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Phase I and II metabolites of benztropine in rat urine and bile

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1. Following oral administration of benztropine (10 mg/kg, body weight), the phase I metabolites, benztropine *N*-oxide, *N*-desmethylbenztropine, tropine, 4′-hydroxybenztropine, *N*-desmethyl-4′-hydroxybenztropine, 4′-hydroxybenztropine *N*-oxide and methoxy-4′-hydroxybenztropine, together with unmetabolized benztropine, were isolated and identified in rat urine and bile by GC-electron impact mass spectrometry (EI GC/MS), microcolumn LC-electrospray mass spectrometry (ES LC/MS) and hplc followed by MS analysis.

2. The mass spectra and chromatographic properties of isolated *N*-desmethylbenztropine, benztropine *N*-oxide and tropine were confirmed by comparison with authentic reference standards.

3. Sufficient quantities of 4′-hydroxybenztropine and *N*-desmethyl-4′-hydroxybenztropine were isolated from the urine by tlc and examined by ^{1}H-nmr, ES/MS and EI/MS. The structure of the methoxy-4′-hydroxybenztropine metabolite was determined by EI/MS. 4′-Hydroxybenztropine *N*-oxide was identified by reacting it with a reducing agent, titanous chloride, to form 4′-hydroxybenztropine, which was then confirmed by comparing its EI/MS and ES/MS behaviour with a previously isolated and ^{1}H-nmr-authenticated sample.

4. In addition, four intact glucuronide conjugates of benztropine were also characterized in bile and urine as phase II metabolites, including 4′-*O*-glucuronylbenzotropine, *N*-desmethyl-4′-*O*-glucuronylbenztropine, methoxy-4′-*O*-glucuronylbenztropine and 4′-*O*-glucuronylbenztropine *N*-oxide by hplc followed by ES/MS analysis. These results provide the first direct evidence of the presence of these metabolites of benztropine in rat.

Introduction

Benztropine, Cogentin®, has been used for many years in the treatment of Parkinson's disease. It is an anticholinergic agent that displays similar pharmacological properties as atropine, and, in adults, the usual therapeutic doses range between 2 and 8 mg daily.

Benztropine is also used widely as an adjunctive therapy to older neuroleptic agents to combat their extrapyramidal symptoms (Stern and Anderson 1979, Keepers *et al.* 1983, Manos *et al.* 1986, Sramek *et al.* 1986, Boyer *et al.* 1987, Goff *et al.* 1991).

Despite the extensive use of benztropine, systematic studies of its pharmacokinetics have been lacking and it seems to have been mainly due to the absence of sensitive and specific methods for assaying this drug in biological fluids. A cholinergic radioreceptor assay has been reported (Tune and Coyle 1981). Because this radioreceptor assay was based on the measurement of total cholinergic receptor binding activity, its results are difficult to interpret pharmacokinetically. The

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previously described gas chromatographic (Jindal *et al.* 1981) and high-performance liquid chromatrographic (Selinger *et al.* 1989) procedures lack sensitivity for determining pharmacokinetics of the drug. Plasma levels of this drug are thought to be too low to be quantified in biological samples by conventional chemical methods, hence there is a need to develop a more sensitive and specific method to study the pharmacokinetics of benztropine.

Radioimmunoassay (RIA) offers an alternative to chemical analyses since the technique can be ultrasensitive. However, specificity of RIA is often questioned. It is well known that knowledge of the metabolism of the drug candidate for RIA can aid in designing a suitable immunogen, antibodies to which can have minimal cross-reactivity to metabolites and hence they can be more specific for the drug. At the time of the present study no information about metabolism of benztropine in any species was available. Thus, prior to the development of RIA for benztropine, a systematic metabolic study to determine metabolic profile of benztropine in rat and man was undertaken. The knowledge from these studies allowed us to synthesize suitable immunogens and then develop a sensitive and specific RIA for benztropine that facilitated a study of the pharmacokinetics of the drug in man following a single, oral, 4-mg dose of benztropine (He *et al.* 1993). The present report describes the systematic isolation of phase I and II metabolites in rat urine and bile, and their identification by means of a variety of techniques, including ^{1}H-nmr, hplc followed by MS, EI GC/MS, and ES LC/MS.

Materials and methods

General chemicals

Benztropine (98% pure), tropine, acetic anhydride and β-glucuronidase type 2 were obtained from Sigma Chemical Co. (St Louis, MO, USA). Tetrahydrofuran, ethyl acetate and methylene chloride were purchased from Fisher Chemical Co. (Edmonton, Alberta, Canada). *N*-methyl-*N*-(tert.-butyldimethylsiyl)-trifluoroacetamide (MTBSTFA) was purchased from Pierce Chemical Co. (Rockford, IL, USA). Methanol, acetonitrile, *n*-heptane, *n*-pentane, *n*-hexane and 2-propanol were obtained from BDH Chemicals (Toronto, Ontario, Canada). 3-Chloroperoxybenzoic acid and titanous chloride were from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals were of the highest commercial grade available. Tlc plates (silica gel $60F_{254}$) were purchased from E. M. Merck (E. M. Science, Cherry Hill, NJ, USA) and CN Sep-Pak^R cartridges were from Scientific Products and Equipment (Concord, Ontario, Canada).

Instruments

Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. The IR spectra was obtained on a Beckman Acculab 4 spectrophotometer and elemental analysis performed on a Perkin-Elmer 2400 CHN instrument analyser. ^{1}H-nmr spectra of synthesized and isolated compounds were recorded on a Bruker AM-300 spectrometer in deuteriochloroform solution with tetramethylsilane as the internal reference. Fast-atom bombardment mass (FAB) spectra were obtained using a VG Micromass 7070HE mass spectrometer equipped with a PDP 11-250J data system. In the electrospray ionization mode, the mass spectrometer was operated at 45 or 90 cone voltage with a source temperature of 60°C.

Electron impact (EI) gas chromatographic mass spectrometry (GC/MS) was performed on a VG Analytical 70 SQ hybrid mass spectrometer equipped with a PDP 11-250J data system. The mass spectrometer was interfaced to a HP 5890 gas chromatograph by a direct inlet system. The gas chromatograph was fitted with a DB-1 capillary column (30 m × 0·32 nm i.d., with a 0·25-μm film thickness). The injection technique was splitless with a purge time of 0·5 min and an injection port temperature of 280°C. The carrier gas was helium with a head pressure of 5 psi, which gave a total column flow of 2 ml/min. The oven temperature was held at 200°C for 1 min, increased to 280°C at a rate of 10°C/min, and held for 10 min. The interface temperature was held constant at 280°C. The mass spectrometer was operated under EI conditions at 70 eV in the positive ion mode with an accelerating voltage of 6 kV. The emission current and the source temperature were held constant at 100 μA and 275°C respectively.

Isocratic hplc for phase I metabolites was carried out using a 20-μl valve loop Rheodyne model 7125 injector (Terochem Laboratories, Edmonton, Alberta, Canada), and a Waters Model 245 pump coupled

to a Waters Model 480 UV detector. The detector was operated at a wavelength of 354 nm. The column (15 cm × 4·6 mm, i.d.) was packed with 3-μm CSC Spherisorb cyano packing material (S.P.E. Rexdale, Ontario, Canada). The mobile phase was a mixture of 5% methanol, 75% acetonitrile, and 20% 0·05 M ammonium acetate. Flow rate was 1·0 ml/min.

Gradient hplc for the analysis for phase II metabolites was performed with a linear gradient system (same equipment as above) after the addition of a model 720 system controller and a second solvent delivery system, Waters Model 510. The mobile phase consisted of distilled deionized water as solvent A and 25% methanol in acetonitrile as solvent B. Flow rate was 1·0 ml/min and the linear gradient system was programmed as follows. For the first 1 min the mobile phase was run isocratically with 100% A and 0% B. During the next 11 min the system controller maintained a linear gradient in which solvent B of the mobile phase gradually changed from 0 to 35%, followed by a final period (1 min) of isocratic elution with 35% solvent B.

During the separation of phase I metabolites, microcolumn ES LC/MS was used and performed using a 2·5-cm i.d. × 10 cm microcolumn packed with 3-μm CSC Spherisorb cyano (S.P.E.). The mobile phase consisted of 80% water and 20% acetonitrile containing 0·1% acetic acid. Flow rate was maintained at 6 μl/min. The effluent from the microcolumn was directly linked to the stainless steel ES needle. Full-scan mass spectra were obtained in the peak centroid mode over the mass range of m/z = 65–635 at 4 s/scan. Spectra were collected with an Intel 302-based data system using LAB-BASE. The individual samples were dissolved in an appropriate volume of methanol and aliquots of 5 μl were analysed by microcolumn ES LC/MS.

Metabolic studies

Female Lewis rats (weight 250–350 g) were obtained from Charles River (Montreal, Quebec, Canada) and housed in plastic cages equipped with stainless steel wire tops. Purina rat chow and water were available *ad libitum* for 2 weeks prior to the study.

Bile samples. Rats (n = 4) were rendered unconscious with diethyl ether, and anaesthesia was maintained with an i.p. injection of urethane (1 g/kg body weight) in saline. The bile duct was cannulated as described by Mulder *et al.* (1981) and Lambert (1965). Briefly, a tube was inserted into the trachea to maintain a clear airway for respiration. The bile duct was exposed and cannulated with polyethylene PE-10 tubing (i.d. 0·28 mm, o.d. 0·61 mm; Clay Adams, Division of Becton Dickinson and Co., Parsippany, NJ, USA). The midline incision in the abdominal wall was then closed with suture clamps. Bile was collected in glass tubes wrapped with foil to exclude light. Bile was collected for 1–7 h from four rats dosed orally with benztropine (10 mg/kg body weight). Rats were maintained under urethane anaesthesia during the collection of bile. The animals were placed under a heat lamp regulated to maintain the rectal temperature at 35°C. Collected bile was stored at − 20°C until analysis. Blank rat bile was also collected before drug administration and stored at − 20°C until analysis.

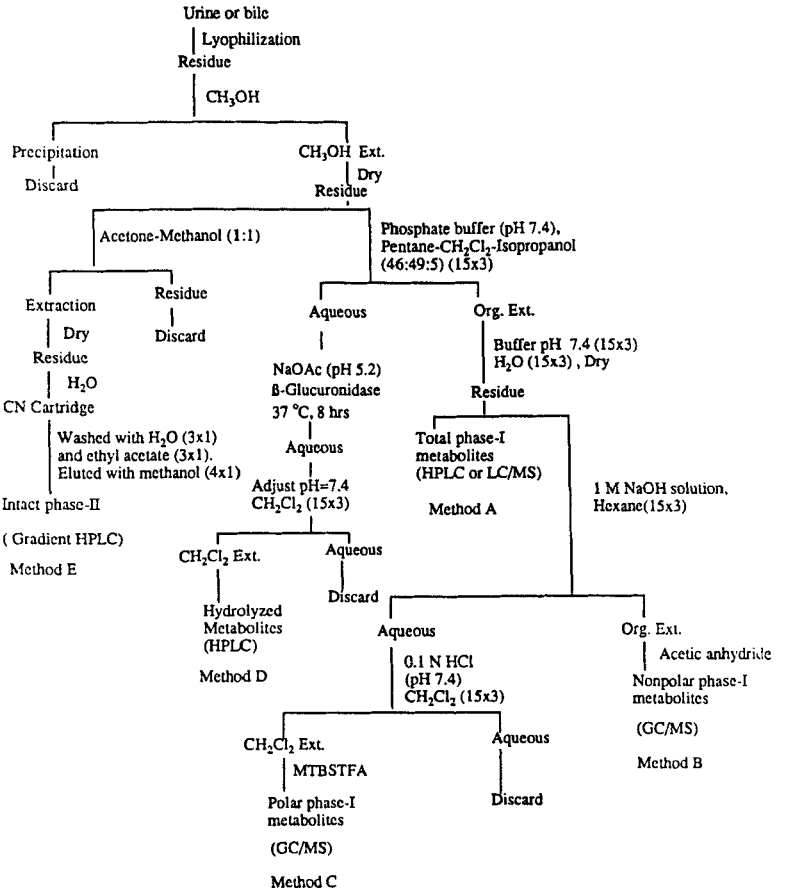
Urine samples. Rats (n = 4) were denied food for at least 12 h before dosing with benztropine. The drug was administered (10 mg/kg) by gastric intubation as its mesylate salt dissolved in 0·1 M phosphate buffer (pH 7·4). Immediately after drug administration, the animals were placed in individual metabolic cages that allowed for the collection of urine. Rats were allowed free access to water at all times and to food 4 h after dosing. Pooled urine was collected for a 24-h period and immediately frozen at − 20°C after collection. In addition, blank urine from rats was also collected and stored at − 20°C until analysis.

Aliquots of frozen samples of urine or bile were lyophilized (Labconco Freezer Dryer-18, Fisher Scientific and Co., Edmonton, Alberta, Canada) and the residues from each biological sample was shaken with methanol (scheme 1). The methanolic solution was filtered to remove any undissolved material, and these solutions were then transferred to a clean flask and evaporated to dryness.

Extraction procedures

Method A (scheme 1). The above evaporated residues from methanolic extracts obtained from rat urine and bile were solubilized in phosphate buffer (0·1 M, pH 7·2) and extracted with a mixture of pentane:methylene chloride:2-propanol (46:49:5, by vol., 3 × 15 ml). The aqueous layer was saved for indirect analysis of phase II metabolites (scheme 1, method D). The combined organic layer was washed (3 × 15 ml) in turn with 0·1 M phosphate buffer (monobasic sodium phosphate and dibasic sodium phosphate, pH 7·4) and water (3 × 15 ml), dried over sodium and evaporated to dryness. One-half of the residue was dissolved in methanol and directly injected into an isocratic hplc system and microcolumn ES LC/MS system as described in Materials and methods. The remaining portion was further processed through the following procedures.

Method B (scheme 1). One-half of the rat urine or bile residue from method A was dissolved in distilled water (20 ml) and treated with 1 M NaOH solution to adjust the pH of the solution to 10. The resultant solution was extracted with hexane (3 × 15 ml) and the organic extracts were combined and dried under nitrogen at 55°C. The dried extract was derivatized with acetic anhydride as described in the section on derivatization procedures.



Scheme 1. Extraction procedures used in studies of the metabolism of benztropine in rat urine and bile.

The remaining aqueous layer was further processed as follows:

Method C (*scheme 1*). The pH of the aqueous solution remaining after method B was adjusted to 7·4. The resultant solution was extracted with methylene chloride (3 × 15 ml) and the organic layer transferred and evaporated under a nitrogen stream at 55°C. The residue obtained was silylated using MTBSTFA as described below.

Method D (*scheme 1*). The presence of phase I metabolites from conjugates in the urine and bile was investigated by enzymatic hydrolysis (Jackson *et al.* 1991) of the aqueous extracts remaining after initial extraction with pentane:methylene chloride:isopropanol. The pH of the aqueous solution remaining after removing phase I metabolites was adjusted to 5·2 with sodium acetate buffer, and a solution containing β-glucuronidase/sulphatase (1 ml, β-glucuronidase activity 100 000 U/ml) and 5 ml 10% w/v aqueous solution of sodium bisulphite were added and incubated at 37°C for 8 h. Then 5 ml 1·0 M potassium phosphate dibasic solution (pH 7·4) was added and the solution extracted with methylene chloride (3 × 15 ml). The combined organic extracts were dried under nitrogen at 55°C and analysed using the isocratic hplc/UV system described in instrumental section.

Method E (scheme 1). The procedure for the extraction and analysis of intact phase II metabolites is based on the utility of the residue obtained after lyophilization and methanolic solubilization of the urine and bile samples from the benztropine-dosed rat. The residue was dissolved in 20 ml methanol and filtered under vacuum. The methanol was evaporated under vacuum and the dried extract was further dissolved in a mixed solvent of acetone:methanol (1:1). The organic solution was again filtered to remove any undissolved material, and this solution was then evaporated to dryness. The dried residue was dissolved in 0·5 ml water and passed through an activated CN Sep-Pak® cartridge. The cartridge was washed with water (3 × 1 ml) and ethyl acetate (3 × 1 ml) and the components eluted with methanol (4 × 1 ml).

The methanolic fractions were concentrated and applied to preparative tlc plates (silica gel, $60F_{254}$, 20×20 cm), which were developed using a solvent system of 1-butanol:acetic acid:water:methanol (7:0·6:2:2, v/v). The tlc plates were allowed to develop for 10 h, whereupon the fractions were made visible with UV light. On segregated portions of the plate, spots were also visualized by spraying with naphtharesorcinol reagent (Parikh *et al.* 1976), which produced blue–purple spots characteristic of uronic acids. The bands corresponding to the glucuronides were scraped from the preparative places and extracted with methanol, filtered and evaporated under vacuum. Any fine silica present in the residue was removed during solid phase extraction using CN Sep-pak cartridges (Pallante *et al.* 1986) and methanol as an eluting solvent. The dried residue was dissolved in methanol and a small portion (5 μl) was injected into the gradient hplc system described in the instrumental section. Individual fractions of the components eluting under the peaks in the hplc UV chromogram were collected and subsequently analysed by ES/MS. The solvents were evaporated at 55° in a flow of nitrogen prior to analysis by ES/MS.

Derivatization procedures

Method I. The residues from method C were dissolved in 200-μl acetonitrile and derivatized with the silylation reagent, *N*-methyl-*N*(tert.-butyldimethylsilyl)-*N*-trifluoroacetamide (MTBSTFA) at 65°C for 10 min.

Method II. The residues from method B were dissolved with ethyl acetate (50 μl) and reacted with acetic anhydride (50 μl) at 65°C for 1 h. In this procedure amines, alcohols and phenols are converted to their amides and acetate respectively.

After each of the above derivatization procedures, the samples were dried under nitrogen at 55°C and redissolved in methanol prior to EI GC/MS.

Chemical synthesis of N-*desmethylbenztropine*

A mixture of benztropine mesylate (1·628 g, 4 mmol) as free base, 2,2,2-trichloroethylchloroformate (1·70 g, 8 mmol), benzene (100 ml) and potassium carbonate (50 mg, 0·3 mmol) was heated under reflux for 60 h. The organic layer was washed with 2 N HCl (2×20 ml) and water until the pH in the aqueous layer reached 7. The organic layer was then dried over magnesium sulphate and evaporated under reduced pressure. The solid residue was recrystallized from methanol to give a *N*-2,2,2-trichlorocarboethoxydesmethylbenztropine (1·49 g, 80% yield) that showed a single spot on the tlc (chloroform: methanol; 14:1; $R_f = 0{\cdot}51$); m.p. 115–117°C; MS (FAB): *m/z* (relative intensity %) = 468/470 (MH^+, 10), 300/302 (5), 284/286 (5), 167 (100); IR (potassium bromide): $\nu = 1790$ (C = O) cm^{-1}; ^{1}H-nmr: $\delta = 7{\cdot}2$–$7{\cdot}4$ (10 H, m, ArH), 5·5 (1 H, s, OCH), 4·8 (2 H, m, $COOCH_2CCl_3$), 3·7 (1 H, m, C_3–H), 3·4 (2 H, m, C_1–H, C_5–H), 1·9–2·4 (8 H, m, C_2–H_2, C_4–H_2, C_6–H_2, C_7–H_2).

Deacylation of *N*-2,2,2-trichlorocarboethoxydesmethyl-benztropine was carried out using zinc dust in either methanol or glacial acetic acid. The above product (2·34 g, 5 mmol) was stirred with zinc dust (0·52 g, 8 mmol) either in 20 ml glacial acetic acid for 4 h or in 40-ml methanol under reflux for 2 h. After the zinc dust was filtered off, the solution was basified with saturated sodium carbonate. The product was extracted with chloroform (3×25 ml), dried over magnesium sulphate and concentrated under reduced pressure to give the desired product, *N*-desmethylbenztropine (0·75 g, 50% yield). The mesylate salt of *N*-desmethylbenztropine was recrystallized from ethanol (m.p. 205–206°C), showing a single spot on tlc (chloroform:methanol; 9:1; $R_f = 0{\cdot}22$). MS (FAB): *m/z* (relative intensity %) = 294 (MH^+, 100), 167 (78), 110 (15), 77 (13), 63 (8); IR (potassium bromide): $\nu = 3300$ (NH) cm^{-1}; ^{1}H-nmr: $\delta = 7{\cdot}2$–$7{\cdot}3$ (10 H, m, ArH), 5·3 (1 H, s, OCH), 4·0 (1 H, m, C_3–H), 3·6 (2 H, m, C_1–H, C_5–H), 2·04 (1 H, NH, D_2O exchangeable), 2·4–1·9 (8 H, m, C_2–H_2, C_4–H_2, C_6–H_2, C_7–H_2); ^{13}C-nmr: $\delta = 142{\cdot}0$ (Ar–C_1), 128·5 (Ar–C_2, Ar–C_6), 127·5 (Ar–C_4), 126·5 (Ar–C_3, Ar–C_5), 82·0 (OCH), 68·0 (C_3), 55·0 (C_1, C_5), 34·0 (C_2, C_4), 27·0 (C_6, C_7). Analysis: calculated for $C_{20}H_{23}NO.CH_4O_3S$: C, 64·10; H, 6·90; N, 3·59. Found: C, 63·44, H, 6·50; N, 3·59.

Chemical synthesis of benztropine N-*oxide*

To a solution of benztropine (2 g, 5 mmol) in purified tetrahydrofuran (Furness *et al.* 1989), which had been cooled to −70°C in a dry ice–acetone bath, was added purified 3-chloroperoxy benzoic acid (0·9 g, 5 mmol) (Fieser and Fieser 1967). The reaction mixture was stirred for 0·5 h after which diethylamine (0·6 ml) was added and stirring continued for an additional 10 min. The solution was then brought to room temperature and the solvent evaporated under reduced pressure. The crude solid was dissolved in methanol and the *N*-oxide of benztropine was isolated from the other components of the crude reaction mixture by elution from a silica gel column (60–200 mesh) using a mobile phase consisting of ethanol:methylene dichloride:ammonium hydroxide (3:6:0·1). The eluted fraction containing the *N*-oxide monitored by tlc was collected from the chromatographic column and evaporated to dryness under reduced pressure. The product showed a single spot on tlc (benzene:methanol:dimethylene chloride; 7·5:1·5:1·0l; $R_f = 0{\cdot}64$). ES/MS: *m/z* (relative intensity) = 324 (MH^+, 100%); EI/MS: 307 (1), 201 (1), 167 (3), 156 (100), 139 (98), 111 (59), 105 (3): ^{1}H-nmr: $\delta = 7{\cdot}4$–$7{\cdot}6$ (10 H, m, Ar H), 5·5 (1 H, s, OCH), 4·0 (2 H, m, C_1–H, C_5–H), 3·7 (1 H, m, C_3–H), 3·4–3·5 (8 H, m, C_2–H_2, C_4–H_2, C_6–H_2, C_7–H_2), 2·6–2·7 (3 H, s, NCH_3).

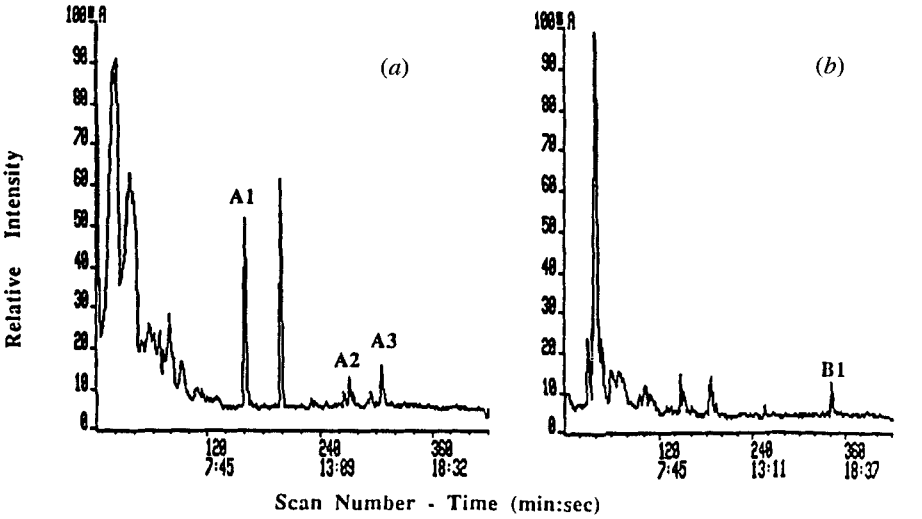


Figure 1. Total ion chromatograms (EI GC/MS) of rat urine extracts derivatized with acetic anhydride (*a*), and MTBSTFA (*b*). A1, benztropine; A2, *N*-acetyl derivative of desmethylbenztropine; A3, *O*-acetyl derivative of methoxy-4′-hydroxybenztropine; and B1, *N*-tert.-butyldimethylsilyl derivative of 4′-hydroxybenztropine.

Results

N-*desmethylbenztropine*

Rat urine extract (scheme 1, method B) was derivatized with acetic anhydride and analysed by EI GC/MS. The total ion chromatogram (TIC) demonstrated the presence of three peaks that were not present in a control urine extract (figure 1 (*a*)). The EI mass spectrum of the component eluting as peak A1 (R_t = 9·3 min) suggested that this material was underivatized benztropine (V; figure 2) since the mass spectrum showed a highest mass ion with low intensity at *m/z* 307 (A1, molecular ion; figure 3 (*a*)) and other diagnostic ions at *m/z* 140 (A1, figure 3 (*b*)) and 167 (A1, figure 3 (*c*)). The EI mass spectrum of the component eluting as peak A2 (R_t = 14·3 min) (A2, figure 1 (*a*)) was rationalized as the *N*-acetyl derivative of desmethylbenztropine. The mass spectrum of this material did not show a molecular ion at *m/z* 335 (A2, figure 3 (*a*)) but the diagnostic ions at *m/z* 168 (A2, figure 3 (*b*)), *m/z* 126 (A2, figure 3 (*d*)) and *m/z* 167 (the other half of the molecule M^+–168) (A2, figure 3 (*c*)) as well as other characteristic ions at *m/z* 110 and 152 (A2, figures 3 (*h*) and (*e*) respectively) (table 1). The component A2 had the same retention time (R_t = 14·3 min) as well as EI mass spectrum as the synthetic *N*-acetyl derivative of *N*-desmethylbenztropine. Figure 4 shows a typical hplc chromatogram of extracts of blank urine (*a*) and urine from rat dosed orally with benztropine (*b*). These extracts were prepared by method A in scheme 1 as described in Materials and methods. *N*-desmethylbenztropine isolated by hplc (IV, figure 4 (*b*)) gave a quasimolecular ion with largest *m/z* at 294 in ES mass spectrum and the spectrum was identical to that of a synthetic sample of *N*-desmethylbenztropine.

Methoxy-4′-hydroxybenztropine

The TIC obtained during EI GC/MS also showed another peak (A3, figure 1 (*a*)), which was not present in the chromatogram of blank urine. The mass spectrum of

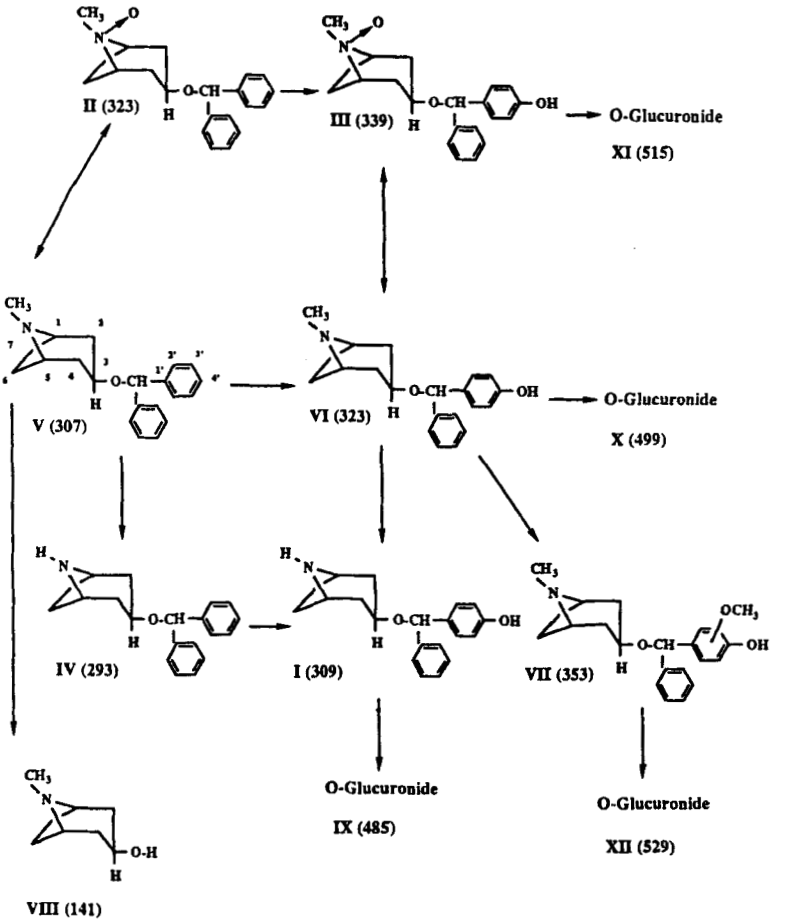


Figure 2. Proposed metabolic pathways of benztropine in rat urine and bile. I, *N*-desmethyl-4′-hydroxybenztropine; II, benztropine *N*-oxide; III, 4′-hydroxybenztropine *N*-oxide; IV, *N*-desmethylbenztropine; V, benztropine; VI, 4′-hydroxybenztropine; VII, methoxy-4′-hydroxy-benztropine; VIII, tropine; IX, *N*-desmethyl-4′-*O*-glucuronyl-benztropine; X, 4′-*O*-glucuronylbenztropine; XI, 4′-*O*-glucuronyl-benztropine *N*-oxide; and XII, methoxy-4′-*O*-glucuronylbenztropine.

the component eluting as peak A3 (R_t = 15·6 min) demonstrated a low diagnostic ion at m/z 395 (A3, molecular ion, figure 3 (*a*)) and other ions at m/z 289, 255 and 140 (A3, figures 3 (*g*), (*c*) and (*b*) respectively), which were consistent with the assignment of A3 as the *O*-acetyl derivative of methoxy-hydroxybenztropine. Additional structural information of this metabolite was obtained from the EI/MS of this material, which gave a diagnostic ion at m/z 289 (A3, figure 3 (*g*)), indicating that the methoxy and hydroxyl groups are both located on the same aromatic ring system. In addition, the underivatized metabolite was isolated by hplc (VII, figure 4 (*b*)) and the residue following evaporation was subjected to ES/MS analysis at 45 eV cone voltage. A quasimolecular ion (MH^+) of the metabolite with a relative abundance of 100% was observed at m/z 354. The ES/MS and EI/MS data are both consistent with the tentative assignment of methoxy-hydroxybenztropine (VII, figure 2) where both the methoxy and hydroxyl groups are located on the same aromatic ring. The

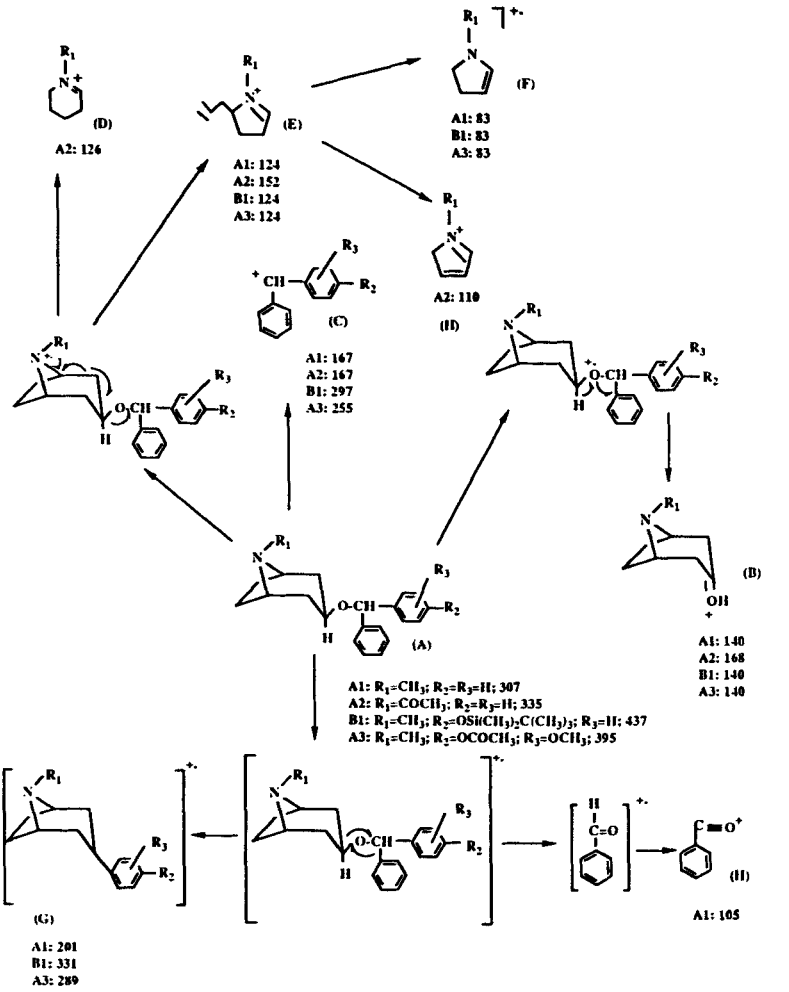


Figure 3. Proposed structures for major fragment ions of benztropine (A1), *N*-acetyl derivative of desmethylbenztropine (A2), *O*-acetyl derivative of methoxy-4′-hydroxybenztropine (A3), and *N*-tert.-butyl-dimethylsilyl derivative of 4′-hydroxybenztropine (B1).

position of hydroxylation can be tentatively assigned to the position 4′ based on data obtained in the next section.

4′-Hydroxybenztropine

Rat urine extract (scheme 1, method C) was derivatized with MTBSTFA and analysed by EI GC/MS. The TIC for the samples shown in figure 1 (*b*) showed the presence of only one peak, which was not present in that of the chromogram from corresponding extract of control urine. The mass spectrum of the component eluting as peak B1 (R_t = 17·4 min) gave a highest mass ion at *m/z* 437 (B1, molecular ion, figure 3 (*a*)) and other ions at *m/z* 331 and 297 (B1, figures 3 (*g*) and (*c*) respectively), which could be rationalized as the *N*-tert.-butyldimethylsilyl derivative of tert.-monohydroxylated benztropine (table 1). In addition, the analysis of the same urine extract by hplc gave a chromatogram which had peak VI (figure 4 (*b*)) with a quasimolecular ion (NH^+) in ES/MS at *m/z* 324. In order to obtain complete

Table 1. EI mass spectra of benztropine (BT), benztropine *N*-oxide (BTNO), 4′-hydroxybenztropine (HOBT), *N*-tertiary-butyldimethylsilyl derivative of HOBT, *N*-acetyl derivative of desmethyl-benztropine (DMBT), and *O*-acetyl derivative of methoxy-4′-hydroxybenztropine (MHOBT).

	$M^{+\cdot}$	Other diagnostic ions*
BT	307 (5)	201 (18), 167 (24), 140 (69), 124 (48), 105 (6), 83 (100)
N-acetyl derivative of DMBT	355 (not observed)	168 (100), 167 (72), 152 (28), 126 (64), 110 (34)
N-tert.-butyl-dimethylsilyl derivative of HOBT	437 (6)	331 (10), 297 (18), 140 (100), 124 (90), 83 (93)
O-acetyl derivative of MHOBT	395 (2)	289 (8), 255 (14), 140 (100), 124 (70), 83 (90)
BTNO	323 (not observed)	307 (2), 156 (100), 167 (3), 139 (98), 111 (59), 75 (49)
HOBT	323 (3)	217 (8), 183 (10), 140 (100), 124 (40), 83 (81)

* Numbers in parentheses are relative abundance (%).

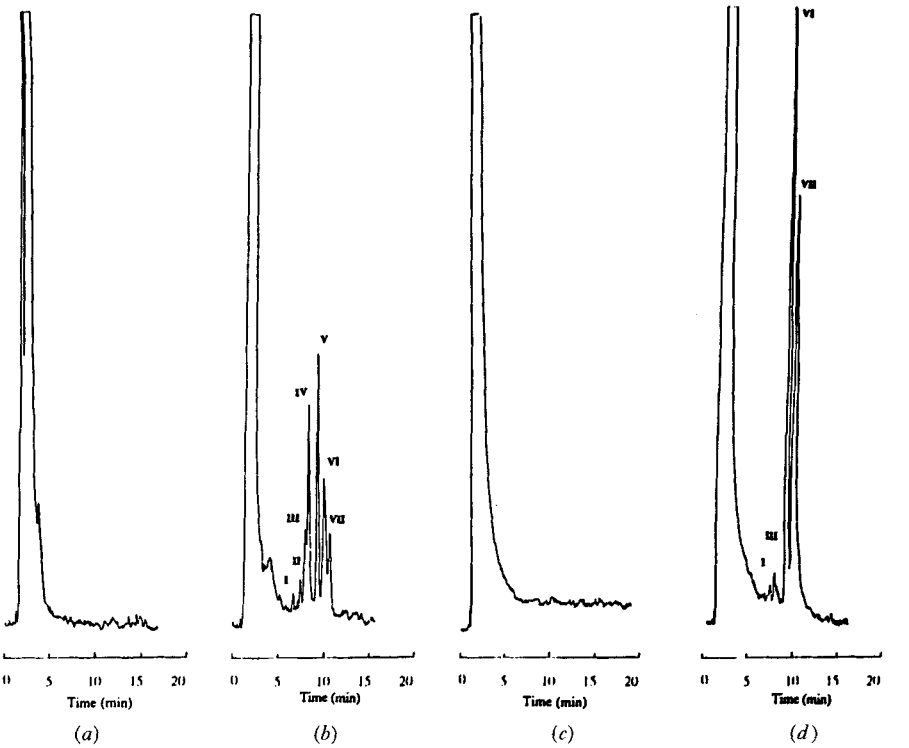


Figure 4. Hplc chromatograms of a blank urine extract (*a*) and an urine extract from rat dosed orally with benztropine (*b*), blank urine extracts after hydrolysis with β-glucuronidase (*c*), and urine extract after hydrolysis with β-glucuronidase from rat dosed orally with benztropine (*d*). I, *N*-desmethyl-4′-hydroxybenztropine; II, benztropine *N*-oxide; III, 4′-hydroxybenztropine *N*-oxide; IV, *N*-desmethylbenztropine; V, benztropine; VI, 4′-hydroxybenztropine; and VII, methoxy-4′-hydroxybenztropine.

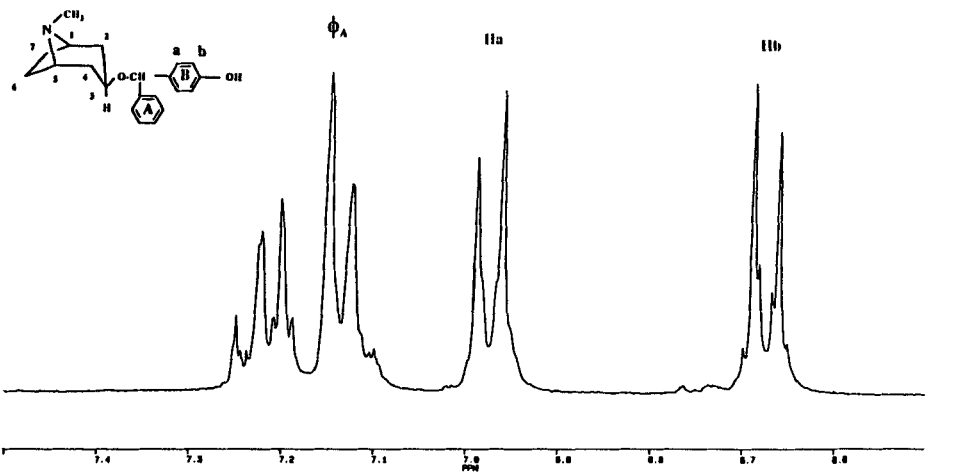


Figure 5. Aromatic proton region of ^{1}H-nmr spectrum of 4′-hydroxybenztropine isolated from rat urine.

characterization, an adequate amount of this metabolite was isolated from urine by preparative tlc (*n*-butanol:acetic acid:methanol:water; 17:0·6:2:2, $R_f = 4{\cdot}2$). EI mass spectrum of the tlc-isolated compound showed the expected molecular ion at *m/z* 323. The most diagnostic fragment ions for the compound in EI/MS were observed at *m/z* 217, 183, 201, 140, 124, 121 and 83 (table 1). The results from EI/MS suggested the presence of hydroxy group in one of the aromatic rings in the isolated metabolite. Furthermore, the ^{1}H-nmr spectrum of the isolated component demonstrated that the aromatic protons in the hydroxylated aromatic ring were observed at higher field ($\delta = 7{\cdot}0$ (Ha); $\delta = 6{\cdot}7$ (Hb)) due to their proximity to the hydroxyl group as compared with the protons ($\delta = 7{\cdot}1$–$7{\cdot}2$) in the other aromatic ring (figure 5). Here a characteristic AB spin coupling pattern ($\mathcal{J}_{ab} = 8{\cdot}5$ Hz) of protons Ha and Hb led to the deduction of the metabolite to be hydroxylated at position 4′ of the aromatic ring (Williams and Fleming 1987).

N-*desmethyl-4′-hydroxybenztropine*

Hplc analysis of rat urine extract for isolating phase I metabolites yielded a number of peaks (figure 4(*b*)). The first peak in this chromatogram labelled peak I was collected and analysed by ES/MS and EI/MS. The ES mass spectrum gave an ion with highest observed *m/z* of 310 at 45 eV cone voltage. The EI mass spectrum of this collected fraction gave fragment ions at *m/z* 126, 110 and 69 (table 1). These spectra are consistent with a monohydroxylated *N*-desmethyl metabolite of benztropine. Furthermore, the identification of this metabolite was confirmed by collecting a sufficient amount of this metabolite from urine by using preparative tlc (*n*-butanol:acetic acid:methanol:water, 7:0·2:1:2; $R_f = 4{\cdot}8$). ^{1}H-nmr analysis of the tlc-isolated material demonstrated that the aromatic protons had the same chemical shifts ($\delta = 7{\cdot}1$ (Ha), $\delta = 6{\cdot}7$ (Hb)) and coupling constant ($\mathcal{J}_{ab} = 8{\cdot}5$ Hz) and 4′-hydroxybenztropine identified above. These results clearly suggested that the isolated metabolite was *N*-desmethyl-4′-hydroxybenztropine (I, figure 2).

4′-Hydroxybenztropine N-*oxide*

The material eluting as peak III in the hplc chromatogram (figure 4 (*b*)) of extract of rat urine when analysed by ES/MS gave an ion whose highest observed *m/z* was at 340. This fraction was reacted with titanous chloride solution (0·2 N) in 1 N hydrochloric acid solution and dried under nitrogen at 55°C (Brooks and Sternglanz 1959). The residue was then dissolved in methanol and the resultant solution was reinjected into the same hplc system. The resultant chromatogram upon repeat hplc analysis using UV detection demonstrated almost complete absence of peak III and the presence of a peak, which had the same retention time and EI mass spectrum as 4′-hydroxybenztropine. These results suggested that the metabolite reduced by titanous chloride was 4′-hydroxybenztropine *N*-oxide (III, figure 2).

Benztropine N-*oxide*

The MS and the hplc retention time of the peak labelled II in figure 4 (*b*) were consistent with those of an authentic sample of benztropine *N*-oxide.

Tropine

Rat urine extract (scheme 1, method A) was analysed by microcolumn ES LC/MS. The selected protonated molecular ions of phase I metabolites were monitored during the ES LC/MS. The reconstructed ion chromatograms diagnostic of various phase I metabolites are shown in figure 6. The complete mass spectrum of the material eluting under the reconstructed ion chromatogram for *m/z* 142 gave a quasimolecular ion (MH^+) with the highest observed *m/z* at 142. Comparison of the retention time and MS with that of an authentic reference sample of tropine clearly indicated that the metabolite was tropine (VIII, figure 2). This metabolite

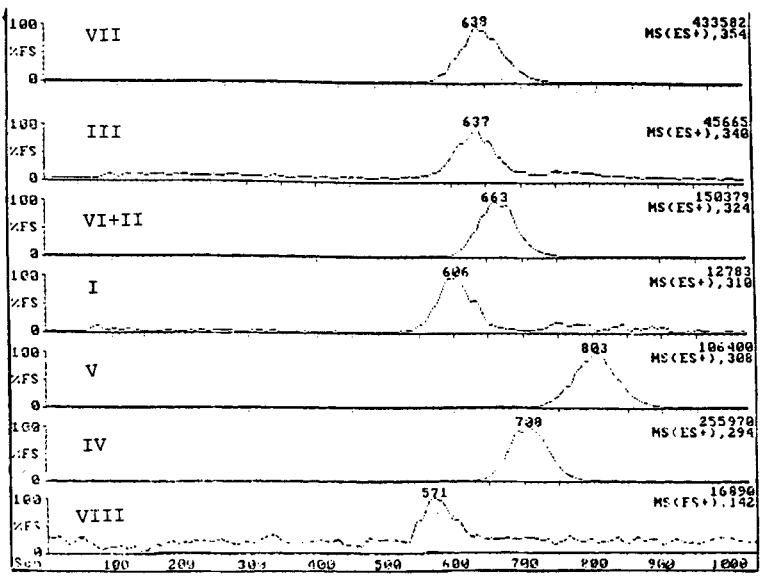


Figure 6. Reconstructed ion chromatograms on microcolumn ES LC/MS analysis of phase I extracts of urine from rat and dosed with benztropine. I, *N*-desmethyl-4′-hydroxybenztropine; II, benztropine *N*-oxide; III, 4′-hydroxybenztropine *N*-oxide; IV, *N*-desmethylbenztropine; V, benztropine; VI, 4′-hydroxybenztropine; VII, methoxy-4′-hydroxybenztropine; and VIII, tropine.

was not detected in the hplc analysis of the extract from rat urine because of the lack of a strong chromophore in this structure. Other reconstructed ion chromatograms observed from the microcolumn LC/MS confirmed data obtained earlier during off-line hplc/MS analysis for phase I metabolites of benztropine.

4'-O-glucuronylbenztropine

Figure 7 shows the linear gradient hplc chromatograms of a blank (*a*) and an extract obtained from urine (*b*) (scheme 1, method E). The component eluting as peak X was collected. After evaporation of solvents, the residue was analysed by ES/MS under the conditions described in Materials and methods. The ES/MS obtained at a cone voltage of 90 V of this material exhibited an ion at m/z 500 (MH^+), which had 56% relative intensity and a diagnostic ion at m/z 324 (loss of 176 amu diagnostic of a glucuronic acid moiety) as well as a prominent natriated ion, $(M + Na)^+$, at m/z 522. These observations indicated that the metabolite was a glucuronide of hydroxylated benztropine. In addition, for further confirmation of the structure of this material, the hydrolysis of the metabolite with β-glucuronidase was carried out. An extraction procedure (scheme 1, method D) for phenolic metabolites was developed and applied to samples after enzymatic deconjugation. The extract obtained after hydrolysis with β-glucuronidase was analysed by hplc

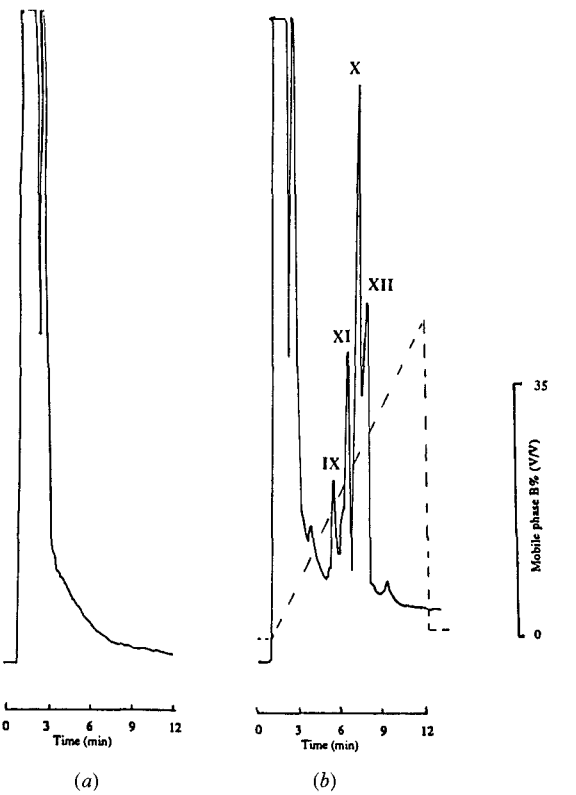


Figure 7. Hplc chromatograms of phase II extracts from blank urine (*a*) and urine sample from rat dosed orally with benztropine (*b*). IX, *N*-desmethyl-4'-*O*-glucuronylbenztropine; X, 4'-*O*-glucuronyl-benztropine; XI, 4'-*O*-glucuronylbenztropine *N*-oxide; and XII, methoxy-4'-*O*-glucuronylbenztropine.

followed by mass spectrometric analysis. The component eluting as peak VI (figure 4(*d*)) had the same retention time and mass spectrum as that of 4′-hydroxybenztropine. Therefore, these results confirm that the metabolite was 4′-*O*-glucuronylbenztropine.

N-*desmethyl-4′*-O-*glucuronylbenztropine*

The gradient hplc chromatogram obtained from analysis of rat urine phase II extract gave other peaks (figure 7(*b*)), not present in the chromatogram of corresponding blank urine extract (figure 7(*a*)). The ES mass spectrum of the component eluting as peak IX gave an ion at m/z 486 (MH^+), and other ions m/z 310 (loss of 176 amu, glucuronic acid moiety) and m/z 508, $(M + Na)^+$. Moreover, the chromatogram of the extract after hydrolysis with β-glucuronidase demonstrated the presence of another peak, which had the same retention time and mass spectrum as that of *N*-desmethyl-4′-hydroxybenztropine (I, figure 4(*d*)). These results suggested that the metabolite was *N*-desmethyl-4′-*O*-glucuronylbenztropine (IX, figure 2).

4′-O-*glucuronylbenztropine* N-*oxide*

The chromatographic peak labelled peak XI in the chromatogram of the urine phase II extract (figure 7(*b*)) was not present in the chromatogram of blank urine (figure 7(*a*)). The ES mass spectrum of the component from this peak exhibited a diagnostic ion peak at m/z 516, (MH^+), a fragment peak ion at m/z 340 (loss of 176 amu, glucuronic acid moiety), and an ion at m/z 538, $(M + Na)^+$, at 90-eV cone voltage. Further hplc (III, figure 4(*d*)) analysis of the extract following enzymatic hydrolysis confirmed that the metabolite was consistent with the assignment of peak XI as containing 4′-*O*-glucuronylbenztropine *N*-oxide (XI, figure 2).

Methoxy-4′-O-*glucuronylbenztropine*

The ES mass spectrum of the component eluting as the final hplc peak (XII, figure 7(*b*)) observed in chromatogram shown in figure 7(*b*) demonstrated an abundant ion at m/z 530 (MH^+), $(M + Na)^+$ at m/z 552 and other important ions with 100% intensity at m/z 354 (loss of 176 amu, glucuronic acid moiety). Further confirmation of the component was achieved by enzymatic hydrolysis. Comparison of the retention time (VII, figure 4(*d*)) and MS of the deconjugated analyte with that of methoxy-4′-hydroxybenztropine clearly indicated that the original metabolite was methoxy-4′-*O*-glucuronylbenztropine (XII, figure 2).

Similar analytical methods were applied to metabolite analysis of bile extracts from rat. All the metabolites previously identified in rat urine were also detected in rat bile with the exception of 4′-*O*-glucuronylbenztropine *N*-oxide, which was indicated to be present in lower amounts in bile than that observed in urine extracts.

Discussion

There have been no reports on the metabolism of benztropine in any species. This report describes a study of the metabolic profile of benztropine in rat and it is the first systematic investigation of the metabolism of benztropine in rat.

In the studies of phase I metabolites, traditional analytical methods such as hplc with UV detection and GC/MS using EI for benztropine were employed. In the EI GC/MS analysis, the extensive fragment ion formation was advantageous to help elucidate the structures of metabolites of benztropine. It was found that the parent

drug, benztropine, produced two prominent ions at m/z 140 (tropine part) (figure 3(*b*)) and m/z 167 (diphenylmethylene part) (figure 3(*c*)) in the EI mass spectrum of benztropine. These ions were considered diagnostic ions in the analysis of metabolites of benztropine. For example, 4′-hydroxybenztropine produced an intensive peak at m/z 140 and lacked an ion at m/z 167, whereas *N*-desmethylbenztropine was characterized by a significant ion at m/z 167 and an absence of the ion at m/z 140. Polar metabolites such as 4′-hydroxybenztropine, methoxy-4′-hydroxybenztropine, and *N*-desmethylbenztropine had to be derivatized before analysis and the thermally labile compounds such as 4′-hydroxybenztropine *N*-oxide and benztropine *N*-oxide were observed to be unstable in the GC conditions employed in this study (Gudzinowicz *et al.* 1964, Hall *et al.* 1982, Jacob *et al.* 1986). Conventional hplc followed by mass spectrometric analysis was more conducive to the identification of these unstable metabolites. All metabolites of benztropine except for tropine were detected during hplc/UV analysis. Hplc followed by MS analysis was very labour intensive and time consuming, especially in the case of metabolites present in low concentrations such as *N*-desmethyl-4′-hydroxybenztropine in urine extracts. In addition, hplc/UV analysis was not useful in deducing metabolites that lacked a suitable chromophore such as tropine. However, on line microcolumn ES LC/MS was more efficient for the analysis of all metabolites of benztropine since it took less time and avoided any additional sample handling prior to MS analysis. The microbore LC system also required less sample than the off-line LC/MS conditions. Although ES/MS has been developed primarily for molecular weight determination of proteins and large biomolecules (Whitehouse *et al.* 1985), this study showed that the technique was also useful for the analysis of low molecular-weight compounds. However, microcolumn LC/MS had lower chromatographic efficiencies for 4′-hydroxybenztropine and benztropine *N*-oxide than conventional hplc. In a system where authentic reference compounds such as 4′-hydroxybenztropine, *N*-desmethyl-4′-hydroxybenztropine and methoxy-4′-hydroxybenztropine were not available, the information of protonated molecular ions obtained during low cone voltage (45 eV) ES/MS was not always sufficient to elucidate the complete structures of all metabolites. Therefore, ^{1}H-nmr spectra provided definitive information to identify the precise location of the hydroxy group in 4′-hydroxybenztropine and *N*-desmethyl-4′-hydroxybenztropine.

Prior to chromatographic analysis of intact phase II metabolites, urine samples were first purified from other water-soluble endogenous compounds. Here the liquid–liquid and solid–phase extraction procedures were used successfully to reduce water-soluble interfering compounds. The phase II metabolites of benztropine were observed to be resolved poorly when isocratic reverse-phase hplc was performed. The reverse-phase hplc system, which utilized gradient elution, was able to provide better resolution for these intact phase II metabolites. The structures of glucuronides were elucidated by identifying pseudo-molecular ions, prominent natriated ions and diagnostic aglycone ions in ES/MS at 90-eV cone voltage. Therefore, use of high cone voltage (90 eV) in ES/MS provided successful analysis of intact glucuronide conjugates. The tentative structural assignments were further confirmed by isolation of products using isocratic hplc following enzymatic hydrolysis and identification of the hydrolysed products by MS. Phase I metabolites and parent drug in urine and bile required two extraction procedures in order to remove them quantitatively. Following the removal of parent drug and phase I metabolites, the remaining aqueous solution was subjected to enzymatic hydrolysis

with β-glucuronidase, which liberated aglycone metabolites. The analysis of the enzymatically hydrolysed products gave indirect evidence for the presence of various glucuronide conjugates.

In summary, the present work suggests that benztropine is metabolized by a variety of routes yielding a large number of metabolites in rat. The oxidative metabolite routes include *N*-demethylation, *N*-oxidation, *O*-dealkylation and aromatic hydroxylation. The hydroxylated metabolites are further biotransformed to glucuronide conjugates. This represents the first systematic study of the metabolism of this drug in rat.

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