the herein described assay is the first HPLC method that permits determination of domperidone and three of its major metabolites in *in vitro* incubation mixtures. This HPLC method is simple, specific and reproducible, requires minimal sample preparation and is suitable for the conduct of *in vitro* drug–drug interaction studies.

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