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High-performance liquid chromatographic methods for the analysis of haloperidol and chlorpromazine metabolism in vitro by purified cytochrome P450 isoforms[1]

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Abstract

Sensitive HPLC methods for the resolution and quantitation of metabolites of both haloperidol and chlorpromazine metabolism have been developed for use in in vitro reconstitution assays with purified P450 isoforms. Separation of haloperidol metabolites was accomplished using a Hypersil CPS column with a mobile phase of 67% acetonitrile and 10 m*M* ammonium acetate, pH 5.4. Separation of chlorpromazine metabolites was achieved using an Ultrasphere cyano column with a mobile phase of 87.5% acetonitrile, 5% methanol, 3% 0.12 *M* sodium acetate, 3% 0.12 *M* ammonium acetate, 0.01% diethylamine and 0.01% triethylamine, pH 9.5. Sharp resolution was observed for haloperidol and three of its major metabolites and for chlorpromazine and five of its major metabolites. Varying levels and combinations of these metabolites are formed during in vitro incubations of parent compound with purified P450 isoforms 1A1 and 2B1 in a reconstituted system.

Keywords: Haloperidol; Chlorpromazine; Cytochromes

1. Introduction

Haloperidol and chlorpromazine are neuroleptic drugs most commonly used for treatment of schizophrenia and other psychotic disorders. Haloperidol is a butyrophenone derivative and chlorpromazine is a phenothiazine compound. They both act as antagonists of the dopamine-2 receptor, which is thought to play a major part in their mechanism of action. Both drugs are routinely given over courses of months to years and both are associated with sometimes severe and sometimes permanent side effects. Much research has been performed over recent years to elucidate the major routes of metabolism of both compounds and to determine which cytochrome P450 (CYP) isoforms are involved in the various pathways [1–3,12–15,17]. Such information in conjunction with recent evidence which points to differential expression of various CYP isoforms in specific brain locations [4–8] leads to the conclusion

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that these drugs could be metabolized to different products in different regions of the brain. The varying levels of expression of CYP isoforms between individuals and the possibility of differential metabolism in the brain, offer one possible reason why there is such a large range of therapeutic effect and adverse effects of these drugs in the patient population.

Haloperidol is currently known to be metabolized by three pathways (see Fig. 1). *N*-dealkylation and ketone reduction have long been considered the major pathways of metabolism and in recent years a third pathway was found in which haloperidol is metabolized to a series of pyridine compounds, one of which is a pyridinium ion, similar in structure and activity to the neurotoxin *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [9,10]. The involvement of CYP3A4 and CYP2D6 has been shown in the *N*-dealkylation and pyridine pathways [11–13].

N-oxidation has been clearly established as the important metabolic pathway of chlorpromazine in man. Subsequent metabolic studies have shown the formation of a 7-hydroxy, an *N*-desmethyl, a sulfoxide, and an *N*-desmethylsulfoxide metabolite in the human, and rat (see Fig. 2) [14,15]. As of this date, specific CYP isoforms have not been assigned to specific steps in the metabolism of chlorpromazine.

The intent of our laboratory is to assess the differential metabolism of these drugs by CYP isoforms specifically expressed in brain tissue. Therefore, as a prelude to this we have modified existing systems to study both haloperidol and chlorpromazine metabolism in vitro using expressed cytochrome P450 isoforms. We report here the use and efficacy of a method for the determination of haloperidol and chlorpromazine metabolism specifically tailored to measuring in vitro metabolism in purified reconstituted isoform systems.

Studies of in vitro metabolism using purified CYP have some advantages over both in vitro studies using microsomes with various CYP inhibitors or inducers, and in vivo studies. By utilizing purified isoforms, the reconstituted system is completely homogenous, with little ambiguity or confounding of results. The experiments are relatively quick and efficient and highly reproducible. Whereas in vivo studies may contribute more directly to the practical biosciences, well-controlled in vitro data are vital as a prelude and validation of the more costly, often difficult in vivo experiments.

2. Experimental

2.1. Chemicals and reagents

Haloperidol [HAL], (4-(4-chlorophenyl)-4-hydroxypiperidine) [CPHP], (4-(4-chlorophenyl)-alpha-

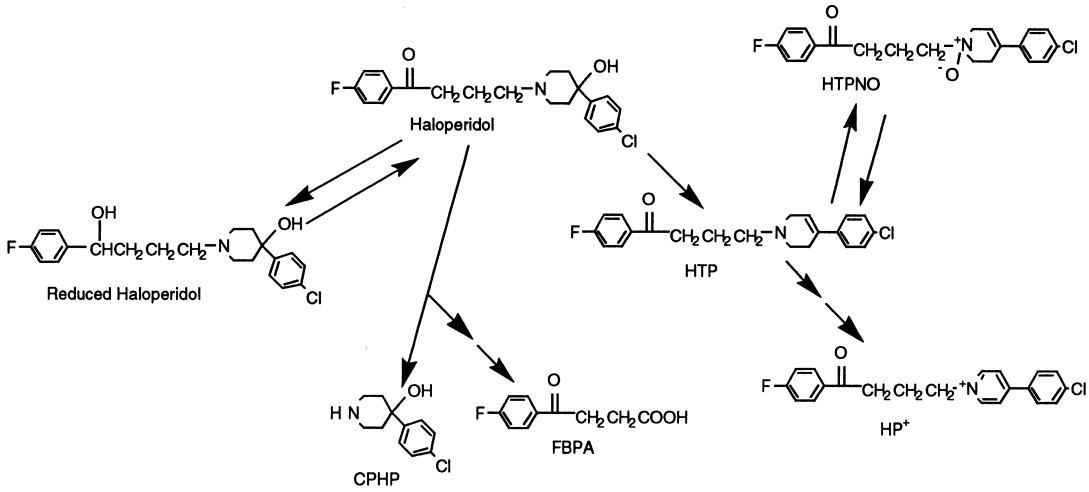

Fig. 1. Major metabolic pathways of haloperidol. CPHP: 4-(4-chlorophenyl)-4- hydroxypiperidine; HTP: Haloperidol 1,2,3,6-tetrahydropyridine; HTPNO: Haloperidol 1,2,3,6-tetrahydropyridine *N*-oxide; HP^+: Haloperidol pyridinium; FBPA: 3-(4-Fluorobenzoyl) propionic acid. Modified from [2].

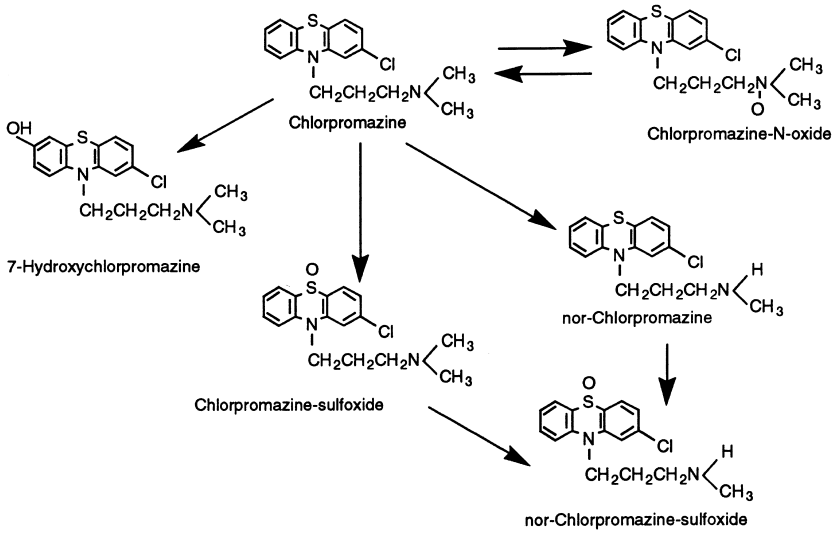

Fig. 2. Major metabolic pathways of chlorpromazine in rat. Modified from [14].

(4-fluorophenyl)-4-hydroxy-1-piperidine butanol [RHAL] and 3-(4-fluorobenzoyl) propionic acid [FBPA] were obtained from Research Biochemicals International (Natick, MA, USA). Pirenzepine was obtained from Sigma (St. Louis, MO, USA).

Chlorpromazine, 7-hydroxychlorpromazine, chlorpromazine-*N*-oxide, nor-chlorpromazine-sulfoxide, chlorpromazine-sulfoxide and nor-chlorpromazine were also obtained from Research Biochemicals International (Natick, MA, USA).

All solvents were HPLC grade from Aldrich (Milwaukee, WI, USA). Other chemicals used were of analytical reagent grade.

CYP1A1 was purified to homogeneity from liver microsomes of β-naphthoflavone-treated rats as described by Saito and Strobel [16]. CYP2B1 was purified to homogeneity from liver microsomes using the same procedure as for CYP1A1 except that rats were treated with phenobarbital.

2.2. Preparation of standards

Stock standard solutions of haloperidol and its metabolites and the internal standard pirenzepine were prepared by dissolving the standard preparations in methanol. Stock standard solutions of chlorpromazine and its metabolites were prepared by dissolving the standard preparations in 0.02 *M* sodium phosphate, pH 5.4.

Each compound was weighed using a Cahn Model C-31 microbalance (Cahn, Cerites, CA, USA) and all stocks were made to a concentration of 1 m*M*. 50 ml of solution were made at a time for each compound and the standard solutions were aliquotted and stored at 4°C in light protected, silanized glass vessels. Working stock solutions were prepared by diluting the stock solution with the respective HPLC mobile phase.

2.3. Chromatographic conditions

Chromatography was performed using a Waters Model 501 solvent pump, Model U6K injector, and Model 486 variable wavelength UV detector (Waters, Milford, MA, USA). Haloperidol and its metabolites were separated by HPLC on a 250×4.6 mm I.D., 5-μm particle size CPS Hypersil Analytical column (Bodman, Aston, PA, USA) with a 10×4.0 mm I.D. guard cartridge containing the same material, using a mobile phase of 67% acetonitrile and 10 m*M* ammonium acetate adjusted to pH 5.4 with approximately 4 ml glacial acetic acid per liter of solution, pumped at a flow-rate of 1.0 ml min^{-1}.

Chlorpromazine and its metabolites were separated by HPLC on a 250×4.6 mm I.D., 5-μm particle size Ultrasphere Cyano Analytical column (Beckman, Palo Alto, CA, USA) with a 45×4.6 mm I.D. guard cartridge containing the same material, using a

mobile phase of 87.5% acetonitrile, 5% methanol, 3% 0.12 *M* sodium acetate, 3% 0.12 *M* ammonium acetate, 0.01% diethylamine and 0.01% triethylamine, adjusted to pH 9.5 with approximately 1 ml of 3 *N* sodium hydroxide per liter of solution, pumped at a flow-rate of 1.0 ml min^{-1}.

The haloperidol HPLC method used is that published by Fang and Gorrod (1993) [17], with modifications in the extraction procedure. The chlorpromazine HPLC method is that published by Jaworski et al. (1988) [14] with modifications in the mobile phase constituents and extraction procedure. All reactions and extractions were performed in silanized 16×125 mm Kimax brand round bottom tubes with rubber-lined caps.

Chromatographs were analyzed using a Waters Baseline data system.

2.4. Metabolism of haloperidol in vitro with purified CYP1A1 and CYP2B1

In vitro metabolism of haloperidol by CYP1A1 and CYP2B1 were determined. Metabolic assays were performed by adding 1.0 m*M* HAL to 0.2 *M* potassium phosphate, pH 7.4, 0.5 m*M* NADPH, 1 unit of cytochrome P450 reductase and 100 pmol of purified P450 isoform in a total volume of 500 μl. 10 μl of 1 mg ml^{-1} L-α-phosphatidylcholine dilauroyl were added to samples containing CYP2B1 to aid reconstitution of enzymatic activity [18]. Lipid was not added to samples containing CYP1A1 since added lipid is not required for efficient reconstitution of enzymatic activity and because the lipid complicates product extraction. Time trials were run to determine the length of time the reaction could run while still remaining linear. After running samples in 15 min increments it was found that the results ceased to be linear after 45 min. Reactions were thus run for 45 min with controls consisting of one sample run without enzyme, one run without NADPH and one run without substrate and terminated by the addition of ethyl acetate (5 ml). By also running a sample of substrate alone, we were able to subtract out the presence of metabolite contaminants in the source haloperidol. A high concentration of HAL is used to assure saturation of enzyme with substrate and to prevent depletion of substrate over the relatively long incubation period. 5.0 μl of 1 m*M* pirenzepine was then added to the samples as an internal standard. Metabolites were extracted from the incubation mixture by first vortex-mixing the tubes and then centrifuging the tubes at 1000×*g* for 10 min to sediment denatured protein. The organic layer was decanted and the samples were dried under nitrogen. Each sample was redissolved in 100 μl of mobile phase and 50 μl was injected on to the HPLC.

2.5. Metabolism of chlorpromazine in vitro with purified CYP1A1 and 2B1

In vitro metabolism of chlorpromazine by CYP1A1 and CYP2B1 was determined in a manner similar to that for haloperidol. Incubations were performed in 0.1 *M* potassium phosphate, pH 7.2 using 100 μ*M* chlorpromazine as substrate, 1.5 m*M* NADPH, 1 unit of cytochrome P450 reductase, and 100 pmol of isoform in a total volume of 500 μl. Lipid was again added to samples containing CYP2B1. Reactions were run for 45 min with the same controls as with haloperidol. As with the haloperidol reaction, a high concentration of chlorpromazine is used to assure saturation of enzyme and to prevent substrate depletion during the incubation. Due to the acidity necessary for chlorpromazine to dissolve into solution only 100 μ*M* concentration is possible without the substrate precipitating out of solution once in the reaction mixture. Thus the concentration used is less than that for haloperidol. The reactions were terminated by addition of 5 ml of ethyl acetate to each sample and the extraction procedure was the same as that used for haloperidol. Each sample was reconstituted in 100 μl mobile phase and 50 μl were injected on to the HPLC for separation.

2.6. Calibration graph

Standards of each available haloperidol and chlorpromazine metabolite were prepared from stock standard solutions at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 n*M* and then extracted using the above method. Calibration graphs were determined from the peak areas of each metabolite over the range of concentrations listed and were found to be linear over the entire range. Extraction

efficiencies of all compounds were determined and varied from 72%–90% with yields only varying 2–5% between experiments.

3. Results and discussion

3.1. Development of the HPLC systems

Haloperidol, RHAL, CPHP and FBPA were quantified by HPLC essentially as described by Fang and Gorrod (1993) [17]. A Hypersil CPS column was used with an isocratic mobile phase consisting of 67% acetonitrile, and 10 m*M* ammonium acetate buffer at pH 5.4, with metabolites detected at 245 nm.

Fig. 3 shows a chromatogram of the separation of haloperidol and three metabolite standards. The retention times (min) were: haloperidol, 9.33; RHAL, 8.18; CPHP, 5.99; FBPA, 4.30. Baseline separation was achieved for all compounds, although quantification of FBPA was difficult since it elutes near the solvent front. A simple, one-step extraction using ethyl acetate was found to be effective with yields varying only 5% between samples on different days.

Chlorpromazine and its five metabolites were quantified by HPLC as described by Jaworski et al. (1988) [14], with some modifications. A Beckman Ultrasphere Cyano column was used, but when samples were run using a mobile phase containing 82.5% acetonitrile and 15.4% methanol, we observed no resolution between chlorpromazine, the 7-hydroxy and the sulfoxide metabolites. By increasing the acetonitrile to 87.5% and decreasing the methanol to 5%, with triple the amount of salts we achieved resolution of all six compounds. The metabolites were detected at 254 nm. Retention times (min) found were: chlorpromazine, 6.68; 7-OH chlorpromazine, 8.09; chlorpromazine-sulfoxide, 13.16; nor-chlorpromazine, 11.46; chlorpromazine-*N*-oxide, 21.05; nor-chlorpromazine-sulfoxide, 23.93. Fig. 4 shows a representative chromatogram of the separation of chlorpromazine and five metabolite standards. The extraction method used was the same as that of the haloperidol assay, and yields varied from 2–4% between experiments on different days.

3.2. Application of the HPLC methods to in vitro metabolism

The in vitro metabolism of CYP1A1 and CYP2B1 were examined using the two HPLC methods presented above. We were able to show evidence of the metabolism of both haloperidol and chlorpromazine,

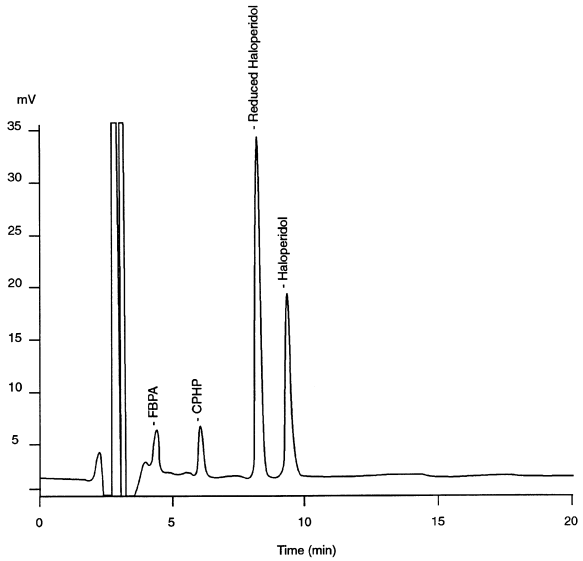

Fig. 3. Chromatogram of the separation of haloperidol and three metabolite standards.

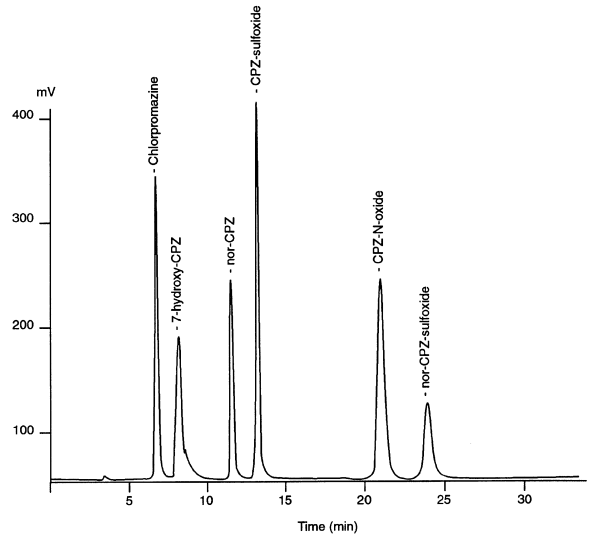

Fig. 4. Chromatogram of the sepration of chlorpromazine and five metabolite standards.

Table 1
Turnover rates of cytochromes P450 1A1 and 2B1 for haloperidol metabolism in vitro

P450 isoform	RHAL (pmol formed min^{-1} $nmol^{-1}$ of protein)	CPHP	U1[a]
1A1	13.3±2.1	68.9±9.4	38.7±5.9
2B1	62.9±11.1	100.7±15.6	–

Metabolic reactions were performed in vitro in the presence of 1 m*M* haloperidol, 0.5 m*M* NADPH, 100 pmol of P450 enzyme and 1 unit of P450 reductase in a total volume of 500 μl. Reaction time was 45 min. Values are the mean of three individual reactions.
[a]Values for unknown peak were estimated from comparing unknown peak areas to those of the other known metabolites, assuming equal molar extinction efficiencies.

in vitro, by both isoforms. The results of the metabolism of haloperidol in vitro are shown in Table 1 and the results of the metabolism of chlorpromazine in vitro are shown in Tables 2 and 3. Fig. 5a and 5b show the chromatograms of the in vitro metabolism of haloperidol by CYP1A1 and CYP2B1 respectively. Fig. 6a and 6b show the chromatograms of the in vitro metabolism of chlorpromazine by CYP1A1 and CYP2B1 respectively.

The major product of in vitro haloperidol metabolism by both CYP1A1 and CYP2B1 was the oxidative *N*-dealkylation metabolite, CPHP. Both CYP1A1 and CYP2B1 showed significant conversion of haloperidol to the ketone reduced metabolite, RHAL,

Table 2
Turnover rates of cytochromes P450 1A1 and 2B1 for chlorpromazine metabolism in vitro

P450 isoform	nor-chlorpromazine (pmol formed min^{-1} $nmol^{-1}$ of protein)	chlorpromazine-sulfoxide	U2[a]
1A1	211.4±19.6	–	–
2B1	258.53±31.8[b]	51.4±11.3	35.0±9.7

Metabolic reactions were performed in vitro in the presence of 0.1 m*M* chlorpromazine, 1.5 m*M* NADPH, 100 pmol of P450 enzyme and 1 unit of P450 reductase in a total volume of 500 μl. Reaction time was 45 min. Values are the mean of three individual reactions.
[a]Values for unknown peak were estimated from comparing unknown peak areas to those of the other known metabolites, assuming equal molar extinction efficiencies.
[b]Value for nor-chlorpromazine formation by 2B1 was calculated at 30 min.

Table 3
Turnover rates over time of cytochromes P450 1A1 and 2B1 for chlorpromazine metabolism in vitro

P450 isoform (time of incubation)		nor-chlorpromazine (pmol formed min^{-1} $nmol^{-1}$ of protein)	chlorpromazine-sulfoxide	U2[a]
1A1	15′	220.1±31.2	–	–
	30′	209.5±30.1	–	–
	45′	211.4±19.6	–	–
	60′	193.9±24.8	–	–
2B1	15′	284.31±22.5	–	–
	30′	258.53±31.8	–	–
	45′	87.3±16.3	51.4±11.3	35.0±9.7
	60′	11.1±7.31	94.4±8.6	60.4±15.2

Metabolic reactions were performed in vitro in the presence of 0.1 m*M* chlorpromazine, 1.5 m*M* NADPH, 100 pmol of P450 enzyme and 1 unit of P450 reductase in a total volume of 500 μl. Values are the mean of three individual reactions.
[a]Values for unknown peak were estimated from comparing unknown peak areas to those of the other known metabolites, assuming equal molar extinction efficiencies.

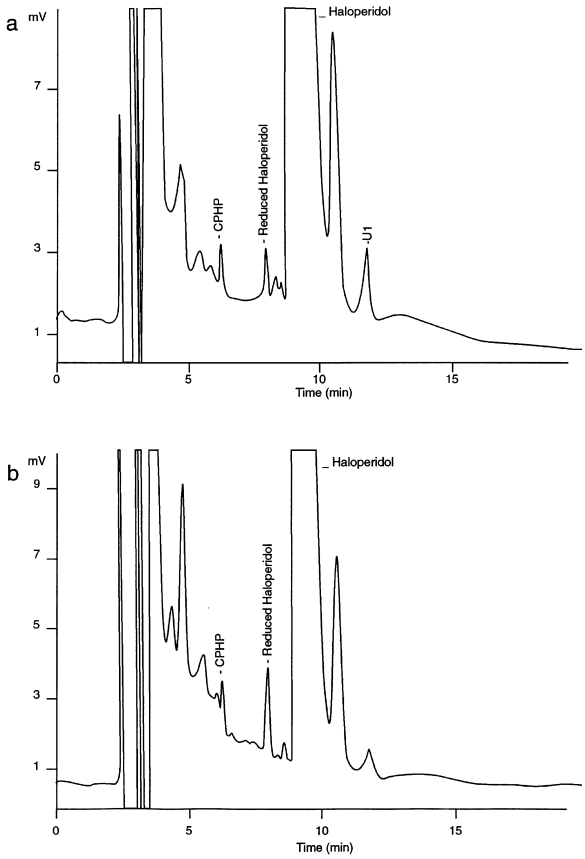

Fig. 5. Chromatograms from the in vitro metabolism of haloperidol by (a) CYP1A1 and (b) CYP2B1.

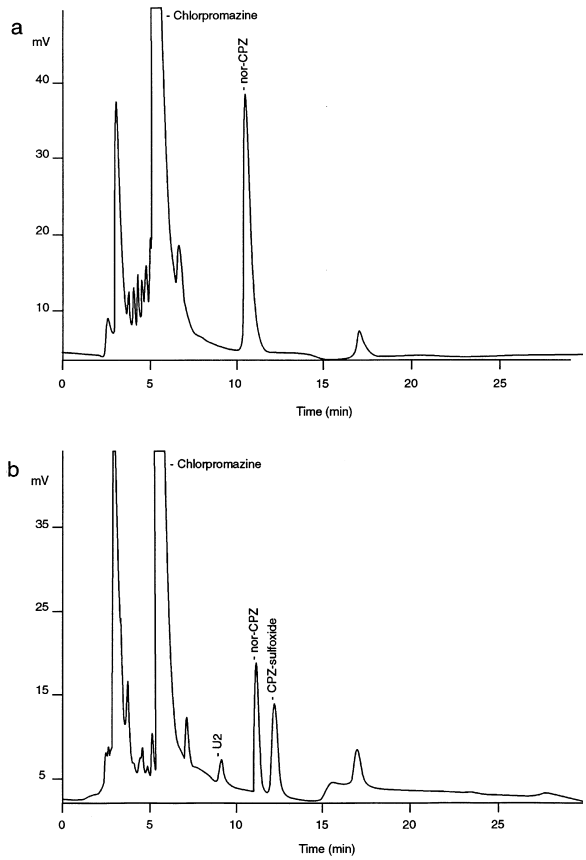

Fig. 6. Chromatograms from the in vitro metabolism of chlorpromazine by (a) CYP1A1 and (b) CYP2B1.

with CYP2B1 having five times the turnover rate of CYP1A1 (Table 1). CYP1A1 was also found to convert haloperidol to an unknown product, U1 (Fig. 5a). These results support the findings of previous studies with liver microsomes in which the *N*-dealkylated and ketone reduced products were shown to be the major products of haloperidol metabolism.

The nor-chlorpromazine metabolite was the only product found in the in vitro metabolism of chlorpromazine by CYP1A1 (Fig. 6a and Table 2). By incubating for increasingly longer periods of time one can see a linear increase in formation of nor-chlorpromazine with almost constant turnover numbers for the first 45 min (Table 3).

CYP2B1, on the other hand, was found to convert chlorpromazine to several different metabolites, with nor-chlorpromazine, and chlorpromazine-sulfoxide being the major metabolites produced. The nor and sulfoxide metabolites were formed in almost equal amounts with the minor formation of an unknown metabolite, U2 (Fig. 6b). When the reaction is followed over time it appears that nor-chlorpromazine is the first product formed. The amount of metabolite increases for the first thirty min, and then actually is depleted over the second thirty min, which allows one to hypothesize that the unknown metabolite is a secondary metabolite of nor-chlorpromazine. The sulfoxide metabolite does not appear in detectable amounts until after the first thirty min of incubation (Table 3). Because of the non-linear product formation in the metabolism of chlorpromazine by CYP2B1 over time, it is difficult to calculate accurate turnover numbers, thus the numbers shown in Table 2 for CYP2B1 are calculated

from the first 30 min of reaction. These results showing *N*-demethylation as the major pathway of metabolism in vitro by CYP1A1 and CYP2B1 are surprising since previous studies measuring chlorpromazine metabolites in plasma after ingestion show the *N*-oxide metabolite to be the major one formed in humans.

4. Conclusions

Two HPLC methods have been developed to study the in vitro metabolism of both haloperidol and chlorpromazine for use with specific P450 isoforms in a reconstituted system. These methods are both sensitive and efficient and should be useful for the characterization of various P450 isoforms as well as for studying drug metabolism in brain.

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References

[1] D. Eyles, J. McGrath, S. Pond, Pyschopharmacology 125 (1996) 214.
[2] J. Gorrod, J. Fang, Xenobiotica 23 (1993) 495.
[3] H. Huang, M. Jann, Y. Tseng, M. Chung, C. Chien, W. Chang, Psych. Res. 57 (1995) 101.
[4] T. Hansson, N. Tindberg, M. Ingelman-Sundberg, C. Kohler, Neuroscience 34 (1990) 451.
[5] K. Hagihara, S. Shiosaka, Y. Lee, J. Kato, O. Hatano, A. Takakusu, Y. Emi, T. Omura, M. Tohyama, Brain Res. 515 (1990) 69.
[6] M. Warner, C. Kohler, T. Hansson, J.-A. Gustafsson, J. Neurochem. 50 (1988) 1057.
[7] J. Kapitulnik, H.V. Gelboin, F.P. Guengerich, D.M. Jacobowitz, Neuroscience 20 (1987) 829.
[8] C. Kohler, J.G. Erikson, T. Hansson, M. Warner, J.-A. Gustafsson, Neurosci. Lett. 84 (1988) 109.
[9] B. Subramanyam, H. Rollema, T. Woolf, N. Castagnoli Jr., Biochem. Biophys. Res. Commun. 166 (1990) 238.
[10] B. Subramanyam, T. Woolf, N. Castagnoli Jr., Chemical Res. Toxicol. 4 (1991) 123.
[11] J. Fang, G. Baker, R. Coutts, P. Silverstone, Cell Mol. Neurobiol. 2 (1997) 227.
[12] E. Usuki, R. Pearce, A. Parkinson, N. Castagnoli Jr., Chem. Res. Toxicol. 9 (1996) 800.
[13] R. Tyndale, W. Kalow, T. Inaba, Br. J. Clin. Pharmacol. 31 (1991) 655.
[14] T. Jaworski, E. Hawes, G. McKay, K. Midha, Xenobiotica 18 (1988) 1439.
[15] T. Jaworski, E. Hawes, G. McKay, K. Midha, Xenobiotica 20 (1990) 107.
[16] T. Saito, H.W. Strobel, J. Biol. Chem. 256 (1981) 984.
[17] J. Fang, J. Gorrod, J. Chromatogr. 614 (1993) 267.
[18] H. Strobel, A. Lu, J. Heidema, M. Coon, J. Biol. Chem. 245 (1970) 4851.