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SHORT COMMUNICATIONS

Excretion balance and urinary metabolites of the *S*-enantiomer of indobufen in rats and mice

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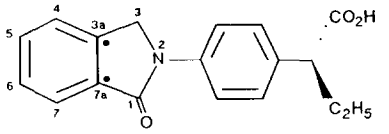
Abstract—The excretion balance and urinary metabolites of the *S*-enantiomer of indobufen, ((*S*)2-[p-(1-oxo-2-isoindolinyl)-phenyl]butyric acid), a platelet aggregation inhibitor, were studied in rats and mice after oral administration. The urinary metabolic profile exhibited a marked species difference. The major metabolic pathway in the mouse was acyl glucuronidation followed by renal excretion, whereas in rat urine 5-hydroxylation and subsequent sulphation at the introduced hydroxyl group accounted for almost all recovered radioactivity. Indobufen glucuronide was the major biliary metabolite in the rat, while very little indobufen glucuronide was present in the urine of intact or bile duct-cannulated rats. A marked dose–effect on the elimination and metabolism of *S*-indobufen was demonstrated in the rat. The recovery (% dose) of 5-hydroxyindobufen and its sulphate after the lower dose of the enantiomer (10 mg/kg) was some 2.8-fold higher compared with the higher dose of 20 mg/kg.



S-Indobufen

Fig. 1. The structure of *S*-indobufen. ● ^{14}C Label.

rac-Indobufen ((±)2-[*p*-(1-oxo-2-isoindolinyl)phenyl]-butyric acid) is an inhibitor of the enzyme cyclooxygenase, thereby inhibiting the production of thromboxane A_2 [1, 2]. *rac*-Indobufen is used therapeutically in Italy as an inhibitor of platelet aggregation in conditions such as peripheral vascular disease and cardiac ischaemic disease, intermittent claudication and in the prevention of venous thrombosis [3–5]. A review of its properties and therapeutic efficacy in several diseases has appeared recently [6].

Indobufen contains a chiral centre and thus exists as a pair of enantiomers. The *S*-enantiomer is almost totally responsible for the *in vitro* and *in vivo* pharmacological activity of indobufen and, like the closely structurally related profen (2-arylpropionic acid) non-steroidal anti-inflammatory drugs, the *R*-enantiomer is essentially inactive [7, 8]. Indobufen possesses an ethyl group α to the carboxyl group in place of the α-methyl group of the profens. The *in vivo* biological activity of *rac*-indobufen in man is also totally accounted for by the *S*-enantiomer [9].

Metabolic chiral inversion from the less active *R*-enantiomer to the active *S*-enantiomer has been observed for several profens, both *in vivo* and *in vitro* [10]. Chiral inversion has, however, not been observed for indobufen after single or repeated doses to animals or man [11–13]. Since *S*-indobufen is the active enantiomer of indobufen, it is of interest to study its excretion and metabolism. A report of the excretion and metabolism of *rac*-indobufen in rats and mice has been completed recently [14]. The present report presents details of the excretion balance and urinary metabolism of *S*-indobufen when administered to rats and mice.

MATERIALS AND METHODS

Chemicals. *S*-[^{14}C]Indobufen ((+)-*S*-2-[*p*-(1-oxo-2-[3*a*, 7*a*-^{14}C]isoindolinyl)phenyl]butyric acid) (Fig. 1), sp. act. 48.07 mCi/mmol, radiochemical purity ≥98% by radioTLC, batch No. EF 7436/95, was supplied by Farmitalia Carlo Erba (Milan, Italy), as were samples of unlabelled racemic and *S*-indobufen, and 3-, 5- and 6-hydroxyindobufen. Other chemicals and reagents were obtained from usual commercial sources and were of the best available quality.

The acyl glucuronides of indobufen and the acyl and phenolic glucuronides of 5- and 6-hydroxyindobufen, for use as HPLC standards, were biosynthesized by incubation of the appropriate substrate with UDP glucuronic acid and rabbit liver microsomes as described previously [15]. The acyl and phenolic glucuronides of 3-hydroxyindobufen were not detected after microsomal incubation of 3-hydroxyindobufen.

Administration of indobufen. Male Wistar rats (200–250 g) and female CD-1 mice (20–25 g) were housed in all glass metabolism cages (Metabowls for rats and Minimetabowls for mice, Jencons Ltd, Leighton Buzzard, U.K.) equipped for the separate collection of urine and faeces, with free access to food (GLP grade diet CRM, Special Diets Services, Cambridgeshire, U.K.) and water. *S*-[^{14}C]Indobufen (*ca.* 5 μCi/animal) was administered p.o. by gastric gavage in propane-1,2-diol in volumes of 0.5 mL containing 10 or 20 mg/kg for rats and 0.2 mL containing 12.5 mg/kg for mice. Urine and faeces were collected daily for 4 days. Urine was collected in glass vessels containing glacial acetic acid (0.1 and 0.04 mL for rats and mice, respectively) cooled with dry ice.

Bile duct cannulation of rats. Three male Wistar rats (250 g) had their common bile ducts cannulated under ether anaesthesia using a polyvinyl cannula, i.d. 0.4 mm (Portex Ltd, Kent, U.K.). The incision was closed with sutures and the cannula led out through the dorsal skin. The rats were held in Bollman restraining cages and allowed to recover for at least 1 hr after surgery to establish bile flow. *S*-[^{14}C]Indobufen (10 mg/kg; *ca.* 5 μCi/rat) was administered p.o. by gastric gavage in 0.5 mL propane-1,2-diol. Bile was collected over ice for 24 hr. The 0–24 hr urine and faeces were also collected. During the experiment the animals had free access only to dextrose-saline solution (0.18% NaCl, 4.3% glucose). Bile and urine were counted for ^{14}C and stored at −20° until further analysis.

Radiochemical techniques. ^{14}C in urine, bile and HPLC eluents was assayed by liquid scintillation counting using Packard TriCarb instruments Models 4640 or 4450

Table 1. Retention times of indobufen and of the synthetic and biosynthetic standards available in the HPLC system used

Compound	Retention time (min)
5-Hydroxyindobufen glucuronide (phenolic)	3.8
6-Hydroxyindobufen glucuronide (phenolic)	5.1
5-Hydroxyindobufen glucuronide (acyl)	6.4
6-Hydroxyindobufen glucuronide (acyl)	8.0
3-Hydroxyindobufen	10.4
5-Hydroxyindobufen	15.1
6-Hydroxyindobufen	16.6
Indobufen glucuronide	18.0
Indobufen	36.8

(Canberra-Packard, Pangbourne, U.K.). Aliquots (0.05–0.5 mL) were counted in triplicate in minivials using a water-miscible biodegradable scintillation cocktail (Ecoscint, National Diagnostics, Watford, U.K.). Quench correlation was achieved by reference to an external standard using a standard curve stored in the instrument which was established at regular intervals. The ^{14}C content of rat and mouse faeces was determined after alkali digestion and bleaching. The faeces from each 24 hr collection were homogenized in water (30 and 15 mL for rats and mice, respectively) using a Stomacher Lab-Blender (Seward Medical, London, U.K.) for 1 min. Two millilitres of the homogenate were mixed with 1 mL 5 M NaOH, 2 mL H_2O_2 and five drops of *iso*-amyl alcohol to control foaming and left overnight. The solution was neutralized with 260 μL glacial acetic acid and the volume made up to 10 mL with ethanol. The solution was then heated to 70° for 30 min to remove excess H_2O_2. The ethanol lost through evaporation was replaced and the solution clarified by centrifugation. Aliquots (0.5 mL) were counted in triplicate in 10 mL of Ecoscint. A quench correlation curve was established using faeces from untreated animals.

HPLC. All HPLC assays were performed using a Shimadzu LC-6A pump, RF-535 fluorescence detector and C-R6A Chromatopac computing integrator (supplied by Dyson Instruments Ltd, Houghton-le-Spring, Tyne and Wear, U.K.). Samples were introduced on to the column with a Waters Associates (Northwich, U.K.) WISP Model 710B autoinjector. The fluorescence detector was set at excitation 290 nm, emission 440 nm. The column was a Merck LiChrosorb C18 cartridge, 250 × 4 mm i.d., and mobile phase was a step gradient of acetonitrile:water, 25:75 by volume containing 0.1% trifluoroacetic acid from 0 to 30 min, then acetonitrile:water, 40:60 by volume containing 0.1% trifluoroacetic acid from 30 to 50 min; flow rate, 2 mL/min throughout at room temperature. Fluorescence detection was used to characterize the chromatographic behaviour of indobufen and the synthetic and biosynthetic standards available. After injection of urine or bile, fractions of column eluent were collected every 30 sec in mini-scintillation vials, using an LKB 2112 RediRac fraction collector (LKB Instruments Ltd, Croydon, U.K.) and counted for ^{14}C as above. Urine and bile were examined by HPLC before and after treatment with β-glucuronidase, sulphatase or mild alkali, the ester glucuronides being cleaved by mild alkali and β-glucuronidase treatment, whereas the phenolic glucuronides were hydrolysed only by β-glucuronidase [16].

Analysis of indobufen metabolites in rat urine and bile and in mouse urine. The metabolites of *S*-[^{14}C]indobufen in rat urine and bile and in mouse urine were analysed using the HPLC system described above. *S*-Indobufen and 5- and 6-hydroxyindobufen were characterized by coelution with authentic standards. Indobufen glucuronide was characterized by coelution with biosynthetic indobufen glucuronide and by selective hydrolysis of urine or bile with β-glucuronidase (glucurase, 5000 U/mL β-glucuronidase *ex* bovine liver, Sigma Chemical Co., Poole, U.K.) or mild alkali (pH 10, 1 hr, room temperature). The HPLC system described above did not allow the analytical separation of the diastereoisomeric indobufen glucuronides. 5-Hydroxyindobufen sulphate was characterized by incubation of urine or bile with sulphatase (Type H-1, *ex Helix pomatia*, sp. act. 18,000 U/g, Sigma). Aliquots of urine (0.5 mL rats and 0.2 mL mice) or bile (0.2 mL) were diluted 2:1 with 0.2 M acetate buffer pH 5.0 and incubated at 37° overnight with either 0.3 vol. glucurase or 0.64 mg sulphatase. Positive and negative control incubations were performed according to Caldwell and Hutt [16].

RESULTS AND DISCUSSION

The retention times of *S*-indobufen and of synthetic and biosynthetic standards in the HPLC system used in the present study are listed in Table 1.

The excretion balance and urinary and biliary metabolite profiles of *S*-[^{14}C]indobufen in rats and mice are presented in Tables 2 and 3, respectively. Recovery of ^{14}C in intact rats given the lower dose or in mice was essentially quantitative with over 95% of the dose being recovered in urine and faeces over four days. Urinary ^{14}C recovery in rats was slightly higher than faecal recovery after the lower dose of 10 mg/kg (55% and 43%), while after the higher dose of 20 mg/kg the reverse was true (37% and 52%). Forty-eight per cent of dosed ^{14}C was recovered in rat bile 24 hr after 10 mg/kg with 33% and 2% being recovered in the urine and faeces, respectively. In mice the bulk of ^{14}C was recovered in the urine (urine 69%, faeces 26%).

The rate of total elimination of ^{14}C in rats given the lower dose of 10 mg/kg was similar to mice given 12.5 mg/kg (69% and 68% during the first 24 hr, respectively) but more rapid compared with the higher dose of 20 mg/kg (only 47% during the same interval).

Table 2. Urinary, faecal and biliary recovery of ^{14}C after oral administration of *S*-[^{14}C]indobufen to rats (10 or 20 mg/kg), bile duct-cannulated rats (10 mg/kg) and mice (12.5 mg/kg)

Time (hr)	Rats (10 mg/kg)		Rats (20 mg/kg)		Bile duct-cannulated rats			Mice	
	Urine	Faeces	Urine	Faeces	Bile	Urine	Faeces	Urine	Faeces
0–24	45.9 ± 4.0	23.4 ± 4.4	20.4 ± 1.4	26.3 ± 4.6	47.9 ± 11.6	33.4 ± 4.9	2.0 ± 1.7	55.9 ± 13.3	12.0 ± 3.7
24–48	7.8 ± 2.3	17.1 ± 6.2	10.8 ± 0.9	18.2 ± 1.3				11.9 ± 6.6	12.6 ± 11.3
48–72	0.8 ± 0.6	1.3 ± 0.9	4.3 ± 0.6	5.7 ± 1.2				0.7 ± 0.3	1.4 ± 1.4
72–96	0.3 ± 0.6	1.0 ± 1.6	1.3 ± 1.5	1.5 ± 0.8				0.2 ± 0.3	0.2 ± 0.3
Total	54.9 ± 1.6	42.8 ± 6.9	37.1 ± 1.5	51.6 ± 3.6				68.7 ± 7.4	26.2 ± 10.9

Each value is the mean % dose excreted ± SD of six animals (three for bile duct-cannulated rats).

Table 3. Urinary and biliary metabolites of indobufen in rats or in bile duct-cannulated rats given 10 or 20 mg/kg and in mice given 12.5 mg/kg *S*-[^{14}C]indobufen p.o.

Metabolite	Rats (10 mg/kg) Urine 0–24 hr	Rats (10 mg/kg) Urine 24–48 hr	Bile duct-cannulated rats (10 mg/kg) Urine 0–24 hr	Bile duct-cannulated rats (10 mg/kg) Bile 0–24 hr	Rats (20 mg/kg) Urine 0–24 hr	Rats (20 mg/kg) Urine 24–48 hr	Mice (12.5 mg/kg) Urine 0–24 hr
Indobufen							
Free	0.8	ND	2.7	2.5	1.2	1.1	1.6
Glucuronide	ND	ND	tr	22.9	0.9	0.3	41.1
5-Hydroxyindobufen							
Free	19.3	3.7	13.8	10.2	4.2	3.4	ND
Sulphate	22.0	3.7	13.8	8.9	5.6	4.4	ND
6-Hydroxyindobufen							
Free	0.8	tr	0.9	ND	ND	ND	ND
Others	3.0	0.4	2.2	1.9	8.8	1.6	13.2
Total	45.9	7.8	33.4	46.4	20.7	10.8	55.9

Each value is the mean % ^{14}C dose of six animals (three for biliary excretion).
ND, not detected; tr, trace.

The major metabolic transformation of *S*-indobufen in rats was 5-hydroxylation and subsequent sulphation of the hydroxyl group [48.7% and 17.6% of the lower and higher dose, respectively, as total 5-hydroxyindobufen (free + sulphated) in the 0–48 hr urine] (Table 3). In the 0–24 hr rat bile, 22.9% of the dose was excreted as indobufen glucuronide and 2.5% as free indobufen, while 10.2% and 8.9% were excreted as free and sulphated 5-hydroxyindobufen, respectively (Table 3). In the mouse the major urinary metabolite was indobufen glucuronide (41.1% of the dose in the 0–24 hr urine). Hydroxylated derivatives, either free or sulphated, were not detected in this species.

The metabolic profiles of *S*-indobufen in the rat and mouse were qualitatively essentially identical to those of indobufen in the same species [14]. The major urinary metabolite in the rat was 5-hydroxyindobufen and its sulphate, while in the mouse indobufen glucuronide predominated. The mouse therefore represents a better model for the metabolism of *S*-indobufen in man, where unchanged indobufen (free + glucuronide) is the major urinary metabolite (77% of the dose in 48 hr) [13]. As with *rac*-indobufen [14], 5-hydroxylation was the major hydroxylated metabolite in rat urine, 6-hydroxyindobufen accounting for less than 1% of the dose, whereas 3-hdyroxyindobufen was not detected.

The results of this study show a clear dose–effect on the excretion and metabolism of *S*-indobufen in the rat. The urinary recovery (as % of dose) of 5-hydroxyindobufen sulphate after the lower dose of 10 mg/kg was some 2.6-fold greater than after the higher dose of 20 mg/kg. The urinary recovery (as % of dose) of free 5-hydroxyindobufen after the lower dose of some 3-fold greater than after the higher dose. This strongly suggests saturation of aromatic hydroxylation rather than sulphate conjugation with increasing dose. Ring hydroxylation is an important metabolic transformation of many profen non-steroidal anti-inflammatory drugs, as well as of other drugs, and saturation of oxidative metabolism has been used to explain the dose-dependent behaviour of, for example, ibuprofen [17] and diphenylhydantoin [18].

Like *rac*-indobufen [14], very little indobufen glucuronide was excreted in rat urine after administration of the *S*-enantiomer. Indobufen glucuronide was, however, the major metabolite in rat bile. The markedly lower faecal excretion of ^{14}C over the first 24 hr in intact rats compared with biliary recovery suggests extensive enterohepatic circulation of indobufen and/or its metabolites. This presumably results from the hydrolysis of the glucuronide by gut microflora and re-absorption of the more lipophilic parent drug. The preferential excretion in the rat of acyl glucuronides in bile and sulphate conjugates in urine has been observed for other compounds, such as diflunisal, a derivative of salicyclic acid [19].

The more rapid recovery of ^{14}C and higher recovery of indobufen glucuronide in mouse urine after administration of the *S*-enantiomer compared with the racemate [14] might be explained by enantioselectivity in the *in vivo* glucuronidation and/or enantioselectivity in the urinary excretion of *S*-indobufen glucuronide. In preliminary experiments (results not shown) the ratio of *S*-indobufen glucuronide to *R*-indobufen glucuronide after oral administration of the racemic compound (25 mg/kg) to mice was some 2.4. However, this is in contrast to the slower elimination of the *S*-enantiomer from mouse plasma [11] and the *in vitro* enantioselectivity of glucuronide formation [15]. Furthermore, it would be useful to have data on ^{14}C recovery after administration of 25 mg/kg *S*-indobufen in the mouse, in order to know whether there is a dose–effect on the excretion and metabolism of *S*-indobufen also in this species, as it is the case in the rat.

In conclusion, these results show that *S*-indobufen is metabolized similarly to the racemic compound, indobufen, in the rat, mouse and man. The mouse is a better model than the rat for the metabolism and disposition of *S*-indobufen in man. In addition, there is clear dose-dependent metabolism and excretion of *S*-indobufen in the rat, probably due to saturation of oxidative metabolism.

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