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Use of electrospray ionization liquid chromatography–mass spectrometry to study the role of CYP2D6 in the in vitro metabolism of 5-hydroxytryptamine receptor antagonists

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Abstract

An electrospray ionization liquid chromatographic–mass spectrometric (ESI–LC–MS) method has been developed to study the involvement of the cytochrome P450 isoenzyme CYP2D6 in the in vitro metabolism of the indole containing 5-hydroxytryptamine (5-HT_3) receptor antagonists tropisetron, ondansetron and dolasetron in human liver microsomes. Compounds were eluted using linear gradients of acetonitrile–20 m*M* ammonium acetate, solvent A, (10:90, v/v) (pH 6.0) and solvent B, (60:40, v/v) (pH 6.0) and a Nucleosil C_4 column. Microsomal incubations were analysed using selected ion monitoring of the molecular ion of parent drug and the molecular ion of hydroxylated metabolites. The involvement of CYP2D6 in drug metabolism was assessed by inhibition studies using quinidine (5 μM), a specific inhibitor of human CYP2D6, as well as by incubating compounds with microsomes prepared from cells transfected with cDNA encoding human CYP2D6. Results showed that the oxidation of all three compounds involved CYP2D6, but only that of tropisetron was inhibited by over 90% in the presence of quinidine. The present method can be applied to pre-clinical compounds, at an early stage of drug discovery, to assess the involvement of CYP2D6 in their metabolism and to screen for those compounds where CYP2D6 is the only isoenzyme implicated in the formation of major metabolites.

Keywords: Cytochrome P450 isoenzyme; CYP2D6; 5-Hydroxytryptamine; Tropisetron; Ondansetron; Dolasetron

1. Introduction

Cytochrome P450 (CYP450) are multiple isoenzymes involved in the oxidative metabolism of a wide range of endogenous and exogenous compounds. Genetic polymorphism(s) of drug oxidations related to specific CYP450 isoenzymes are now well documented [1]. For example, the CYP2D6 isoenzyme is responsible for the human defect in drug oxidation known as the "debrisoquine/sparteine" polymorphism [2,3]. This polymorphism occurs in approximatively 5–10% of the White European and North American population but only 1–2% in Asian or Oriental populations [1]. Individuals can be phenotyped into "extensive" (EM) and "poor" metabolizers (PM) by administering debrisoquine, sparteine or dextromethorphan and collecting urine in order to calculate the metabolic ratio [4]. More recently, polymerase chain reaction (PCR) methods have become available [5], and individuals can be genotyped using a small sample of blood. A remark-

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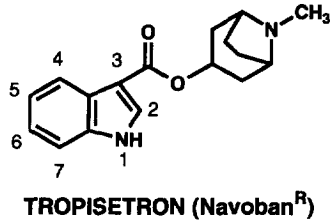
able characteristic of substrates of this isoenzyme is that they all possess one or more basic nitrogen atoms and have a site of oxidation 5–7 Å from the basic nitrogen [6,7]. As genetic polymorphism can lead to inter-individual variability in therapeutic drug response it is important to identify, at an early stage of drug development, the involvement of CYP2D6 in drug metabolism. This is especially true if CYP2D6 is the only isoenzyme implicated in the formation of major metabolites.

In the present paper we describe an electrospray ionization–liquid chromatography–mass spectrometric (ESI–LC–MS) method to study the in vitro metabolism of three indole-containing 5-hydroxytryptamine (5-HT_3) receptor antagonists in human liver microsomes. Tropisetron, ondansetron and dolasetron (Fig. 1) are in clinical use for the treatment of chemotherapy-induced emesis [8–10]. As dolasetron is rapidly reduced by carbonyl reductase to form its major metabolite reduced-dolasetron (red-dolasetron) which is then further metabolized by CYP450 [11], it was thought more appropriate to study the in vitro metabolism of red-dolasetron. Potential CYP2D6 metabolism was assessed by inhibition studies using quinidine, a specific inhibitor of human CYP2D6, as well as by incubating compounds with microsomes prepared from cells transfected with cDNA encoding human CYP2D6.

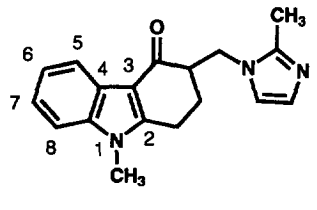
2. Experimental

2.1. Chemicals

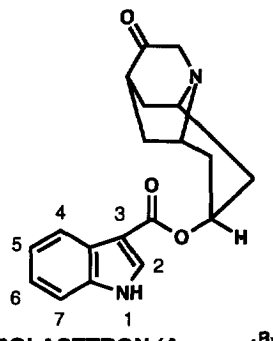
Tropisetron (1*H*-indole-3-carboxylic acid (1αH, 5αH)-8-methyl-8-azabicyclo(3.2.1)oct-3α-yl-ester), tropisetron N-oxide, ondansetron (1,2,3,4-tetrahydro-9-methyl-3-((2-methyl-1*H*-imidazol-1-yl)-methyl)-carbazol-4-one), red-dolasetron ((2α,6α, 8α,9aβ)-octahydro-3-hydroxy-2,6-methano-2*H*-quinolizin-8-yl-1*H*-indole-3-carboxylate,monomethane-sulphonate), 5-, 6- and 7-hydroxy- red-dolasetron and red-dolasetron N-oxide were synthesised in the laboratories of Marion Merrell Dow (Strasbourg, France). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, ammonium acetate and quinidine were obtained from Sigma Chemicals (St. Quentin-Fallavier, France). Acetonitrile was purchased from SDS (Peypin, France). All other re-



TROPISETRON (Navoban^R)



ONDANSETRON (Zofran^R)



DOLASETRON (Anzemet^R)

Fig. 1. Chemical structures of tropisetron, ondansetron and dolasetron.

agents were purchased from commercial sources and were of the purest grade available.

2.2. Microsomes

Human liver samples from two male subjects were obtained under strict ethical conditions from Hautepierre Hospital (Strasbourg, France). Livers were genotyped [5] and were shown to be homozygous 'wild type' extensive metabolisers (EM) for the CYP2D6 polymorphism. Samples were stored at −80°C until use. Microsomes were prepared by homogenising the liver (1:3, w/v) in ice-cold 50 m*M* (pH 7.4) Tris-buffer, 154 m*M* KCl using a Potter–Elvejehem Teflon-glass homogeniser. The homogenate was centrifuged at 10 000 *g* for 30 min at 4°C and the supernatant was then centrifuged at 105 000 *g* for 60 min at 4°C. The resulting pellet was resuspended, to the same volume of supernatant, in homogenising buffer and again centrifuged at 105 000 *g* for 60 min at 4°C. The washed pellets were resuspended in 100 m*M* potassium phosphate buffer (pH 7.4) containing 20% glycerol and the microsomal suspensions were stored in 500 μl aliquots in Eppendorf tubes at −80°C until use.

Human CYP2D6 microsomes were purchased from Gentest Corporation (Woburn, MA, USA). The CYP2D6 microsomes derive from a human AHH-1 TK +/− cell line which was transfected with complementary DNA that encoded human CYP2D6. As native human AHH-1 TK +/− cells contain small amounts of CYP1A1, compounds were also incubated with control microsomes prepared from these native cells

The protein content of the microsomal preparation was measured by the method of Bradford [12] with bovine serum albumin as standard.

2.3. Microsomal incubations

Tropisetron, ondansetron and red-dolasetron metabolism was studied in human liver microsomes, CYP2D6 microsomes and control microsomes at a concentration of 100 μ*M*. The following conditions were used: 1 mg microsomal protein /ml, potassium phosphate buffer (80 m*M*, pH 7.4), $MgCl_2$ (5 m*M*), NADP (1 m*M*), glucose-6-phosphate (10 m*M*) and glucose-6-phosphate dehydrogenase (2 units/ml) in a total volume of 200 μl.

Incubations were performed in duplicate in the presence or absence of quinidine (5 μ*M*), which is a selective inhibitor of CYP2D6 [13]. The inhibitor was added to incubates in Milli-Q water. The reaction was started by the addition of the NADPH generating system and stopped after 60 min at 37°C with an equal volume of methanol and by cooling on ice. Proteins were sedimented by centrifugation at 15 000 *g* for 5 min, and 40 μl of the supernatant were injected into the ESI–LC–MS. The percent peak area of each metabolite formed was calculated with respect to total area of metabolites and parent drug, and the total percentage of all hydroxylated metabolites calculated. The effect of quinidine on the metabolism of the compounds was assessed by comparing the total percentage of hydroxylated metabolites formed in the presence of quinidine with that in corresponding controls, and the mean percent inhibition of the formation of hydroxylated metabolites was calculated. This was used to assess the contribution of CYP2D6 to overall drug hydroxylation.

2.4. Instrumentation

2.4.1. HPLC conditions

The HPLC system consisted of a HP1050 quaternary solvent delivery pump and a HP1050 autosampler (Hewlett-Packard, Les Ulis, France). The HPLC conditions were adapted from a previously published method [14]. A stainless steel (250 × 4.6 mm I.D.) Nucleosil C_4 column (5-μm particle size; Macherey Nagel, Strasbourg, France) was used with a flow-rate of 0.5 ml/min, and column temperature was set at 35°C. Eluants used were solvent A, acetonitrile–20 m*M* ammonium acetate (10:90, v/v) (pH 6.0) and solvent B, acetonitrile–20 m*M* ammonium acetate (60:40, v/v) (pH 6.0). The eluents were filtered through a 0.2-μm Zetapor® membrane (Cuno, Meriden, CT, USA) before use and the pH was adjusted by drop-wise addition of glacial acetic acid.

Red-dolasetron was separated using initial conditions of 100% A followed by a linear gradient to 70% B, in a total run time of 30 min. Tropisetron and ondansetron were eluted using initial conditions

of 20% B followed by a linear gradient to 80% B, in a total run time of 30 min. In both cases, the column was re-equilibrated at initial conditions for 10 min before the next analysis.

2.4.2. ESI–LC–MS

ESI–LC–MS was carried out with a Finnigan SSQ-700 single stage quadrupole instrument (Finnigan, San Jose, CA, USA), fitted with a Finnigan atmospheric pressure ionization (API) ion source which was run in the positive-ion ESI mode. A potential of 4.8 kV was applied to the ESI needle. The pressure of the sheath gas (nitrogen) was set at 40 p.s.i. and the flow-rate of the auxillary gas (nitrogen) was set at 15 ml/min. The temperature of the heated capillary was 230°C. Selected ion monitoring was used to quantify metabolites formed in microsomal incubations.

The $[M+H]^+$ ions of red-dolasetron, and hydroxy or N-oxide metabolites, at respectively m/z 327 and 343 were monitored, whereas for tropisetron, and hydroxy or N-oxide metabolites, the $[M+H]^+$ ions at respectively m/z 285 and 301 were monitored. For ondansetron, and hydroxy ondansetron the $[M+H]^+$ ions at respectively m/z 294 and 310 were selected.

2.5. Reproducibility, recovery, and linearity using red-dolasetron metabolites

As authentic standards of the metabolites of red-dolasetron were available they were used to assess reproducibility, recovery, and linearity of the method. Repeated injections of aqueous solutions of 5-, 6- and 7- hydroxy and N-oxide red-dolasetron (1 nmol/ml) were carried out to assess the variation in detector response, as no internal standard was used. Recovery was determined, at a concentration of 1 nmol/ml, by comparing the area of the 5-, 6- and 7-hydroxy red-dolasetron and red-dolasetron N-oxide peaks in spiked inactivated microsomal preparations to those of directly injected aqueous standard solutions. Calibration curves were obtained by spiking inactivated microsomal preparations with metabolites. Concentrations ranging from 0.25–5 nmol/ml were used for the calibration curves. Linear regression analysis of the peak area versus the concentration of red-dolasetron metabolites was performed.

2.6. Within-series precision of the complete method

Replicate microsomal incubations ($n=6$) of red-dolasetron were carried out on the same day, as described above, to assess the overall within-series precision of the method.

3. Results

3.1. Reproducibility, recovery, and linearity using red-dolasetron metabolites

Repetitive injection ($n = 6$) of an aqueous solution containing 1 nmol/ml of 5-, 6-, 7-hydroxy and red-dolasetron N-oxide showed a C.V. (%) of 2.07, 1.25, 0.96 and 1.49% respectively for each compound. Recoveries of the metabolites at a concentration of 1 nmol/ml from inactivated liver microsomes were greater than 78.5% except for 7-hydroxy red-dolasetron, which appeared lower, but could not be established with high precision because this concentration was close to the limit of detection for this compound (Table 1). Calibration curves were linear over the concentration range of 0.25–5.0 nmol/ml in inactivated microsomal preparations, with correlation coefficients greater than 0.99. As the variation in detector response was low, and calibration curves were linear, it was considered unnecessary to include an internal standard, especially as samples were injected directly after protein precipitation.

3.2. Within-series precision of the complete method

Results, expressed as percent total peak area for each metabolite formed, are shown in Table 2. Replicate microsomal incubations ($n=6$) of red-

Table 1
Recovery of red-dolasetron metabolites

Metabolites	Recovery (%) (mean ± S.D., n =5)
5-OH	95.6 ± 18.2
6-OH	78.5 ± 15.1
7-OH	67.5 ± 20.9
N-Oxide	108.7 ± 18.5

Table 2
Within-series precision for the measurement of the formation of red-dolasetron metabolites using the described method. Replicate microsomal incubations (n=6) of red-dolasetron were carried out on the same day. (Results are expressed as percent total peak area for each metabolite formed with respect to total area of metabolites and parent drug)

No.	5-OH	6-OH	7-OH	N-Oxide	Red-dolasetron
1	0.67	5.17	0.23	4.60	89.27
2	0.60	5.07	0.23	3.70	90.37
3	0.63	5.10	0.20	4.23	89.80
4	0.73	5.47	0.17	4.87	88.80
5	0.60	5.30	0.27	4.10	89.73
6	0.83	5.93	0.30	4.37	88.60
Mean	0.68	5.34	0.23	4.31	89.43
S.D.	0.09	0.33	0.05	0.40	0.67
C.V.(%)	13.44	6.11	20.20	9.39	0.74

dolasetron showed an overall C.V.(%) of less than 14% for the formation of metabolites, except for 7-hydroxy red-dolasetron where the C.V.(%) was around 20%.

3.3. Incubation of red-dolasetron, tropisetron and ondansetron with microsomes

All the metabolite peaks of red-dolasetron were identified by co-chromatography of reference compounds on ESI–LC–MS. Human liver microsomes produced 5-, 6- and 7-hydroxy red-dolasetron and also red-dolasetron N-oxide (Fig. 2A). The major metabolite formed was 6-hydroxy red-dolasetron. It can be clearly seen in Fig. 2B, that quinidine (5 μM) decreased the formation of 5-, 6- and 7-hydroxy red-dolasetron. When control incubations were compared with incubations in the presence of quinidine, 73% of the overall formation of hydroxylated metabolites was inhibited. CYP2D6 is thus the major isoform that oxidizes red-dolasetron. Red-dolasetron was also metabolised by microsomes from human CYP2D6 transfected cells (Fig. 2C) to give the same three hydroxylated metabolites. Reduced dolasetron N-oxide was not detected in this incubation system.

After incubation with tropisetron, human liver microsomes formed one major and two minor metabolites characterized by ions at m/z 301 corresponding to the addition of 16 a.m.u. (Fig. 3A). As authentic tropisetron N-oxide had a different retention time, these compounds were considered to be hydroxylated metabolites. Tropisetron has been previously shown to be hydroxylated at positions 5, 6 and 7 of its indole ring [15]. It can be clearly seen in Fig. 3B, that quinidine (5 μM) dramatically decreased (91%) the overall formation of hydroxylated metabolites. Thus CYP2D6 is almost exclusively involved in tropisetron hydroxylation. Tropisetron was also metabolised by microsomes from human CYP2D6 transfected cells (Fig. 3C) to give the same three hydroxylated metabolites.

Ondansetron was metabolised by human liver microsomes (Fig. 4A) to form two major metabolites characterised by ions at m/z 310, again corresponding to the addition of 16 a.m.u. As no N-oxidation of ondansetron has been reported [15,16], these compounds were considered to be hydroxylated metabolites. Quinidine, as shown in Fig. 4B, decreased the formation of the hydroxylated metabolites by 36%. Ondansetron was also metabolised by microsomes from human CYP2D6 transfected cells to form the same hydroxylated metabolites (Fig. 4C). Thus, for ondansetron, CYP2D6 plays a minor role in drug hydroxylation.

No metabolism was seen when the 5-HT_3 antagonists were incubated with control microsomes prepared from native cells, which contain small amounts of CYP1A1.

4. Discussion

Mass spectrometry is a useful tool for drug metabolism studies. Liquid chromatography–mass spectrometry (LC–MS) combines the temporal resolution of HPLC with the mass resolution and sensitivity of mass spectrometry and permits the analysis of small amounts of non-volatile drugs or polar metabolites in complex biological matrices. The ESI technique is extremely versatile and allows the analysis of compounds of widely varying polarity [17,18]. The present ESI–LC–MS method has also been applied to studies on the metabolic pathways of pre-clinical compounds.

The involvement of CYP2D6 in a metabolic pathway was confirmed in two different ways. Firstly, the use of quinidine, which is a specific inhibitor of CYP2D6 [13], permits the assessment of the relative importance of CYP2D6 in the formation

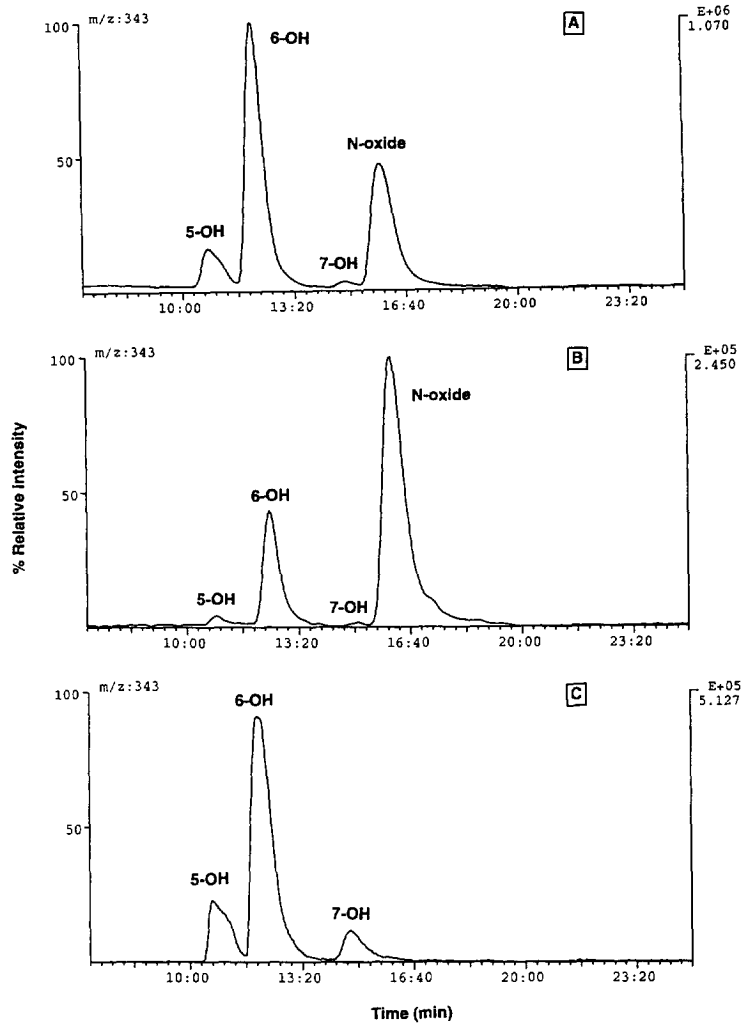


Fig. 2. ESI–LC–MS selected ion chromatograms (m/z at 343) of red-dolasetron metabolites formed by (A) human liver microsomes, (B) human liver microsomes in the presence of quinidine (5 μM) and (C) microsomes from CYP2D6 transfected cells, incubated with red-dolasetron (100 μM).

of major metabolites. Secondly, the use of microsomes from CYP2D6 transfected cells allows the identification of CYP2D6 substrates, as well as confirming the formation of a particular metabolite by CYP2D6. These two techniques can thus help the selection of suitable compounds for further drug development. The present method can be applied to pre-clinical compounds, at an early stage of drug discovery, to assess the involvement of CYP2D6 in their metabolism and to screen out those compounds where CYP2D6 is the only isoenzyme implicated in the formation of major metabolites. As genetic polymorphism can lead to inter-individual variability in therapeutic drug response which may cause adverse drug reactions, it is important to identify, at this early stage, the relative importance of CYP2D6 in drug metabolism.

Although CYP2D6 is implicated in the metabolism of all three compounds, the impact on their elimination in vivo will depend on the extent of metabolic clearance compared to renal clearance and, assuming that metabolic clearance is carried out by CYP450 isoenzymes, to the relative importance of CYP2D6 in metabolic clearance. Tropisetron and

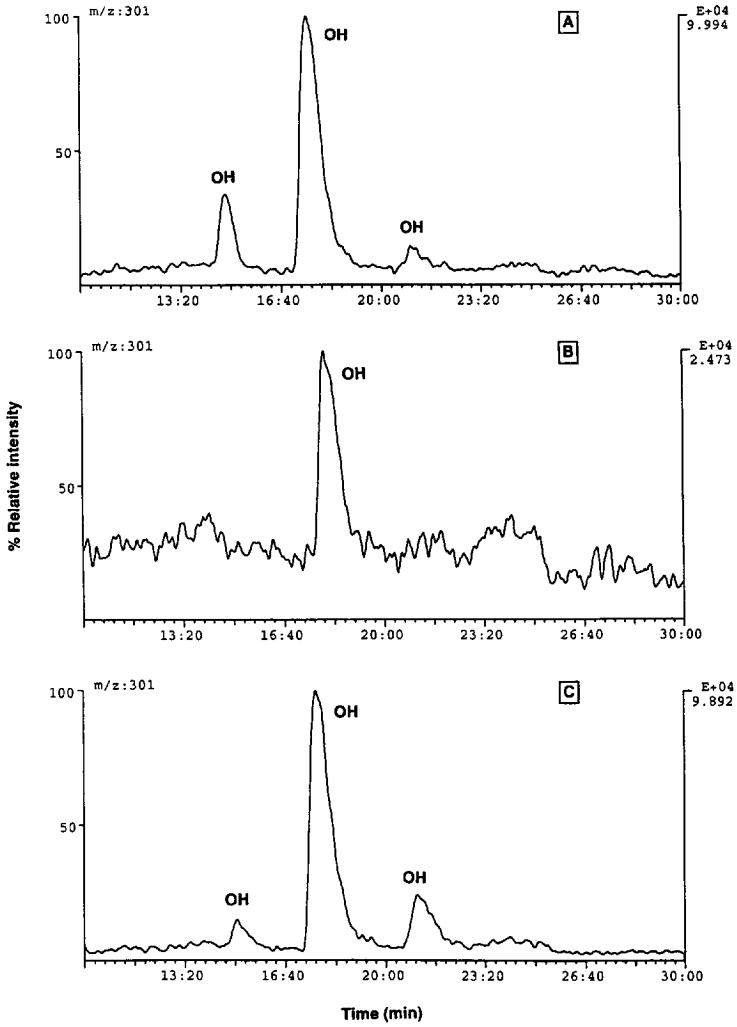


Fig. 3. ESI–LC–MS selected ion chromatograms (m/z at 301) of hydroxylated metabolites of tropisetron (OH) formed by (A) human liver microsomes, (B) human liver microsomes in the presence of quinidine (5 μM) and (C) microsomes from CYP2D6 transfected cells, incubated with tropisetron (100 μM).

ondansetron both have low renal and high metabolic clearance [8,19], but only tropisetron, which was over 90% metabolized by CYP2D6, shows pharmacokinetic consequences of lower clearance and increased half-life in PM subjects [8]. As ondansetron is only partially metabolized by CYP2D6, polymorphic drug metabolism is not evident, and no pharmacokinetic consequences are seen in PM subjects [19]. Dolasetron is rapidly reduced in man by carbonyl reductase [11] to give the major metabolite, red-dolasetron, which has high renal clearance as well as being largely metabolized by CYP2D6. As renal clearance of red-dolasetron can compensate for decreased metabolism in PM subjects, polymorphic drug metabolism of dolasetron is not apparent [20].

In conclusion, the present method, using human liver microsomes in the presence of a specific inhibitor of CYP2D6 as well as microsomes prepared from cells transfected with cDNA encoding for CYP2D6, allows the study of the relative involvement of this isoenzyme in the metabolism of indole containing 5-HT_3 antagonists. Although in

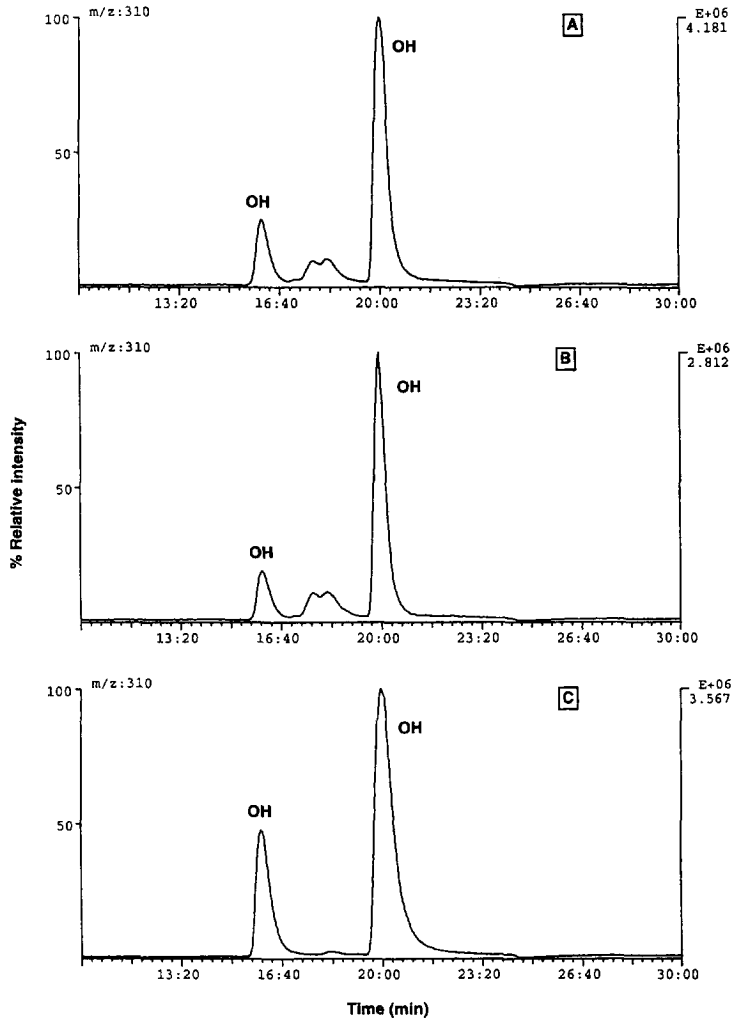


Fig. 4. ESI–LC–MS selected ion chromatograms (m/z at 310) of hydroxylated metabolites of ondansetron (OH) formed by (A) human liver microsomes, (B) human liver microsomes in the presence of quinidine (5 μM) and (C) microsomes from CYP2D6 transfected cells, incubated with ondansetron (100 μM).

vitro results predicted potential CYP2D6 polymorphic drug metabolism, correlations from in vitro to in vivo need to be done with caution, as in vivo both metabolic and renal clearance can be involved in drug elimination. If a compound is seen to demonstrate polymorphic drug metabolism it may be necessary to complete these in vitro studies with in vivo studies where the involvement of renal clearance can be assessed. The present ESI–LC–MS methodology can then be used to measure parent drug and metabolites in urine.

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