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Metabolism and excretion of centbutindole (neuroleptic) in rats after oral administration

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Abstract

The metabolism and excretion of centbutindole was studied in Sprague-Dawley rats after oral administration. The percentage of dose excreted was monitored over 4 days. Efforts were also directed towards evaluating the stability of centbutindole in various in-vitro biomatrices. Centbutindole was found to be metabolised in rats and only a negligible amount of parent drug was excreted. The percentage of the dose excreted in bile and faeces was 0.2 % and 0.6 %, respectively. No drug was found in urine after oral administration, but after an intravenous dose only 0.0012 % of the dose was eliminated through urine. Two metabolites, dealkylated metabolite and hydroxy metabolite, were identified by ion spray LC/MS/MS, using a combination of parent ion and product ion scanning techniques. The major routes of metabolism of centbutindole include reduction of the carbonyl functional group in the butyrophenone side chain and *N*-dealkylation of the butyrophenone side chain attached to the pyrazinopyridoindole ring nitrogen at position 2. The hydroxy metabolite was excreted negligibly in bile, although it was present in the form of glucuronide conjugates more in comparison to its free form.

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Introduction

Centbutindole, (±)-2-[1-(*p*-fluorobenzoyl)propyl]-1,2,3,4,6,7,12,12a-octahydropyrazino(2′,1′:6,1)pyrido[3, 4-b]indole, is a new neuroleptic developed by Central Drug Research Institute, Lucknow, India (Saxsena et al 1972, 1973; Singh et al 1977). It is well reported in the literature that most of the currently available neuroleptics are highly lipophilic and are subject to extensive biotransformation before their elimination from the body (Rudorfer & Potter 1997). Centbutindole is a dopamine antagonist (Singh et al 1977; Doongangi et al 1983) and is indicated in syndromes involving agitation, hyperactivity, hallucinations and paranoid delirium, particularly schizophrenia. Its unique structural configuration confers certain pharmacological properties, which differ from those of other neuroleptic agents (Kumar et al 1982). In experimental studies, centbutindole has been found to exert pronounced neuroleptic activity in doses lower than other available drugs. In rats, the molecule showed an oral bioavailability of 22%. The elimination half-life after intravenous administration ($2\ mg\ kg^{-1}$) was 128 min, with a total body clearance of $67\ mL\ min^{-1}\ kg^{-1}$ which was twice the normal hepatic plasma flow rate of $36\ mL\ min^{-1}\ kg^{-1}$ (unpublished data). The objective of this study was to evaluate the metabolism and excretion of centbutindole in rats after administration of an oral dose and also to identify the various metabolites formed in-vivo (i.e. in bile, urine and faeces) and in in-vitro biomatrices. LC-MS/MS (liquid chromatography-mass spectrometry/mass spectrometry), and PDA (photodiode array) detection techniques were used for the characterisation of the various metabolites.

Materials and Methods

Chemicals and reagents

Reference standard centbutindole (purity >99%) was obtained from Pharmaceutics Division of the Institute. The hydroxy metabolite of centbutindole (purity >99%) was

synthesised in this laboratory. Anaesthetic ether (T. K. M. Pharma, Hyderabad, India) was used as received in animal experiments as an anaesthetic, but was purified by distillation for use as an extraction solvent in sample processing. Acetonitrile, methanol, n-hexane and ethyl acetate were of HPLC grade (Ranbaxy Laboratories Ltd, India). Biochemicals like β-glucuronidase (from Helix Pomatia, type V), nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate and nicotinamide were obtained from Sigma Chemical Co. (St Louis, MO). All other reagents were of analytical grade and were used without further purification.

One of the metabolites in the treated rat bile was identified by mass spectrometric analysis as the reduced form of centbutindole. This metabolite was synthesised as an authentic sample as follows. Sodium borohydride (four equivalent to parent molecule) was added to a solution of centbutindole (1.35 mM) in chloroform (7 mL) and methanol (15 mL). The mixture was stirred at room temperature for 15 min and the progress of the reaction was constantly monitored on TLC (using 10% methanol in chloroform) for the appearance of the final product and disappearance of centbutindole. The mixture was quenched with ice-cold water (~ 5 mL) and the cloudy suspension was extracted with ethyl acetate (2×30 mL). The ethyl acetate was evaporated to dryness under reduced pressure, and recrystallised in 1,2-dichloroethane and dried in vacuum. The off white product, 2-γ-[(*p*-fluorobenzyl-ol)propyl]-1,2,3,4,6,7,12,12a-octahydropyrazino (2′,1′:6,1) pyrido [3,4-b] indole, showed yield greater than 90%, m.p. 160–162°C. ESI-MS/MS (electron spray ionization-mass spectrometry/mass spectrometry) m/z: 394 $(M+H)^+$, 376, 251, 233, 165, and 144. Infra-red (IR), ν_{max} (KBr) cm^{-1}: 3305 (NH str.), 3066 (-CH str.), 3671 (-OH str.) and 1606 (C=C str.). In ^1H NMR data, a secondary CH-proton (>C*H*OH) as a multiplet of one proton and secondary alcholic proton (>CHO*H*) as a broad signal of one proton appears at δ 3.56 and 5.45 ppm, respectively.

Animals

Healthy, young, male Sprague-Dawley rats, 225±25 g, were procured from Laboratory Animal Division of the Central Drug Research Institute, and acclimatised to a 12-h day–night cycle for at least 2 days before the study. The rats were fasted overnight with free access to water before dosing and food was allowed freely 2 h after the dosing. All animal work adhered to the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985).

Metabolism studies

Microbial metabolism in intestinal contents
Freshly excreted faeces collected from non-fasted rats was pre-soaked for 30 min in normal saline (pH 7.4) and dispersed mechanically (Bramer et al 1993). Centbutindole ($2\ \mu g\ mL^{-1}$) was incubated with 14% w/v faecal homogenate at 37°C at 80 oscillations per min. Serial samples (100 μL) were withdrawn at 0, 1, 2, 3, 4, 6, 8 and 10 h and 200 μL acetonitrile was added to each sample. The samples were vortex mixed, centrifuged at 2000 *g* for 10 min and analysed by HPLC (System A).

Metabolism with S-9 fraction of intestine and liver
The procedures of Bramer et al (1993) were followed with slight modifications. Two male Sprague-Dawley rats were exsanguinated and sacrificed after cervical dislocation. Small intestine (duodenum to ileum) was immediately removed and the mucosal side of the intestine was flushed/rinsed with ice cold 1.15% KCl in 10 mM K_2HPO_4 buffer, pH 7.4. All subsequent operations were conducted at 4°C. The intestine was slit open and the mucosal layer was peeled off. This layer was homogenised in four volumes of ice-cold KCl/phosphate buffer and centrifuged at 9000 *g*. The supernatant was used as the S-9 fraction of intestine. Liver was also processed in a similar manner to a 20% w/v homogenate. The homogenate was centrifuged at 9000 *g* at 0°C for 20 min to give the S-9 fraction of liver. The metabolic activity was initiated by addition of 5 mL of S-9 fraction of the intestine or liver to separate conical flasks containing 25 μL of centbutindole solution in methanol (2.5 μg of centbutindole) and 5 mL of the NADPH-generating system (1.85 μmol NADP, 20 μmol glucose-6-phosphate, 100 μmol nicotinamide and 20 mg magnesium chloride) in pH 7.4 K_2HPO_4 buffer. The mixture was thoroughly mixed by hand and incubated at 37°C at 80 oscillations per min for 4 h. Samples (100 μL) of incubated mixture were withdrawn at 0, 10, 20, 30, 60, 120, 180 and 240 min and the enzymatic reaction was terminated by snap-freezing the sample in liquid nitrogen. The samples were analysed for centbutindole and its metabolites using HPLC (System B). The incubation time at time zero served as control.

Excretion studies

The excretion of centbutindole was studied in normal and bile-duct-cannulated rats. The rats were divided into three groups, 1, 2 and 3, comprising three rats in each. Groups 1 and 3 comprised of rats with normal bile duct (NBD) whereas group 2 comprised of bile-duct-cannulated (BDC) rats. A group of three rats from group 2 were implanted with a PE-47 cannula into the common bile duct under ether anaesthesia. The rats were housed in individual modified Bollman cages and were allowed to recover overnight before drug administration. The rats were administered centbutindole orally ($4\ mg\ kg^{-1}$, 20% PEG and 80% sodium acetate buffer, pH 4.5). All rats were fed at 2 h after the dose and received electrolyte solution throughout the study. Urine, bile and faeces were collected for 4 days at 0–24, 24–48, 48–72 and 72–96 h post dose. The volumes of bile and urine samples were recorded and stored at −60°C until analysis. Faeces samples were dried in desiccators under vacuum and stored at room temperature. Rats belonging to group 1 were also orally dosed with centbutindole, $4\ mg\ kg^{-1}$, and were similarly housed in individual

modified Bollman cages. Urine and faeces were collected on similar basis as for group-2 rats. However, rats belonging to group 3 were administered with 2 mg kg^{-1} intravenous dose and only urine was collected until 96 h and analysed for centbutindole by HPLC (System A).

Sample analysis

Bile

To drug-free or drug-spiked bile (200 μL) was added 20 μL of internal standard (20 μg mL^{-1}) and 100 μL of 1 M potassium hydroxide. The sample was extracted with 2×4 mL ether for 1 min and centrifuged at 2000 *g* for 5 min. The organic phase was separated after snap-freezing in liquid nitrogen and the combined ether extract was evaporated to dryness in a Speed Vac Concentrator (Savant Instrument Inc. Farmingdale, NY). The residue was acidified with 250 μL of 0.5 M hydrochloric acid and washed with 2×2 mL n-hexane. The aqueous layer was basified with 200 μL of 1 M potassium hydroxide solution and extracted with 2×3 mL ether. The organic phase was separated after snap-freezing in liquid nitrogen and the combined ether extract was evaporated to dryness in a Speed Vac Concentrator. The residue was reconstituted in 100 μL methanol and analysed by HPLC (System A).

Urine

Samples (200 μL) of drug-free or drug-spiked urine were processed as mentioned for bile. The residue was reconstituted in 200 μL methanol and analysed by system A.

Faeces

Blank or spiked faeces samples (0.05 g) were wetted with 300 μL pH 4.5 buffer and were allowed to stand aside for 30 min at room temperature. Acetonitrile (2 mL) was added to the samples which were vortex mixed for 1 min and kept at room temperature for 15 min. The samples were then centrifuged at 2000 *g* for 10 min at 0°C and the supernatant was analysed by HPLC (System A).

Intestine or liver incubation mixture

The intestine or liver incubation mixture (100 μL) was precipitated with 200 μL of acetonitrile, vortex mixed and centrifuged at 2000 *g* for 5 min. The supernatant was injected directly onto the column and analysed by HPLC (System A).

Isolation of metabolites from urine

Urine samples (0–24 h collection) after a centbutindole oral dose of 4 mg kg^{-1} to rats were pooled and extracted using diethyl ether under alkaline conditions. The ether layer was separated, evaporated to dryness under reduced pressure and the residue was diluted in 2 mL 0.5 M HCl. The solution was mixed thoroughly, washed with n-hexane and the hexane layer was stored at 4°C. The acid layer was basified and re-extracted with 3×10 mL ether. The combined ether layer was washed with water till neutral. The ether layer was evaporated to dryness and the residue was dissolved in methanol for further analysis. The remaining aqueous layer was re-extracted with ethyl acetate for higher recovery of polar metabolites. A portion of the ether extract was analysed by HPLC and the UV spectra of the metabolites was recorded using online photodiode array detector (PDA), whereas the remainder was analysed by LC-MS/MS.

Enzyme hydrolysis

Pooled rat bile and urine samples (1 mL each) were adjusted to pH 5 with glacial acetic acid and treated with 2500 U of β-glucuronidase. The mixture was incubated at 37°C under constant shaking conditions for 12 h. The samples were processed as mentioned under isolation of metabolites from bile. Incubation of bile and urine samples without enzyme served as a control.

Chromatographic conditions

HPLC-UV-fluorescence (system A)

A high-pressure isocratic HPLC, Shimadzu, Japan (LC-10ATvp) with SCL-10Avp (Communication Bus Module) was used to pump the mobile phase at 1 mL min^{-1} flow rate. The mobile phase comprised acetonitrile–phosphate buffer (25 mM, pH 3.5) in the ratio 55:45 (% v/v) for bile and urine analysis; for analysis of faeces and in-vitro samples the mobile phase was acetonitrile–phosphate buffer (25 mM, pH 4.5) in the ratio 70:30 (% v/v). Optimum chromatographic separations were performed on a C_{18} reversed-phase column (Spheri-5, 5 μm, 220×4.6 mm i.d.) preceded by a guard column (30×4.6 mm i.d.) (Perkin Elmer, Norwalk, CT) of the same material. Mobile-phase solvents were filtered and degassed before use. A model 7725i syringe loading sample injector (Rheodyne, Cotati, CA) with a fixed 20-μL sample loop was used to inject the samples onto the HPLC system. After elution the compounds were monitored using a model RF-10A spectrofluorimetric detector set at $\lambda_{Ex/Em}$ 285/345 nm and SPD-10Avp dual wavelength UV-VIS spectrophotometric detector set at 230 and 270 nm. Chromatographic peaks were integrated using Class VP series (Ver 5.22) work station (Shimadzu, Japan).

Recovery, linearity and variations in intra- and inter-batch accuracy and precision were determined for bile, and urine at low, medium and high concentrations (31.25, 125 and 500 ng mL^{-1} for centbutindole and 15.625, 62.5 and 250 ng mL^{-1} for the hydroxy metabolite). Recovery, accuracy and precision for both analytes from faeces was assessed at 5, 10 and 20 μg g^{-1} for centbutindole and 2.5, 5 and 10 μg g^{-1} for the hydroxy metabolite. Each of the validation samples was in triplicate (n = 3).

LC-MS conditions (system B)

An HPLC pump (Jasco PU-980 intelligent pump) was used to pump the mobile phase (70:30% v/v acetonitrile–ammonium acetate buffer (10 mM, pH adjusted to 4.5 with

glacial acetic acid) at a flow rate of 1 mL min^{-1}. Chromatographic separations were performed using the same column as mentioned above. A model 7725i syringe loading sample injector (Rheodyne, Cotati, CA) with a fixed 20-μL sample loop was used to inject the samples onto the HPLC system. The eluates containing centbutindole and its metabolites were analysed by an online MICROMASS QUATTRO II triple quadrupole mass spectrometer with electron spray ionisation source. The electro spray interface was operated at 3500 V and the mass spectrometer was operated in positive ion mode. The spectra were collected in 2-s scans. The eluates were monitored using a Jasco 875-intelligent UV/VIS detector, set at 250 nm. The CID (collision-induced dissociation) studies were performed using argon gas (2×10^{-3} mbar) at a collision energy of 20 eV.

Data analysis

The concentrations of centbutindole and its hydroxy metabolite were read from the calibration standards drawn in the same biomatrix. However, the concentrations of centbutindole and the hydroxy metabolite in faeces were read from the mobile phase standard curve of centbutindole and hydroxy metabolite. Excretion of centbutindole and its hydroxy metabolite through urine, bile and faeces was expressed in terms of percent of administered dose. The cumulative amount of dose excreted was plotted versus the midpoint of collection time. Renal clearance (CL_r) after an intravenous dose was calculated as Ae_∞/AUC, where Ae_∞ is the amount excreted in urine up to time infinity (this was assumed to equal the total amount excreted in 24 h since no detectable centbutindole could be found in urine collected later) and AUC is the area under the serum concentration–time curve. Non-renal clearance (CL_{nr}) was calculated as $CL_{nr} = CL_{total} - CL_r$, where CL_{total} and CL_r are total body clearance and renal clearance, respectively, after administration of a single intravenous dose (Lee & Chiou 1983).

Results

Bioanalysis

The assay methods developed were specific and the chromatograms of the blank matrices showed no interfering peaks in the eluting regions of either of the analytes. The standard curves in bile and urine were linear over the range 31.25–1000 ng mL^{-1} for centbutindole and 15.625–500 ng mL^{-1} for the hydroxy metabolite. The recovery of centbutindole and its hydroxy metabolite, variations in intra- and inter-batch accuracy and precision in bile, urine and faeces are summarised in Table 1. These variations were within acceptable limits (Shah et al 1992). Recovery of centbutindole and its hydroxy metabolite from spiked liver and intestinal homogenates was greater than 90% and was thus read directly from the standard curves drawn in methanol.

Microbial metabolism in intestinal contents

Degradation of centbutindole by bacterial metabolism was examined by incubating faecal contents of non-fasted rats with centbutindole solution under aerobic conditions. This shows the time course of degradation of centbutindole in the suspension of faecal contents. The data show that centbutindole is stable in the presence of microbes during 10 h of incubation under continuous shaking.

Table 1 Assay validation parameters of centbutindole and its hydroxy metabolite in bile, urine and faeces.

Concn	Recovery (%)		% Bias				% RSD inter-batch		% RSD intra-batch	
	CB	HM	CB		HM		CB	HM	CB	HM
			Inter-batch	Intra-batch	Inter-batch	Intra-batch				
Bile										
31.25/15.625	85.2±5.55	80.6±9.81	−1.58	−5.88	0.004	−0.26	3.75	4.66	1.28	3.45
125/62.5	84.9±8.72	88.5±7.83	−6.23	−6.71	0.32	0.59	4.65	1.05	12.0	2.96
500/250	89.3±5.18	88.2±2.40	−1.81	−1.68	−3.23	−5.51	6.74	4.96	1.82	1.61
Urine										
31.25/15.625	93.8±3.23	76.3±6.25	0.40	−1.43	3.57	5.91	6.97	9.07	3.63	3.91
125/62.5	82.4±0.10	73.4±4.47	−7.96	−7.41	−5.91	−6.41	2.61	3.52	1.93	3.84
500/250	93.6±1.88	89.0±2.75	4.9	4.59	6.18	7.19	7.43	12.03	2.20	2.05
Faeces										
5/2.5	97.2±1.72	81.1±0.95	2.10	1.15	7.28	8.17	3.09	2.65	2.71	3.20
10/5	89.2±2.49	78.6±1.75	−5.96	−7.10	4.51	4.75	5.06	4.52	3.30	0.70
20/10	91.5±1.27	80.7±1.64	−3.11	−2.87	7.42	6.37	2.26	3.45	1.48	1.29

Concn (centbutindole/hydroxy metabolite) is presented as ng mL^{-1} for bile and urine and as $\mu g\ g^{-1}$ for faeces. Recovery is presented as means±s.d., n = 3. CB, centbutindole; HM, hydroxy metabolite.

Metabolism with S-9 fraction of intestine and liver

Liver HPLC-UV profiles obtained after incubation of centbutindole with S-9 fraction of rat liver suggested the formation of one major metabolite (Figure 1). The decline and formation of centbutindole and the hydroxy metabolite, respectively, at 0.5 μg mL^{-1} concentration is shown in Figure 2. Major decline in concentration of the parent molecule was observed up to 2 h (Figure 2). This indicates either that the enzyme may be active only up to 2 h or that the reaction may be saturated. The data show that nearly 40% of the drug undergoes conversion in 240 min using a 20% w/v homogenate. The effect of substrate (centbutindole) concentration on the formation of metabolites was studied at 0.5 and 1.5 μg mL^{-1}. Similar metabolites were formed at both concentrations implying that there was no effect of substrate concentration on the formation of metabolites. The metabolism, however, did not proceed in the absence of NADPH, depicting that the formation of the hydroxy metabolite from centbutindole is not spontaneous but is enzymatic in nature. The protein content of the S-9 fraction was determined to be 7.35 mg mL^{-1}. Analysis of a sample of the S-9 fraction of liver by LC-MS/MS revealed formation of the dealkylated metabolite, which could not be detected using PDA detector, probably due to the concentration being lower than its lower limit of quantitation. Incubation of centbutindole with small intestinal mucosa homogenate for 4 h also resulted in the formation of hydroxy metabolite (Figure 3).

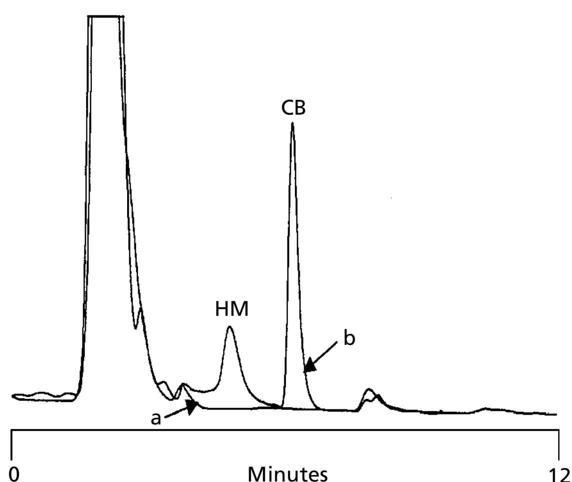

Figure 1 Representative chromatograms of in-vitro metabolism studies in S-9 fraction of liver blank liver homogenate (a) and centbutindole-spiked liver homogenate (b). CB, centbutindole; HM, hydroxy metabolite.

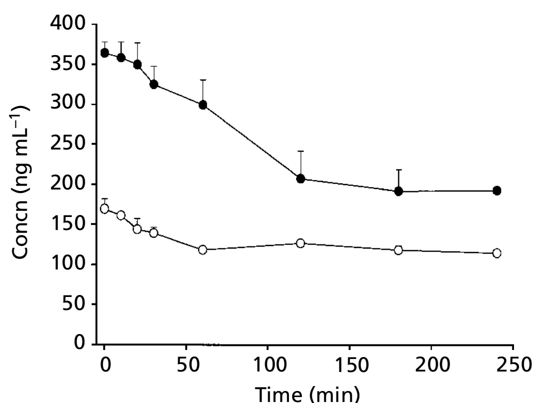

Figure 2 Formation of hydroxy metabolite (open circles) and disappearance of centbutindole (closed circles) after rat liver S-9 fraction incubation at 0.5 μg mL^{-1} in 10 mM K_2HPO_4 buffer (pH 7.4) at 37°C. Results are means±s.d. of five readings.

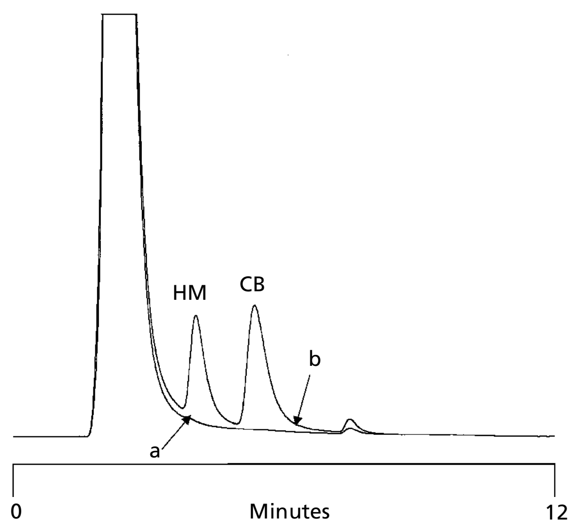

Figure 3 Representative chromatograms of blank intestinal wall homogenate (a) and centbutindole-spiked intestinal homogenate (b). CB, centbutindole; HM, hydroxy metabolite.

Identification of metabolites

The retention times of the dealkylated and hydroxy metabolites observed in S-9 fraction of rat liver were 5.52 and 7.03 min, respectively. The characterisation of the

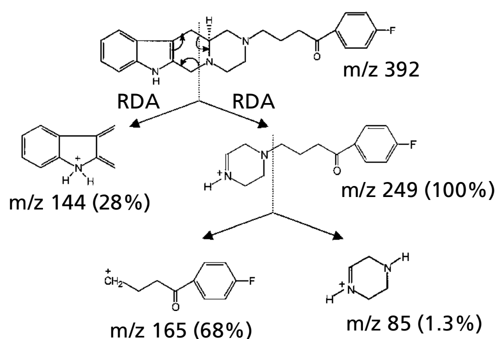

Figure 4 Proposed scheme for fragment ions generated by collisionally induced dissociation of parent compound (centbutindole). RDA, retro-Diel Alder fragmentation.

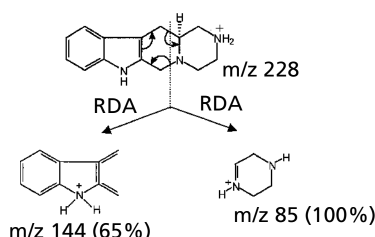

Figure 5 Proposed scheme for fragment ions generated by collisionally induced dissociation of the dealkylated metabolite of centbutindole. RDA, retro-Diel Alder fragmentation.

metabolites were based on the comparison of mass fragmentation and UV spectral similarities to centbutindole and the synthetic standard of hydroxy metabolite. The dealkylated metabolite was transparent to UV, hence spectral matching could not be performed. The structures of centbutindole and its dealkylated and hydroxy metabolites observed in-vitro and in-vivo were elucidated by ESI/MS/MS using positive ion mode. The identification details of centbutindole and its metabolites are given below.

Centbutindole (*Figure 4*)

The parent compound showed λ_{max} at 224, 245 nm and λ_{min} at 243 and 272 nm in UV spectra. The IR data show the following frequencies (cm^{-1}) at 3300 (-NH str.), 2889 (-CH str.), 1682 (C=O str.) and 1408 (CH_2-CO-CH deformation). The parent molecule showed a strong protonated molecular ion peak $[M+H]^+$ at 392. The product ion spectrum of m/z 392 showed retro-Diel Alder (RDA) peak signals at m/z 249 and 144. The m/z ions at 249 further lost 84 Da to give the fragment at m/z 165. The fragment ions at m/z 249, 144 provide valuable information about the presence of intact pyrazinopridoindole ring nucleus. The CID spectra of the various metabolites were compared with that of the parent drug.

Dealkylated metabolite (*Figure 5*)

This metabolite could not be detected by PDA detector, thus the UV spectra of the metabolite could not be obtained. The polar *N*-dealkylated metabolite showed a protonated molecular ion peak $[M+H]^+$ at m/z 228 in the ESI mass spectrum. The MS/MS spectrum of the ion at m/z 228 showed RDA peak signals at m/z 144 and m/z 85. The chromatographic retention and LC-MS fragmentation pattern indicated the tentative structure of dealkylated metabolite as 1,2,3,4,6,7,12,12a-octahydropyrazino(2′,1′:6,1)pyrido[3,4-b]indole.

Hydroxy metabolite (*Figure 6*)

The hydroxy metabolite showed λ_{max} 1 at 223 and λ_{max} 2 at 278 nm in UV spectra. However, there was shift in the second wavelength from 245 nm to 278 nm, whereas λ_{max} 1 remained unaltered at 223 nm indicating no significant change in spectra. The spectra of this metabolite exhibited similarity of more than 90% with centbutindole. IR spectroscopy parameters of the hydroxy metabolite are 3305 (-NH str.), 3066 (-CH stretching), 3761 (-OH str.) and 1606 (C=C str.). The mass spectrum of the major metabolite exhibited protonated molecular ion peak $[M+H]^+$ of 394, 2 Da greater than that of centbutindole, suggesting that the molecule had undergone addition of two hydrogens via reduction of the ketonic function. This route was analogous to that observed with haloperidol and related drugs (Janicki & Gilpin 1978; Janicki & Ko 1978). The MS/MS reaction of the molecular ion peak at m/z 394 also showed

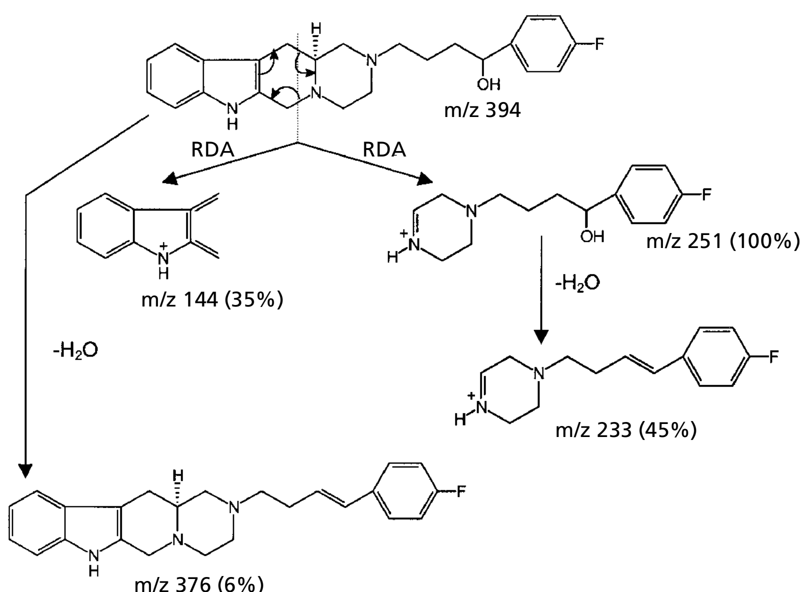

Figure 6 Proposed scheme for fragment ions generated by collisionally induced dissociation of the hydroxy metabolite of centbutindole. RDA, retro-Diel Alder fragmentation.

RDA peak signals at m/z 251 and 144. The mass unit of m/z 251 was 2 units greater compared with the daughter ions formed in the case of the parent molecule. Hydroxy metabolite m/z 394 and the daughter ion m/z 251 gave further fragments by loss of water molecule to give m/z 376 and 233, respectively. Based on these data, UV spectrum and comparison with the synthetic standard, the hydroxy metabolite was identified as the reduced product of the parent molecule. The metabolite was identified as 2-γ-[(*p*-fluorobenzyl-ol)propyl]-1,2,3,4,6,7,12,12a-octahydro-pyrazino(2′,1′:6,1)pyrido[3,4-b]indole.

Metabolites in bile

The 0–24-h bile samples were pooled and extracted for the identification of metabolites. Only one metabolite, the hydroxy metabolite, was identified in bile.

Excretion

About 0.6% of the administered dose of centbutindole as parent drug was recovered in faeces during the first 24 h; thereafter, no parent drug could be detected and thus its excretion was insignificant. No parent drug was present in bile, urine or faeces up to 72 h; an insignificant amount of hydroxy metabolite could be found in bile only. Urine from group 3 rats was collected and analysed for centbutindole and its metabolite(s). About 0.0012% of the total administered dose (as parent and metabolite(s)) was excreted in urine during the first 24 h, and thereafter no drug or metabolite could be detected up to 72 h. Renal clearance was calculated to be 0.017 mL min^{-1} kg^{-1}, which was insignificant when compared with the total body clearance of 66.7 mL min^{-1} kg^{-1} (discussed elsewhere).

Enzymatic hydrolysis

The urine and bile samples were subjected to enzymatic hydrolysis using β-glucuronidase (type V). The hydrolysis of bile sample showed the presence of the hydroxy metabolite in its free form. The hydroxy metabolite was present as glucuronide conjugate to a greater extent than its non-conjugated form; about 0.2% of the dose was excreted in bile as hydroxy metabolite glucuronide, compared with 0.07% as non-conjugated form. No drug or metabolite could be detected after hydrolysis of urine with β-glucuronidase.

Discussion

This study demonstrates the identification and characterisation of the in-vivo metabolites of centbutindole in male Sprague-Dawley rats. Efforts were also directed towards evaluating the stability of centbutindole in various in-vitro biomatrices. The drug was found to be stable to microbes, present in the intestinal contents, under aerobic conditions. However, the possible role of anaerobic metabolism by enteral flora cannot be ruled out since it is shown that azetirelin is metabolised by oxygen-sensitive bacteria localised mainly in the intestine (Sasaki et al 1994). Moreover, the data obtained from the preclinical in-vitro metabolism studies can not be directly extrapolated to man. Although all the members of the super family CYP 450 possess highly conserved regions of amino acid sequence, there are considerable variations across the species on the primary sequence (Kawataki 1995). Even a small change in the amino-acid sequences can give rise to profound differences to substrate specificity (Lindberg & Negishi 1989). This fact has been clearly demonstrated by the qualitative and quantitative differences in the metabolism of losartan (Stearus et al 1992) and azidothymidine (Blum et al 1988) across the various species.

Metabolism studies using in-vitro liver and intestinal mucosa wall homogenates indicated that the carbonyl reductases have a significant role to play in the biotransformation of centbutindole. Two common phase-I metabolites were observed in both in-vitro and in-vivo preparations. The structures of one of these two metabolites (i.e. dealkylated metabolite) obtained from in-vitro liver metabolism studies and from treated rat urine was identified by ion spray LC/MS/MS, using parent-ion and product-ion scanning techniques. With the dealkylated metabolite, the appearance of a RDA peak signal at m/z 144, similar to that obtained from centbutindole, confirmed that the pyrazinopyridoindole nucleus of this metabolite was intact. For the hydroxy metabolite, a similar RDA peak signal at m/z 251 was observed for molecular ion peak at m/z 394, which was 2 Da greater compared with the daughter ions formed in the case of the parent molecule, thus confirming the reduction of the carbonyl function of centbutindole. The loss of a water molecule by the molecular ion at m/z 394 and daughter ion m/z 251 (Figure 6) also confirmed the reduction of the ketone group of centbutindole. However, the RDA fragment m/z 144 (2,3 dimethyleneindoline) was common for both the hydroxy metabolite and centbutindole, which suggests that no alteration occurred in this part of the molecule. Moreover, the IR analysis of the hydroxy metabolite showed absence of carbonyl stretching frequency at 1682 cm^{-1} and CH_2-CO-CH stretching frequency, as seen for centbutindole at 1467 cm^{-1}, also suggesting that the C=O group had been converted into a secondary alcoholic group (-OH), which showed its stretching frequency at 3761 cm^{-1} further confirming the reduction of the carbonyl function. The structure of the hydroxy metabolite was unambiguously confirmed by comparison with that of synthetic standard. The dealkylated metabolite was tentatively assigned a structure based on the fragmentation pattern observed (Figure 5) and by comparing it with that of the parent molecule. From the metabolites identified, it appears that the metabolism of centbutindole occurred by two routes. The two routes, carbonyl reduction (hydroxy metabolite) and *N*-dealkylation reaction were analogous to those for structurally related drugs (Janicki & Gilpin 1978; Janicki & Ko 1978). Thus only two metabolites, hydroxy and dealkylated, were identified in-vivo.

A single oral dose of 4 mg kg^{-1} centbutindole to NBD and BDC rats showed that the contribution of the urinary,

biliary and faecal routes for excretion of centbutindole was non-significant. Although, biliary excretion of the hydroxy metabolite was less than 0.1%, the profile of the hydroxy metabolite in bile was not similar after treatment with β-glucuronidase suggesting that the elimination of the drug was not only due to phase-I but also due to the phase-II metabolic pathways. The absence of parent drug and its metabolite(s) in urine after oral administration and an insignificant presence after intravenous administration to rats indicates the distribution of the drug and its metabolite(s) to the deep tissue compartments of the body, thus prolonging their residence. Moreover, the insignificant renal clearance of 0.017 mL min^{-1} kg^{-1} obtained after intravenous administration of centbutindole to rats also suggests the role of other organs in the elimination of the drug.

In conclusion, bioanalytical methods for the determination of centbutindole and its hydroxy metabolite in bile, urine and faeces were developed and validated. The stability of centbutindole in various in-vitro biomatrices was assessed and was found to be prone to pre-systemic metabolism. Two metabolites were identified in-vivo and in S-9 fraction of liver and were characterised by LC-MS/MS technique. The role of biliary, urinary and faecal routes for the excretion of parent drug and its metabolite(s) was found to be insignificant. These results point towards the possibility of the presence of drug and its metabolite(s) in the tissues, for which further work is in progress.

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