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High-performance liquid chromatographic method for the analysis of imipramine metabolism in vitro by liver and brain microsomes

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Abstract

A new sensitive method for resolution and quantitation metabolites of in vitro imipramine metabolism has been developed for use in liver and brain microsomes. Separation of metabolites was done using a Supelcosil PCN column with a mobile phase of acetonitrile–methanol–potassium phosphate dibasic (40:35:25, v/v/v), pH 7. Resolution is achieved for 2- and 10-hydroxyimipramine, didesmethylimipramine, and desipramine. Varying levels of these metabolites formed during in vitro incubations of rat liver and brain microsomes following treatments.

1. Introduction

Imipramine is a tricyclic antidepressant which has been used for treating the symptoms of major depression since the 1950s. It is a dibenzazepine compound structurally similar to the phenothiazine antipsychotic agents, but pharmacologically distinct for having efficacy in the treatment of depression. Imipramine and related compounds are believed to work by blocking the re-uptake of biogenic amines into neurons, thereby increasing the concentration of neurotransmitters. They are given over a course of weeks to months; a time corresponding to the alleviation of the patient's symptoms. Thus, in order to ensure proper medication, drug levels must be monitored closely. In the clinical setting a number of assays exist to measure the level of imipramine in blood, serum, and plasma [1,2]. These systems, however, are designed to detect a single metabolite of imipramine and are not well suited for in vitro metabolic studies.

Imipramine is metabolized primarily by two pathways: N-demethylation and hydroxylation (Fig. 1). Removal of one methyl group from the aliphatic side chain results in the formation of desipramine, an active metabolite also efficacious for treatment of depression. Hydroxylation of imipramine on the aromatic ring produces 2-hydroxyimipramine, the major hydroxylated metabolite. In humans, formation of this metabolite is associated with the polymorphic cytochrome P-450 2D6 isozyme of the liver [3,4]. Additional pathways of imipramine metabolism are also possible. These include: hydroxylation of the aliphatic portion of the ring to form 10-hydroxy-

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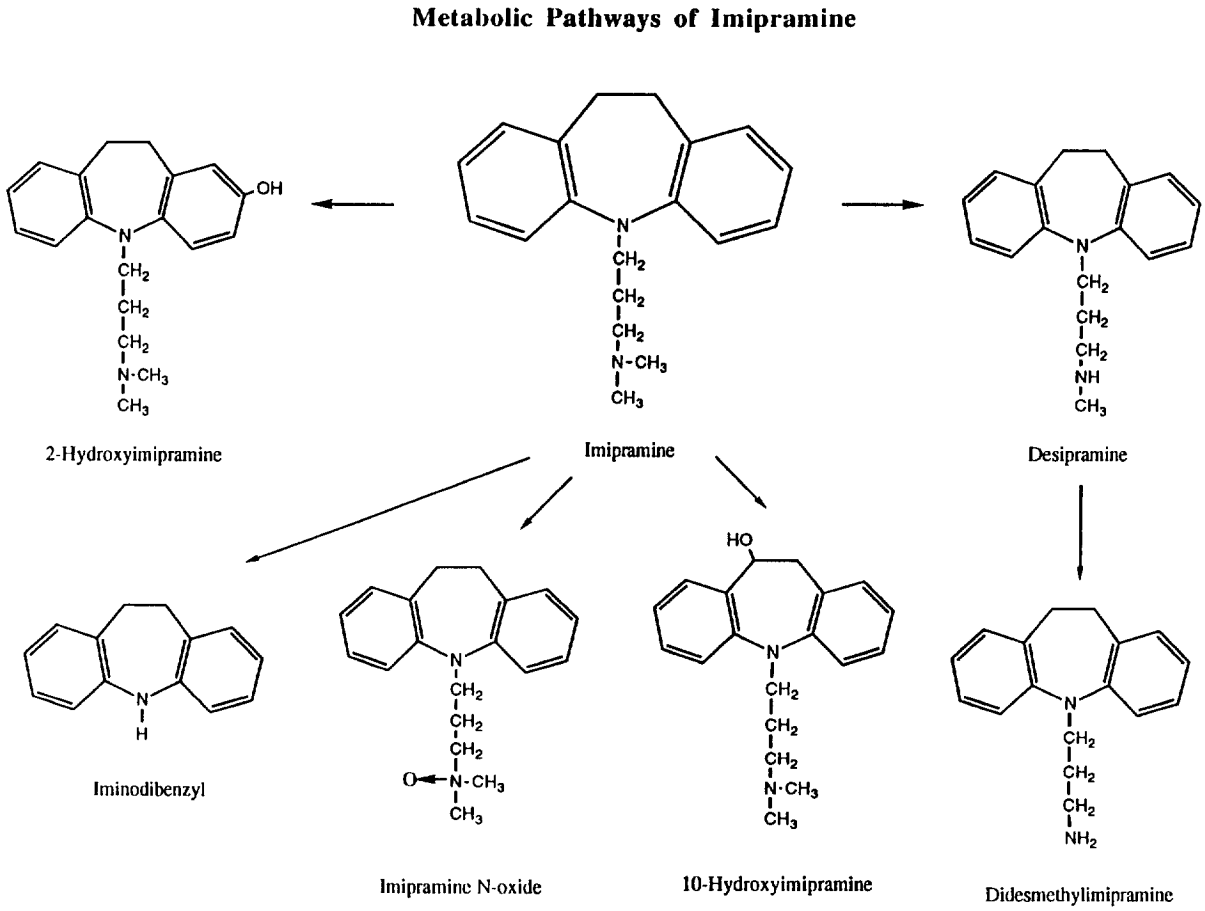

Fig. 1. Structures of imipramine and metabolites.

imipramine; removal of the second methyl group from the aliphatic side chain to form didesmethylimipramine; oxide formation to imipramine N-oxide; and cleavage of the entire aliphatic side chain to make iminodibenzyl (Fig. 1). Forms of cytochrome P-450, in addition to cytochrome P-450 2D6, are responsible for the formation of many of these metabolites in liver [5]. It is the intent of this laboratory to study the metabolism of imipramine not only in the liver, but also in extrahepatic tissues such as brain. Therefore, we have developed a system to study imipramine metabolism in vitro in microsomes from liver and brain. Although a number of methods have been published for the determination of imipramine and one or two metabolites, few look at imipramine metabolism in vitro in extrahepatic tissues. This new method is an effective and highly sensitive means for measuring the metabolism of imipramine in vitro in extrahepatic tissues.

2. Experimental

2.1. Chemicals and reagents

Imipramine hydrochloride and desipramine hydrochloride were obtained from Sigma (St. Louis, MO, USA). 10-hydroxyimipramine, 2-hydroxyimipramine, didesmethylimipramine, and imipramine N-oxide were gifts from Ciba Geigy (Basle, Switzerland). Iminodibenzyl was purchased from Aldrich (Milwaukee, WI, USA). All solvents were HPLC grade (EM Science, Gibbs-

town, NJ, USA). Other chemicals and solvents used were of analytical reagent grade.

2.2. Preparation of standards

Stock standard solutions of imipramine, desipramine, 10-hydroxyimipramine, 2-hydroxyimipramine, and didesmethylimipramine were prepared by dissolving the standard preparations in methanol. Each compound was weighed using a Cahn Model C-31 microbalance (Cahn, Cerritos, CA, USA), and methanol was added to make the stocks 1 m*M*. Working stock solutions were prepared by diluting the stock solution with the HPLC mobile phase. The standard solutions were stored in the refrigerator in sealed silanized glass vessels.

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). The parent drug and metabolites were separated by HPLC on a 250 × 4.6 mm I.D., 5-μm particle size Supelcosil LC-PCN column (Supelco, Bellefonte, PA, USA) using a mobile phase of acetonitrile–methanol–0.01 *M* K_2HPO_4, pH 7 (40:30:25), pumped at a flow-rate of 1.4 ml/min.

Chromatographs were analyzed using a Waters Baseline data system.

2.4. Microsomal sample collection and treatment

Male Sprague Dawley rats weighing between 175 and 200 g were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). The rats were housed in wire-bottomed stainless-steel cages in a temperature- and light-controlled room (25°, 12 h light/dark cycle). Rats were allowed to acclimate for 5 days prior to treatment, and were maintained on an unrestricted diet of rodent chow (Ralston Purina, St. Louis, MO, USA) and water. Rats given phenobarbital received one daily interperitoneal (i.p.) injection of phenobarbital (80 mg/kg) for 4 days. β-naphthoflavone (β-NF) was given as a single i.p. injection at 80 mg/kg β-NF in corn oil for 3 days. Imipramine was administered for 10 days at the dose of 10 mg/kg body weight via daily i.p. injections. All rats were killed by decapitation 24 h following the last injection of treatment. Brains and livers were excised and immediately placed in liquid nitrogen (brain). Liver microsomes were prepared according to the method of Dignam and Strobel [6]. Prior to the microsomal preparation for whole brain, the tissue was thawed in a solution of 1.14% KCl–0.01 *M* EDTA–0.25 m*M* PMSF and the microsomes were previously described [7,8].

2.5. Metabolism of imipramine in vitro by liver and brain microsomes

Metabolism of imipramine was determined in vitro using rat liver and brain microsomes. Imipramine metabolism was performed in 100 m*M* KH_2PO_4, pH 7.25 using 1 m*M* imipramine as substrate, an NADPH-generating system (0.5 m*M* $NADP^+$, 5 m*M* glucose-6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase, and 10 m*M* $MgCl_2$), and 3 mg/ml microsomal protein from liver or brain in a total volume of 1 ml. The reactions were run for 5 and 60 min in liver and brain microsomes, respectively, and terminated by freezing. Metabolites were extracted from liver and brain microsomes using a modification of the method of Suckow and Cooper [9] for extraction of imipramine metabolites from plasma. Following termination of the reaction, 0.5 ml of 2 *M* Na_2CO_3, pH 12 and 5 ml of anhydrous ether were added to each tube. The tubes were vortex-mixed and centrifuged at 1000 *g* for 10 min. The ether layer was decanted and back-extracted into 1 ml of 0.1 *M* HCl. The tubes were again vortex-mixed and centrifuged followed by a second extraction using 0.1 ml of 2 *M* Na_2CO_3, pH 12 and 1 ml ether. The ether layer was decanted and the samples were dried under nitrogen. Each sample was reconstituted using 100 μl of the mobile phase, and 50 μl was used for analysis.

2.6. Calibration graph

Standards at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 n*M* of each imipramine metabolite were prepared from stock standard solutions. A 50-μl volume of these solutions was injected onto the column. Calibration graphs were calculated based upon the peak areas of each compound.

3. Results and discussion

3.1. Development and optimization of the analytical HPLC system

Imipramine and each metabolite standard was dissolved in the HPLC mobile phase and the UV spectrum was measured. The maximum UV absorption of imipramine and its metabolites occurred at 200 nm (Fig. 2). In the majority of HPLC methods using UV detection of tricyclic antidepressants the wavelength usually used is 254 nm. This wavelength is of relatively low sensitivity, and considering that metabolism in extrahepatic tissues is comparably much lower than in liver [10,11], a more sensitive wavelength was needed. Therefore, a wavelength of 214 nm was chosen. This wavelength provides high sensitivity for tricyclic antidepressants, and avoids the problems of using a wavelength such as 200 nm where a number of biological compounds absorb.

Reversed-phase HPLC chromatography of imipramine and its metabolites was investigated on a Supelcosil LC-PCN column. This column is pre-conditioned for analysis of many common tricyclic antidepressants such as imipramine, desipramine, amitriptyline, nortriptyline, and doxepin [12]. It has not been utilized to separate metabolites of imipramine other than desipramine. In order to achieve this, the conditions

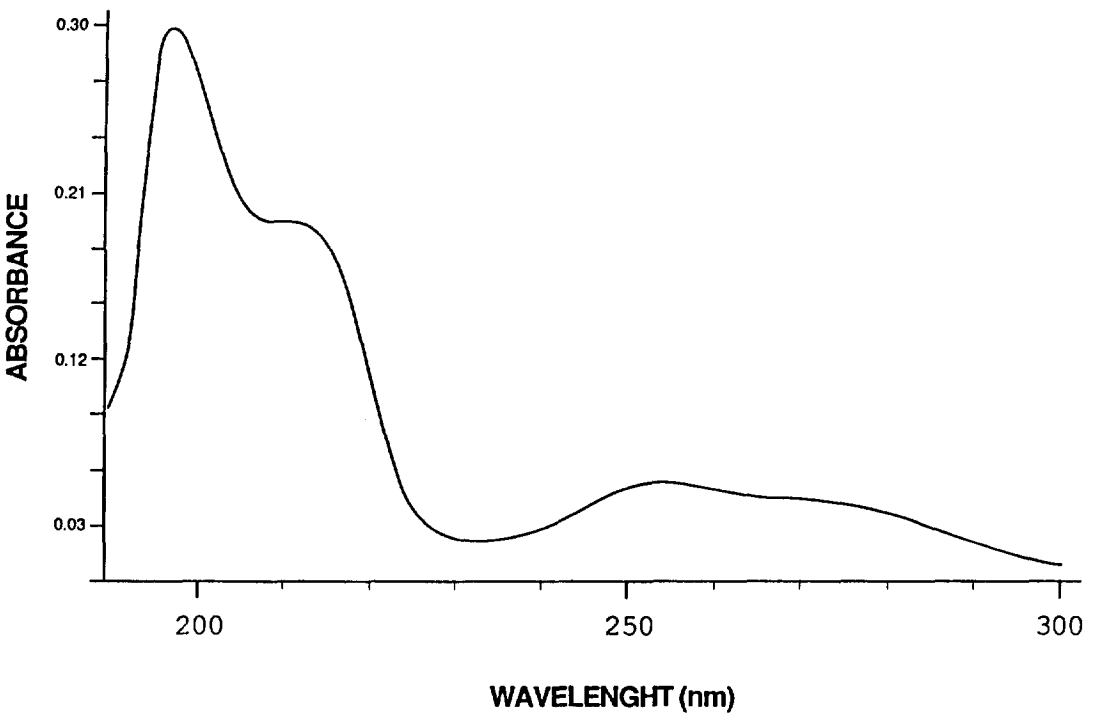

Fig. 2. Absorption spectrum of 10 μM imipramine in the mobile phase. Imipramine was dissolved in acetonitrile–methanol–potassium phosphate dibasic (40:35:25, v/v/v), pH 7. The solution was placed in a quartz cuvette and an absorbance spectrum scanned from 190 to 300 nm using a Hewlett Packard 845A diode-array spectrophotometer.

for separation of imipramine and desipramine were altered to separate the hydroxylated metabolites. Conditions for effective separation were determined by examining changes in the content of mobile phase on the capacity factors, separation factors, and resolution for 2-hydroxyimipramine and 10-hydroxyimipramine (data not shown). Retention of both compounds decreased when acetonitrile and methanol content in the mobile phase were lowered and raised, respectively. Increasing methanol to 35% and decreasing acetonitrile to 40% provided the best resolution of 2-hydroxyimipramine and 10-hydroxyimipramine. Therefore, a mobile phase composed of acetonitrile–methanol (40:35) in 0.01 *M* KH_2PO_4, pH 7 was used for the chromatography. Fig. 3 shows a chromatograph of imipramine and six of its metabolites. The retention times (min) were: iminodibenzyl, 3.03; imipramine N-oxide, 3.43; 10-hydroxyimipramine, 6.18; 2-hydroxyimipramine, 6.58; imipramine, 7.83; didesmethylimipramine, 10.4; and desipramine, 12.9. Baseline separation was achieved for all compounds except 10-hydroxyimipramine and 2-hydroxyimipramine, which was separated to 75% baseline resolution.

3.2. Application of the HPLC method to in vitro metabolism

The HPLC technique described above was used to examine in vitro metabolism of imipramine. Standards were extracted from tissue homogenates containing 3 mg/ml microsomal protein. Due to the limitation of tissue, especially brain microsomes, a single standard concentration (0.1 nmol) of each imipramine metabolite was used to calculate extraction efficiency. Recoveries from microsomal suspensions for the hydroxylated metabolites were 85% and 68% in brain and liver, respectively, and 68% and 60% for demethylated metabolites in brain and liver, respectively.

During the design of this method a suitable internal standard was not found. Tripramine, protriptyline, and clomipramine were each ex-

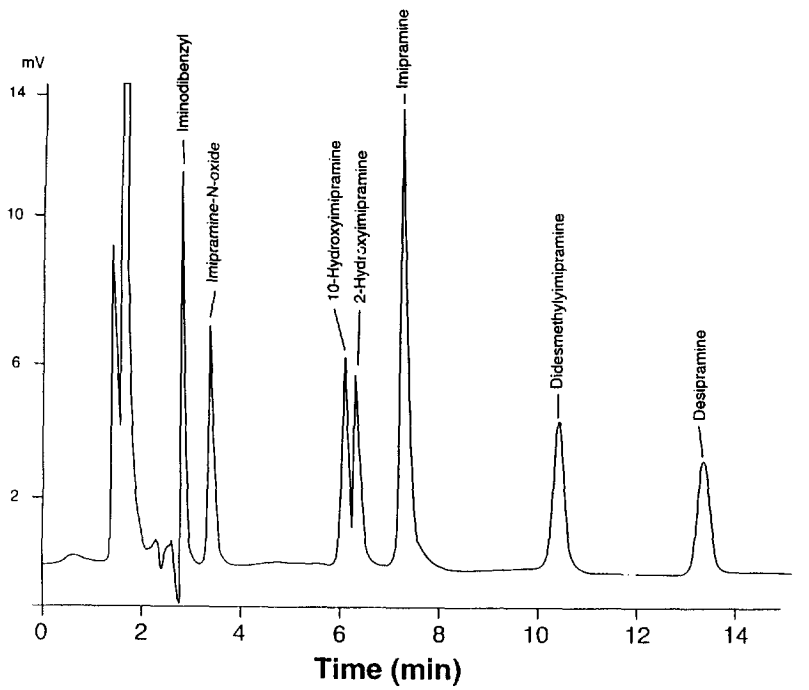

Fig. 3. Chromatogram of the separation of imipramine and six metabolite standards. Mobile phase: acetonitrile–methanol–potassium phosphate dibasic (40:35:25, v/v/v), pH 7, 1.4 ml/min, detection, UV at 214 nm; sample loading 50 pmol.

Table 1
Effects of inducers of cytochrome P-450 on imipramine metabolism in brain microsomes

Treatment	Rate of metabolite formation (pmol/min per mg microsomal protein)			
	10-Hydroxy-imipramine	2-Hydroxy-imipramine	Didesmethyl-imipramine	Desipramine
Control	4.14 ± 0.61	3.56 ± 0.41	–	23.8 ± 4.3
β-Naphthoflavone	3.09 ± 0.23	2.81 ± 0.41	–	37.4 ± 0.76
Phenobarbital	3.54 ± 0.75	2.97 ± 0.81	–	28.6 ± 3.6
Imipramine	4.45 ± 0.40	3.65 ± 0.45	–	28.1 ± 2.4

Imipramine metabolism was performed in vitro in the presence of 1 m*M* imipramine, an NADPH-generating system, and 3 mg/ml of brain microsomes. Reaction time was 1 h. Values are the mean of four determinations on an individual microsomal sample.

amined and determined unacceptable due to incomplete resolution with imipramine metabolites of interest or interference due to unknown peaks in the chromatographs. Therefore, the efficiency of the extractions was calculated by comparing peak areas from directly injected authentic standards of imipramine metabolites to those that were extracted from microsomal tissue. In samples that served as blanks (no imipramine), any unknown peaks resulting from the extraction procedure did not interfere with the separation or resolution of the imipramine metabolites in question (not shown). The limit of detection was 20 pmol/l for 2-hydroxyimipramine and 10-hydroxyimipramine, and 50 pmol/l for desipramine and didesmethylimipramine, (signal-to-noise ratio = 3). The sensitivity of this procedure is similar to that reported by Nielsen and Brøsen [13] for the analysis of imipramine metabolites in human plasma and urine, and for in vitro analysis in human liver microsomes described by Zeugin et al. [14]. Although imipramine metabolism has been examined in vitro in human liver microsomes [14], the purpose of this study was to develop a system capable of monitoring metabolism in brain tissues. Using this method we are able provide evidence for the metabolism of imipramine, in vitro, after incubation with brain microsomes.

The results of imipramine metabolism in vitro using brain and liver microsomes are shown in Tables 1 and 2. Conversion of imipramine to both hydroxylated and N-demethylated products occurred in both brain and liver microsomal suspensions. In brain, both the 2- and 10-hydroxylated metabolites were detected; however, only a single N-demethylated metabolite (desipramine) was formed (Table 1, Fig. 4A). This supports, in part, the in vivo finding by Sugita et al. [15] of the detection of 2-hydroxyimipramine

Table 2
Effects of inducers of cytochrome P-450 on imipramine metabolism in liver microsomes

Treatment	Rate of metabolite formation (nmol/min per mg microsomal protein)			
	10-Hydroxy-imipramine	2-Hydroxy-imipramine	Didesmethyl-imipramine	Desipramine
Control	1.42 ± 0.89	7.88 ± 1.5	–	6.81 ± 2.9
β-Naphthoflavone	1.38 ± 0.21	6.78 ± 1.3	–	4.02 ± 0.82
Phenobarbital	1.87 ± 1.1	5.97 ± 3.0	0.19 ± 0.08	12 ± 6.8
Imipramine	1.54 ± 0.45	6.35 ± 1.2	0.35 ± 0.09	13 ± 3.5

Imipramine metabolism was performed in vitro in the presence of 1 m*M* imipramine, an NADPH-generating system, and 3 mg/ml of liver microsomes. Reaction time was 5 min. Values are the mean of four determinations on an individual microsomal sample.

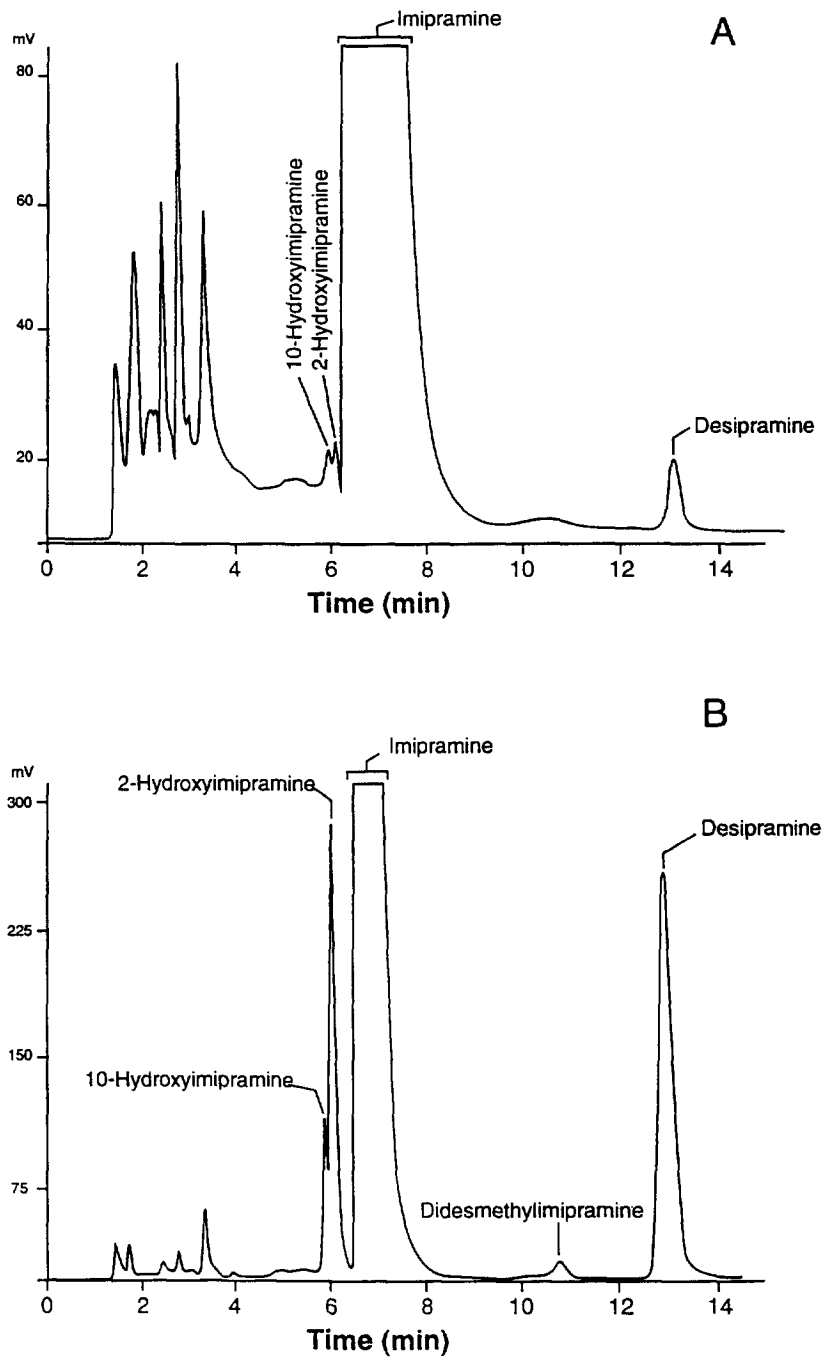

Fig. 4. Chromatograms from in vitro metabolism of imipramine by (A) brain and (B) liver microsomes. Experimental conditions are described in the text.

and desipramine in brain extracts. In contrast to the study of Sugita et al., using our system we were able to detect the formation of 10-hydroxyimipramine in brain in addition to 2-hydroxyimipramine and desipramine.

Liver metabolism of imipramine resulted in formation of 2-hydroxyimipramine, 10-hydroxyimipramine, desipramine, and didesmethylimipramine (Table 2, Fig. 4B). In addition, the rate of 2-hydroxylation was about 5-fold greater than that of 10-hydroxylation. However, unlike the brain where desipramine was the major metabolite detected, the rates for 2-hydroxyimipramine and desipramine formation did not differ greatly. It appears that use of rat microsomes provides an effective in vitro model for imipramine metabolism, since in studies using human liver microsomes a similar metabolic paradigm occurs [14]. Inducers of cytochrome P-450 did not cause any great change in the overall metabolism of imipramine; however, a small increase did occur in formation of desipramine in liver following imipramine treatment.

4. Conclusions

A sensitive and efficient HPLC method to study the in vitro metabolism of imipramine has been developed for use in liver and brain microsomes. The procedure provides baseline separation of most of the imipramine examined, and is useful for studying drug metabolism in brain.

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