CHAPTER 11

OXIDATIVE METABOLISM OF THE ANTIPARKINSON AGENT N-0923 BY RAT-, MONKEY-, AND HUMAN LIVER MICROSOMES.

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ABSTRACT

Oxidative metabolism of the S(-) enantiomer of the potent dopamine D2 agonist 2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin (N-0437) has been investigated using rat-, monkey-, and human liver microsome preparations. The S(-) enantiomer is known as N-0923. Microsomes from monkey showed the highest cytochrome P-450 activity with 8.3 $nmol.min^{-1}.mg^{-1}$ protein, the human material resulted in the lowest capacity of 1.2 $nmol.min^{-1}.mg^{-1}$ protein. Metabolic profiling showed that the three species produced the same metabolites, yet to different extents. The liver microsomes metabolized N-0923 to 2-(N-2-thienylethylamino)-5-hydroxytetralin, 2-(N-propylamino)-5-hydroxytetralin, and 2-(N-propyl-N-2-thienylethylamino)-5,6-dihydroxytetralin, a catechol metabolite.

Dopaminergic affinity of N-0923 and its phase I metabolites was measured *in-vitro* using racemic standards. The N-dealkylated derivatives showed an affinity towards the radioligand, ^{3}H-N-0437, of 99 nM for the despropyl and 1.3 μM for the desthienyl metabolites, which were far lower as the affinity of 4.9 nM of the corresponding racemate of N-0923 which is known as N-0437. The racemic catechol had an affinity of about 7.3 nM. Due to the weak affinities of the N-dealkylated compounds and the high metabolic conversion of the catechol no therapeutic effects of these three compounds may be expected *in-vivo*.

INTRODUCTION

N-0437, 2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin is a highly potent and selective dopamine D2 agonist[1,2]. The highest activity was found to reside in the

S(-) enantiomer, known as N-0923. The structure of the drug is given in Fig. 4. N-0923 is active at both pre- and post-synaptic dopaminergic receptors and therefore has possible therapeutic potentials in Parkinson's disease[3]. N-0923 reduced Parkinson like syndromes in MPTP treated marmosets[4] and it showed pharmacological activity in 6-OHDA lesioned rats[5]. Both pharmacological test models were studied after dermal application.

The metabolic fate of ^{3}H-N-0437 was studied in rats as well as in monkeys[6-8]. The major metabolites were the 5-O-glucuronide- and 5-sulfate conjugates of the phenolic parent structure. Some minor metabolites were found but were not identified due to lack of sufficient material. N-dealkylated metabolites were not found which was partly hampered by the radioactive label in the propyl side chain.

The pharmacokinetics of N-0923 were studied in freely moving Albino Wistar rats. The drug's elimination half-life was found to be in the order of 100 min following an i.v. injection of 10.0 μmol.kg^{-1}. However a high gastrointestinal metabolic conversion to the glucuronide resulted in an oral bioavailability of less than 0.5 percent. Pharmacokinetic studies after intragastric administration of 10.0 μmol.kg^{-1} N-0923 in which blood samples were taken from the portal vein showed that maximally 1% of the dose reached the liver unchanged[9,10]. Absorption and disposition studies using the racemate ^{3}H-N-0437 have shown that, after oral administration, about 50% of the radioactive dose was excreted into the bile and about 5% was excreted into the urine within 8 hours after dosing[6].

The extensive first pass effect after oral administration could be circumvented by means of other administration routes. Following dermal, nasal-, rectal-, or buccal administration bioavailabilities up to 10% were achieved[11,12]. Dermal application and buccal administration showed prolonged absorption and were therefore the most attractive routes for further development. Initial buccal studies in cynomolgus monkeys resulted in similar bioavailability data as found in rats. The bioavailability was about 7% (Swart et al., unpublished results).

Previous investigations on the metabolism of N-0923 in isolated rat liver perfusions showed that N-dealkylation of both the propyl group as well as the thienylethyl group were important metabolic pathways[13].

This paper describes the oxidative metabolism of N-0923 by rat-, monkey-, and

human liver microsomes in an attempt to investigate possible species differences and to identify hitherto unknown metabolites. Metabolic profiles were run by means of reversed phase gradient high performance liquid chromatography with UV detection and off line radioactivity counting. Metabolic identifications were obtained by on line HPLC-atmospheric pressure ionization mass spectrometry.

MATERIALS AND METHODS

Chemicals

The drug S(-)-2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin hydrochloride (N-0923.HCl, I) was obtained from Whitby Research (Richmond, VA, USA). The enantiomer was prepared according to the method described by Ten Hoeve and Wijnberg[14] and converted to the HCl. The enantiomeric purity of N-0923 was found to be higher than 99.9%, by the method of Witte et al.[15]. The pK_a value for the tertiary amine group and the drugs octanol/water partition coefficient (log P) were 7.9 and 3.3 respectively. Tritiated N-0437 was obtained from Amersham (Little Chalfont, UK) with a specific activity of 98.0 Ci.mmol^{-1} and a radiochemical purity of at least 95%, checked by HPLC. The tritium labels were present in the propyl side chain. Unfortunately, ^{3}H-N-0923 and ^{3}H-N-0437 were unstable, giving a chemical disintegration of at least 10% per month. Due to the fact, that no sufficient tritium labelled N-0923 was available, the radioactive incubations were performed using ^{3}H-N-0437.

The compounds (±) 2-(N-2-propyl-N-2-p-fluorophenylethylamino)-5-hydroxytetralin (IS), (±) 2-(N-2-thienylethylamino)-5-hydroxytetralin (II), (±) 2-(N-propylamino)-5-hydroxytetralin (III) and (±) 2-(N-propyl-N-2-thienylethylamino)-5,6-dihydroxytetralin (IV) were synthesized in our laboratory. These compounds were found to be at least 98% pure according to HPLC and TLC.

NADP, glucose-6-phosphate and glucose-6-phosphatedehydrogenase were purchased from Boehringer Mannheim (Mannheim, FRG). Trifluoroacetic acid was from Pierce (Rockford, IL, USA) and human serum albumin fraction V (A-1653) was obtained from Sigma (St. Louis, MO, USA). All other chemicals were analytical reagent grade and obtained from Merck (Darmstadt, FRG). Throughout the study de-ionized water was used (Milli-Q, Purification system, Millipore, Bedford, MA,

USA).

Microsome preparation

Human liver was kindly donated by the Groningen Liver Cell Research Group. Monkey liver (Macaca arctoides) was obtained from TNO (Rijswijk, The Netherlands). The livers were stored in small portions at -80°C for maximally 6 months. Male Albino Wistar rats (CDL, Groningen, The Netherlands) were killed by cervical dislocation and the livers were rapidly removed.

The livers were minced, homogenized at 5°C in a fourfold volume of 0.154 M KCl solution (pH 7.0 by the addition of 0.154 M KOH) by means of a Potter-Elvejehem homogenizer with a teflon pestle (Janke and Kunkel KG, Staufen im Breisgau, FRG). The homogenate was centrifuged at 5°C, 9,000 g for 20 minutes and the supernatant was centrifuged at 5°C, 100,000 g for 60 minutes. The microsomal pellet was suspended in 0.1 M sodium phosphate/0.154 M potassium chloride solution pH 7.4 and again centrifuged for 60 min, 5°C, at 100,000 g. The supernatant was decanted and the pellet was resuspended in 0.1 M sodium phosphate buffer pH 7.4 containing 0.154 M KCl and 1 mM EDTA to provide a protein concentration of about 10 $mg.ml^{-1}$ determined according to Lowry et al.[16]. These suspensions were stored at -80°C in small vials for maximally 6 months.

Incubation procedure

Incubations were performed in polyethylene cups (Eppendorf®, 1.5 ml). The incubation medium in a final volume of 1.0 ml consisted of 0.1 M potassium phosphate pH 7.4, 10 mM $MgCl_2$, 48 mM KCl, 1.0 mM NADP, 10 mM glucose-6-phosphate, 0.1 $U.ml^{-1}$ glucose-6-phosphatedehydrogenase and a substrate concentration of 0.28 mM. The incubations were initiated by the addition of the microsomal protein in a final concentration of about 1 $mg.ml^{-1}$.

The mixtures were incubated for 60 min at 37°C in a water bath with slight agitation. The reactions were terminated by placing the vials for 10 min on ice. After centrifugation of the incubation mixtures at 10,000 rpm for 10 min in a Biofuge A (Heraeus Christ, Osterode am Harz, FRG) a microsomal pellet was obtained. The clear supernatant was decanted and 900 μl were mixed with 100 μl 50 $\mu g.ml^{-1}$ IS

and 100 μl of this mixture were analyzed by HPLC. The IS was added in order to correct for possible changes in retention times during the chromatographic separations. Similar incubations were performed with a substrate concentration of 0.28 mM N-0923 spiked with 111 kBq tritiated N-0437.

Chromatography

a. Apparatus

The chromatographic system consisted of a Spectroflow 400 solvent delivery system (ABI Analytical Kratos Division, Manchester, GB) coupled to a variable wavelength UV spectrophotometer model Spectraflow 757 (Kratos). The UV chromatograms, at 225 nm, were recorded using a Omnischribe recorder (Houston Instruments, USA). Injections were made using a Rheodyne 7125 injection valve, fitted with a 200 μl injection loop (Rheodyne, Cotati, CA, USA). Separations were performed on a stainless steel column (150*4.6 mm i.d.), packed with Nucleosil® 5 C18 (Macherey-Nagel, Düren, FRG), which was thermostatted at 30°C using a column oven (Spark Holland, Emmen, The Netherlands).

b. Gradient elution

Before and during a gradient run the eluent components 0.1% TFA in water (A) and acetonitrile (B) were purged with helium. For the metabolic profiling a linear gradient of 40 min was performed, starting with 90% A/10% B and finishing with 50% A/50% B. In the analysis a flow rate of 1.0 $ml.min^{-1}$ was used. A standard procedure between two subsequent analyses was maintained: The column was washed for 20 min with 50% A/50% B and thereafter, regenerated to the initial conditions by means of a linear 5 min reverse gradient run, followed by at least 10 min of isocratic flushing with 90% A/10% B solution.

c: Radioactive profiling

Microsomes of the three different species were incubated with 0.28 mM N-0923 and 111 kBq tritium-labelled N-0437. After centrifugation of the microsomal proteins 100 μl of the clear supernatant was injected on the HPLC. Detection was performed by UV and off line radioactivity counting. Eluent fractions of 30 sec were

collected and mixed with 2.5 ml Luma Safe (Packard Instruments, Groningen, The Netherlands) and measured in a model LS 1800 scintillation counter (Beckman Industrial Corp., La Habra, CA, USA) for 5 min or 10,000 counts, whatever came first. Quenching was corrected by the H-number method and radioactive profiles were printed after background correction.

d:. Mass spectrometric profiling

The on line LC/MS analyses were performed on a modified R 3010 triple quadrupole mass spectrometer (Delsi-Nermag, Argentueil, France), equipped with a custom-built prototype atmospheric ionization source. The clustering of ions with polar solvent molecules during expansion into the vacuum system was prevented by the use of a curtain of dry nitrogen gas. Nitrogen was also used as the nebulizing gas for the ionspray LC/MS interface. This interface is a pneumatically assisted electrospray system, constructed as a concentric pneumatic nebulizer floating at +3 kV for the production of positive ions[17]. A 10 cm long 0.3 mm o.d. x 0.15 mm i.d. stainless steel capillary was used as the centre tube for the nebulizer. A butt connection was made to an approximately 1 m long x 50 μm i.d. fused silica capillary, which in turn was connected to a pneumatically controlled splitter (Jeol Application Note MS49), mounted on the outlet of the UV detector of the HPLC system. The flow rate into the LC/MS interface was approximately 5 μl.min^{-1}. The ionspray nebulizer was positioned at about 4 cm distance from the sampling orifice of the ion source and about 2 cm off axis. Mass spectra were recorded by repetitive scanning from m/z 150 to m/z 650 at 5.2 sec cycle time. Total ion current traces, reconstructed ion current traces of specific ions, and mass spectra were generated by the NERMAG SIDAR data system.

Dopaminergic affinity

Membrane fractions of calf caudate were prepared and the assays were performed according to the method described by Ensing et al.[18]. Competition experiments were performed by incubating 11 concentrations of the parent drug and the metabolites (up to 10^{-4} M) with a fixed amount of 1 nM ^{3}H-N-0437. Equilibrium dissociation constants were calculated with the ligand curve fitting program

RIARRA[19].

RESULTS

The cytochrome P-450 activity of the various microsome preparations were determined according to the method of Greenlee and Poland[20]. The activities were found to be 3.8, 8.3 and 1.2 $nmol.min^{-1}.mg^{-1}$ microsomal protein for rat, monkey and human liver microsomes, respectively. Oxidative incubations of N-0923 with the three types of microsomes showed comparable patterns in metabolic conversion.

HPLC-UV-RA profiling

Analysis of the incubation mixtures using HPLC-UV and off line radioactivity counting showed no differences in the profiles for the three species. A representative example of a radioactive profile obtained after incubation with monkey liver microsomes and examples of UV chromatograms obtained for the three species are depicted in Fig. 1. It can be seen from the radioactive profile that N-0923 is metabolized to at least five metabolites in which the propyl side chain is still present. The large amount of radioactivity during the first 10 min (peak VII and VIII, see Fig. 1[a] in the RA chromatogram is probably propanal and/or propionic acid, which can be formed after N-dealkylation of the propyl side chain. The UV-detection mode seems to indicate that in addition there is a substantial number of phase I metabolites that do no longer bear the radioactive label. From the UV chromatograms it can be concluded that the various species produced the same metabolites, yet to different extents.

HPLC-UV-MS

Under the experimental conditions used, N-0923 and its phase I metabolites associate with H^+ ions in solution. For each previously observed phase I metabolite as well as newly hypothesized phase I metabolites the ion current profiles were reconstructed for the ion $M.H^+$. Profiles which showed a chromatographic peak clearly discernible from the background noise were investigated further. For each metabolite found in a reconstructed ion current profile, a good quality mass spectrum was obtained from the same data. Reconstructed ion current profiles of

N-0923 and its metabolites are given in Fig. 2. The peaks in Figs. 2[c], 2[d] and 2[e] with $M.H^+$ values of 206, 274 and 332 corresponded to the metabolites III, II and IV in

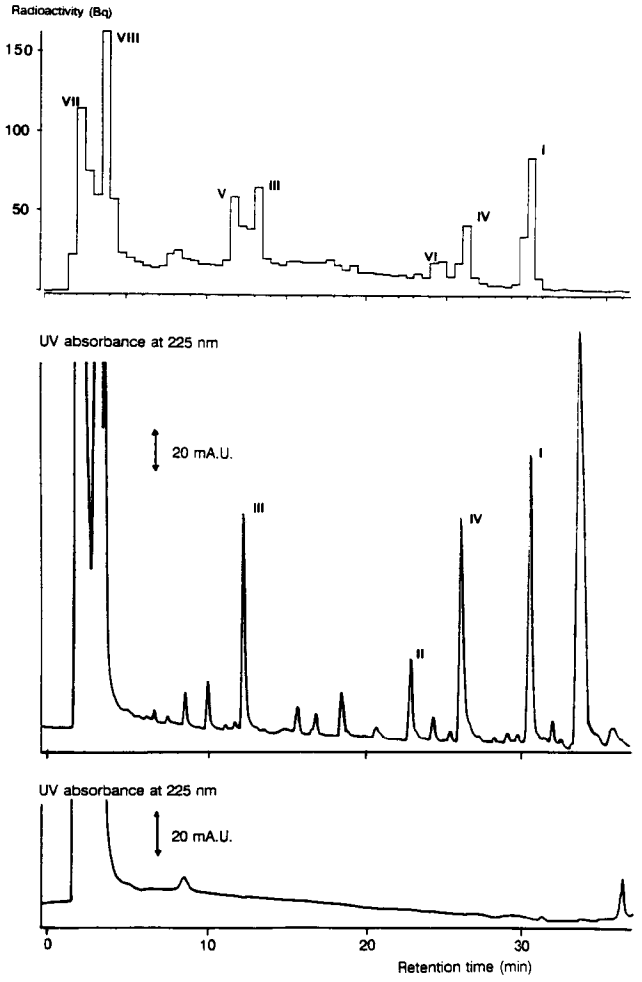

Fig. 1[a]. Radioactive (**top**) and UV (**middle**) and blank UV (**bottom**) profiles after incubation of N-0923 with monkey liver microsomes.

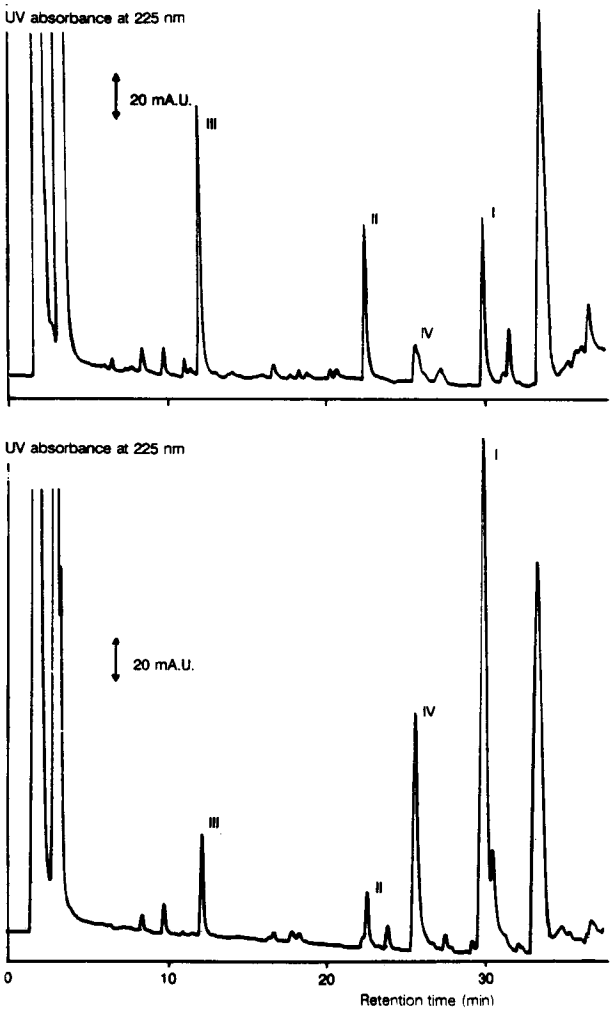

Fig. 1[b]. UV profiles after incubation of N-0923 with rat (**top**) and human (**bottom**) liver microsomes.

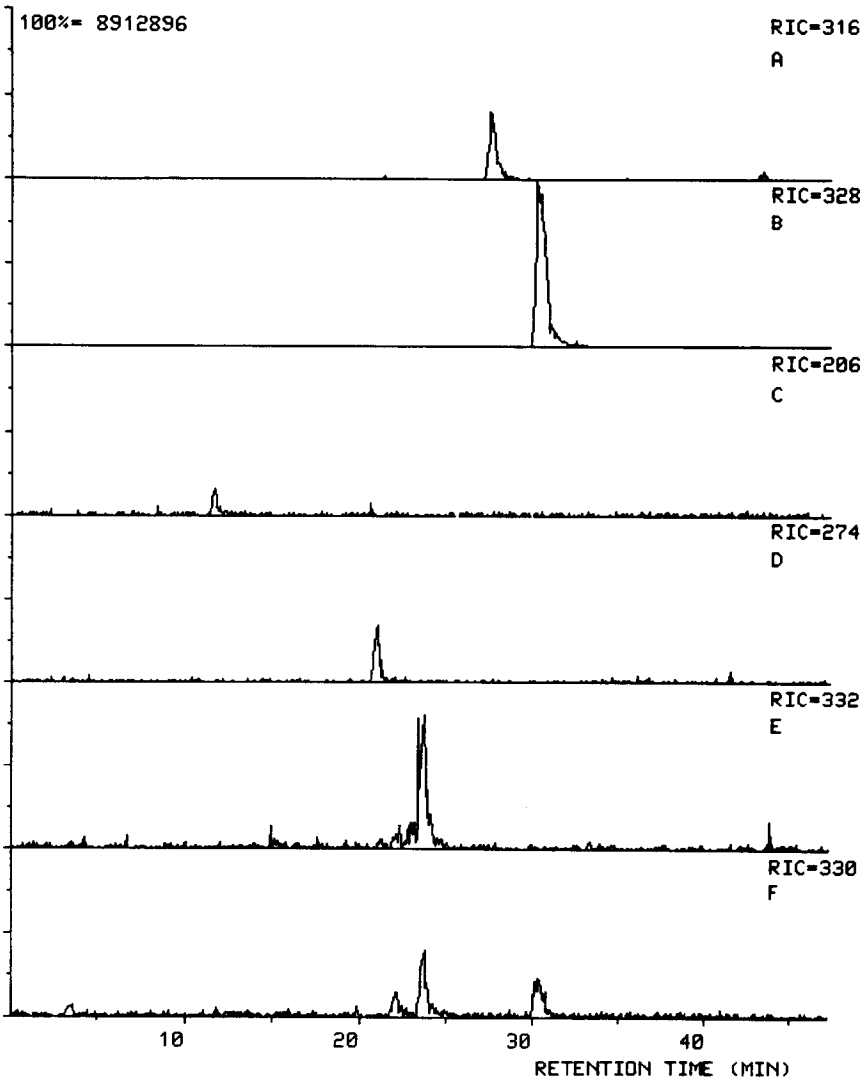

Fig. 2. Reconstructed ion profiles ($M.H^+$) of N-0923 and its phase I metabolites after incubation of N-0923 with monkey liver microsomes. Intensities of the profiles C, D, E and F are multiplied by 5.

Fig. 1. The profile in Fig. 2^b with *m/z* of 328 represents the internal standard, which was added to the incubation mixture prior to the HPLC analysis for comparative purposes, when small changes in retention times of the eluting peaks occurred. In Fig. 2^f a *m/z* of 330 was found at the retention time of the internal standard. This value is due to the occurrence of about 4% of double ^{13}C in the substance.

The parent compound (I), showed metabolic conversion to a catechol, resulting in 6-OH-N-0923 (IV) and to two dealkylated products, namely 2-(N-2-thienylethyl-amino)-5-hydroxytetralin (II) and 2-(N-propylamino)-5-hydroxytetralin (III). As expected, these tertiary and secondary amines are easily protonated at the amino function. A representative mass spectrum of metabolite II is shown in Fig. 3. Retention times, reconstructed ion current profiles and mass spectra of N-0923 and the metabolites II, III and IV as found in this study were compared to those of the synthesized racemic products. No differences were found in the three parameters. An overview of the various metabolic pathways is shown in Fig. 4. The other peaks found in the UV chromatograms were screened for their *m/z* values. Based on this parameter no possible structure could be hypothesized.

Due to the fact that MS is a very selective ion monitoring method two peaks with a *m/z* value of 330 were observed in the chromatogram at 21 and 23 min. (see Fig. 2^f). The peak at 23 min co-eluted with metabolite IV. Up till now we were not able to identify these two peaks.

Dopaminergic activity

The characteristics of the calf caudate membrane fractions for ^3H-N-0437 binding were a B_{max} of 25 pmol.g^{-1} wet tissue and a K_d of 4.92 nM and were in line with previous observed values[18]. Displacement studies were performed using racemic standards. The N-dealkylated compounds were found to be weak displacers of ^3H-N-0437 with K_d values of 99 nM for II and 1.3 μM for III respectively. The racemic catechol 6-OH-N-0437 showed an affinity of 7.3 nM, which was comparable to that of N-0437.

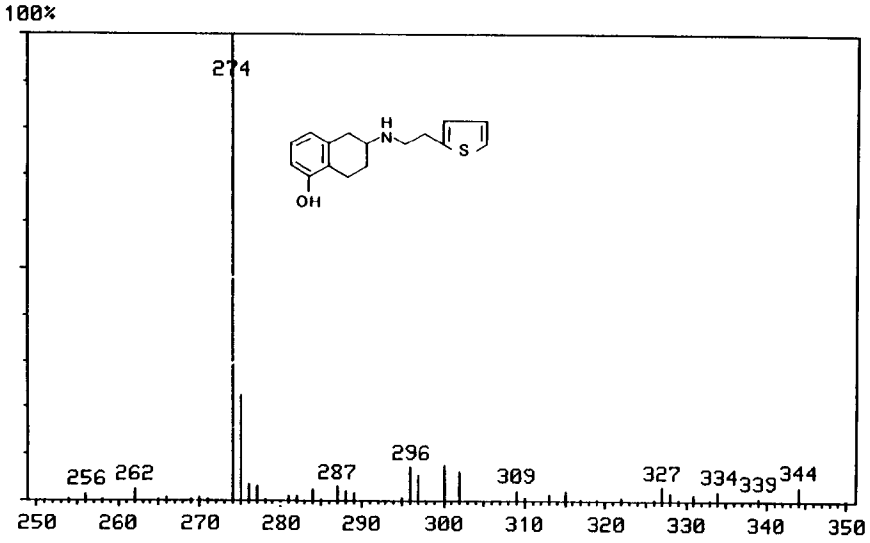

Fig. 3. Background corrected mass spectrum of II.

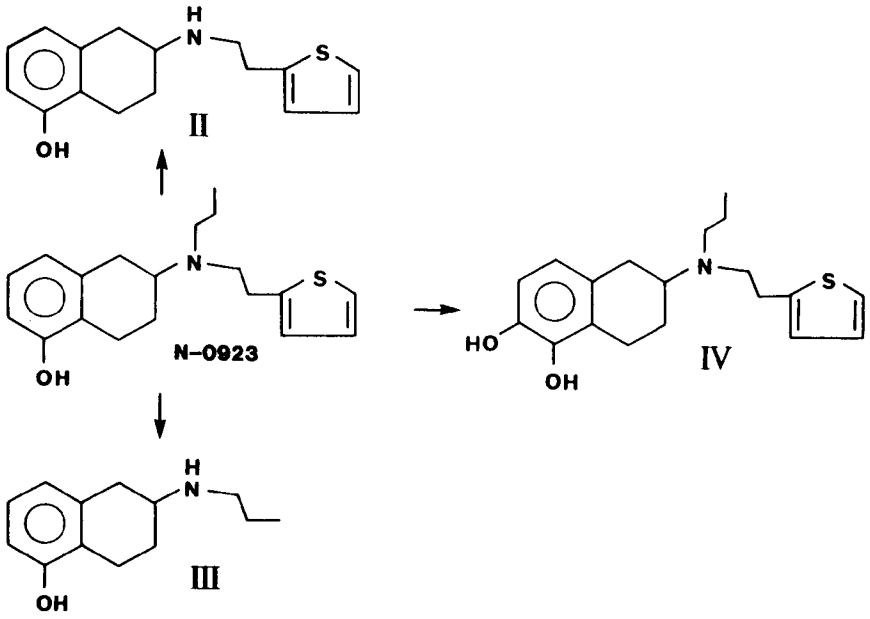

Fig. 4. Oxidative biotransformation pathway of N-0923.

DISCUSSION

Previous investigations on the *in-vivo* metabolism of N-0437 were performed using an alkaline mobile phase[6-8]. In this study we used acid conditions, because an eluent containing 0.1% TFA resulted in more selective and faster chromatographic separations. Microsomal incubations were preferred to e.g. isolated rat liver perfusions because it resulted in relatively clean samples and the incubations can be more selective as a result of properly chosen incubation conditions and co-substrates. In this study the conditions were chosen so as to focus on oxidative phase I metabolism.

The *in-vitro* oxidative metabolism of N-0923 was studied by liver microsomes from rat, monkey and man, and resulted in at least five different metabolites. Three of them could be identified. The large amount of radioactivity found in peak V and VI (see Fig. 1[a]), whilst the peaks were hardly detectable using UV, may be explained by the isotope effect of the radiolabelled drug[21].

N-depropylation is a well known oxidative pathway in N,N-disubstituted-2-aminotetralins[13,22]. Cleavage of the thienylethyl chain is quite remarkable and to our knowledge it has not been described before. Preliminary investigations on the *in-vitro* oxidative metabolism of an analog of (I), 2-(N-propyl-2-N-phenylethylamino)-5-hydroxytetralin (N-0434) showed also a cleavage of the phenylethyl chain (Swart et al. unpublished results). Masumoto et al.[23] reported the metabolic removal of a phenylmethyl chain in 1-bezylpiperidine and dipropylbenzylamine.

Aromatic hydroxylation of N-0923 produced 6-hydroxy-N-0923, a catechol metabolite, but the catechol metabolites of the N-dealkylated compounds could not be found.

In conclusion, oxidative metabolism of N-0923 by liver microsomes of rat, monkey and man resulted mainly in the metabolites II, III and IV, which were identified as 2-(N-2-thienylethylamino)-5-hydroxytetralin, 2-(N-propylamino)-5-hydroxytetralin and 2-(N-propyl-N-2-thienylethylamino)-5,6-dihydroxytetralin respectively. The N-dealkylated metabolites showed weak affinities in dopaminergic receptor binding studies whereas the catechol had an affinity comparable to the parent compound. However, due to the high further metabolic conversion in intact organs as well as *in-vivo* it seems unlikely that the catechol metabolite can contribute to the

therapeutic efficacy of the parent drug. This possibility is even lower for the N-dealkylated metabolites because of their low receptor affinities.

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