Evaluation of the Pharmacological Actions and Pharmacokinetics of BOF-4272, a Xanthine Oxidase Inhibitor, in Mouse Liver

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Abstract

BOF-4272 ($\pm$)-8-(3-methoxy-4-phenylsulphinylphenyl) pyrazolo[1,5-a]-1,3,5-triazine-4-(1*H*)-one, a new synthetic anti-hyperuricaemic drug, which has a chiral centre and exists as racemates, is a potent inhibitor of xanthine oxidase/dehydrogenase in the purine catabolism pathways. The present studies using mice demonstrated that BOF-4272 was specifically distributed in the liver, which is the main organ of uric acid production. Therefore, a decrease in uric acid concentration in the liver, rather than the plasma, was identified as a pharmacological action of BOF-4272.

The ratio of liver to plasma concentrations of BOF-4272 increased from 2·5 to 6·3 over time, up to 8 h after oral administration. The elimination half-life of BOF-4272 in the liver was 5·1-fold longer than that in the plasma. High concentrations of BOF-4272 were observed in the liver up to 8 h after oral administration. Furthermore, the influx of BOF-4272 into hepatocytes occurred in a temperature-dependent manner.

The liver concentrations of uric acid from 1 h to 8 h after the oral administration of BOF-4272 ($0.34-0.75\,\mu$g (g tissue)$^{-1}$) were significantly lower than those in control animals ($5.03-10.96\,\mu$g (g tissue)$^{-1}$). BOF-4269 (the sulphide metabolite of BOF-4272) was the only metabolite detected in plasma or faeces after intravenous or oral administration. BOF-4269, which has no inhibitory action on the uric acid biosynthesis system, is generated by the metabolism of BOF-4272 in the intestinal tract.

In conclusion, this work using the liver as the target organ has allowed us to identify the pharmacological actions of BOF-4272 in mice. The long-lasting effect of BOF-4272 in reducing levels of hepatic uric acid was consistent with the prolonged high BOF-4272 concentrations in the liver. These results also demonstrate that the mouse is a suitable animal species for evaluating the clinical pharmacology and pharmacokinetics of BOF-4272.

Inhibitors of xanthine oxidase (XO)/xanthine dehydrogenase (XDH), enzymes which catalyse the last step of purine catabolism, can be used for the treatment of hyperuricaemia. However, no new inhibitors have been used clinically since the introduction of allopurinol (Elion et al 1963; Rundles et al 1963; Elion 1966; Hille & Massey 1981). BOF-4272, a derivative of pyrazolotriazine, is a new drug that has been developed for the treatment of hyperuricaemia and ischaemic reperfusion injury (Sato et al 1991; Yamamoto et al 1993; Iwahara et al 1994; Uematsu & Nakashima 1994). BOF-4272 inhibits the de-novo biosynthesis of uric acid by blocking the XO/XDH system in the liver and small intestine,

which are the target organs of the pharmacological actions of BOF-4272 (Sato et al 1991; Uematsu & Nakashima 1994). This mechanism of inhibition by BOF-4272 has been elucidated by an in-vitro study using milk XO/XDH (Okamoto & Nishino 1995). BOF-4272 also significantly decreases the concentration of free radicals generated by xanthine oxidase and consequently reduces cellular necrosis (Suzuki et al 1994).

We have previously reported the metabolic pathways and pharmacokinetic properties of BOF-4272 in rats, mice, and dogs (Naito & Nishimura 1999; Naito et al 1999a, b). BOF-4269, which is the sulphide metabolite of BOF-4272 and has no pharmacological activity (Okamoto et al 1993), is generated from the metabolism of BOF-4272 by the intestinal flora and absorbed from the intestinal

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tract in rats (Naito & Nishimura 1999). Since high bioavailability of BOF-4272 was observed in fed mice and humans, the mouse was thought to be a suitable animal species for evaluating the clinical pharmacokinetics of BOF-4272. Furthermore, previous in-situ studies showed that the hepatic elimination of BOF-4272 was quite substantial (Nishimura et al 1994, 1995, 1996).

Studies in healthy male volunteers were conducted to determine the effect of BOF-4272 in reducing serum levels of uric acid, a final product of xanthine metabolism, as a pharmacological action of this XO/XDH inhibitor. The results showed that serum concentrations of uric acid were reduced both during and after the period of high plasma BOF-4272 levels (Uematsu & Nakashima 1994). However, the serum level of uric acid was only slightly changed when rodents were used as the experimental animals. It is known that there are species differences between humans and rodents in uric acid metabolism involving the tissue distribution of XO/XDH and the presence of uricase (Sato et al 1991). This might be one reason that no pharmacological action of BOF-4272 could be detected in rodents when serum concentrations of uric acid were followed.

The present study was designed to measure the concentration of uric acid in the liver rather than in the plasma after the administration of BOF-4272 to mice, because the liver is a target organ for BOF-4272 and may thus reveal its anti-hyperuricaemic action. This paper describes the detailed pharmacological activity and disposition of BOF-4272 in the mouse liver.

Materials and Methods

Materials
BOF-4272, ($\pm$)-8-(3-methoxy-4-phenylsulphinyl-phenyl) pyrazolo [1,5-a] - 1,3,5-triazine-4(1H)-one), and its metabolites used in this study were synthesized at Otsuka Pharmaceutical Factory Inc. (Tokushima, Japan). The chemical purity was greater than 99·0%.

Carboxymethylcellulose sodium salt (CMC), acetonitrile, ethyl acetate, collagenase and perchloric acid (PCA) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Polyethylene glycol 400 (PEG 400) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Bovine serum albumin (Fraction V) (BSA) and trypsin inhibitor were purchased from Sigma Chemical Co. (St Louis, MO). Trypan blue was purchased from Flow Laboratories Ltd (Irvine, UK). Eagle's MEM

Nissui (type 1) was purchased from Nissui Pharmaceutical Co. Ltd (Tokyo, Japan). All other chemicals and reagents used were of analytical reagent grade.

Animals
The mice used were male ICR mice, 8 weeks of age and weighing 36·0–42·0 g (n = 91), supplied by Japan SLC Inc. (Shizuoka, Japan). During the experiment, the mice were housed individually in metabolic cages in a room maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 10% with a 12-h night–day cycle. The mice were allowed free access to food (CRF-1-R, Oriental Yeast Co. Ltd, Tokyo, Japan), except during periods of fasting. The mice were allowed free access to water throughout the study period.

Chromatography
Uric acid in the liver was measured using high-performance liquid chromatography (HPLC) systems as described in the literature (Sakuma et al 1987). The concentrations of BOF-4272 and its metabolites were measured using HPLC systems (CCP & 8010 Series, Tosoh Co., Tokyo, Japan) with a stationary phase of TSKgel ODS-120T (250 × 4·6 mm i.d., Tosoh Co.). The HPLC systems consisted of a system controller (PX-8010), pump (CCPM), autosampler (AS-48), UV detector (UV-8010), and integrated data analyser (C-R4AX Chromatopac, Shimadzu Co., Kyoto, Japan). The detector wavelength and the flow rate were 323 nm and 1·0 mL min^{-1}, respectively. The column temperature was ambient. The mobile phase was a mixture of solution A (10 mM NH$_4$H$_2$PO$_4$, pH 3·0) and solution B (acetonitrile and solution A, 80 : 20 v/v), with a gradient from 70%/30% to 0%/100% over 31 min.

Animal studies
In the intravenous and oral administration studies, the mice were fasted from 16 h before to 4 h after drug administration. BOF-4272 was dissolved in 50% PEG 400 for intravenous administration and in 0·5% CMC solution for oral administration. BOF-4272 was administered to the mice intravenously at a dose of 5 mg kg^{-1} and orally at doses of 0 mg kg^{-1} (0·5% CMC solution alone, control group) and 5 mg kg^{-1}.

The mice were anaesthetized with pentobarbital (50 mg kg^{-1}, i.p.; Nembutal, Abbott Laboratories, North Chicago, IL), and blood samples were obtained from the abdominal inferior vena cava. Blood samples were drawn into heparinized test

tubes at 0 (pre-dose), 0·083, 0·25, 0·5, 1, 2, 4 and 8 h after bolus intravenous administration and at 0 (pre-dose), 1, 2, 4 and 8 h after oral administration. All blood samples were immediately centrifuged to obtain plasma. The livers at 0 (pre-dose), 1, 2, 4 and 8 h after the oral administration of BOF-4272 at 0 mg kg^{-1} (control group) and 5 mg kg^{-1} were perfused with saline solution (20 mL) at 4°C and then frozen on dry ice.

Urine and faeces samples were collected daily for 4 days after intravenous or oral administration.

Preparation of isolated hepatocytes
The liver was perfused at 37°C for 4 min with Ca^{2+}- and Mg^{2+}-free Hanks' solution (pH 7·4) containing 0·5 mM EGTA. The liver was then removed from the body, and the hepatocytes were dispersed by gentle shaking for 8 min with Hanks' solution (pH 7·4) containing 0·1% collagenase and 0·05% trypsin inhibitor. After filtration through gauze, the hepatocytes were separated from non-parenchymal cells by centrifugation three times, with the cells washed and resuspended in Ca^{2+}- and Mg^{2+}-free Hanks' solution (pH 7·4) each time. Finally, the hepatocytes were resuspended in Eagle's MEM medium (pH 7·4) containing 0·5% BSA and 2 mM L-glutamate. Cell suspensions with a viability greater than 90% as assessed by trypan blue exclusion were used in the experiments. The number of cells was counted using a Coulter. For incubation, the cell suspensions were diluted to a final concentration of 2×10^6 cells mL^{-1}.

Hepatic uptake studies
After pre-incubation of 1 mL of the cell suspension containing 2×10^6 cells mL^{-1} for 5 min at 4°C or 37°C, the reaction was started by the addition of 1 mL of the incubation medium containing BOF-4272 at 2 μg mL^{-1} pre-incubated for 5 min at 4°C or 37°C. The reaction time was set at 15 min. After incubation, 1·5 mL of the reaction mixture was transferred to another tube and centrifuged at 10 000 rev min^{-1} for 10 s (CR15T centrifuge, Hitachi Koki Co. Ltd, Tokyo, Japan) at 4°C to stop the reaction by separating the cells from the incubation medium. The cells were washed twice with 1·5 mL of ice-cold PBS(−) and dissolved by adding 1·5 mL of 0·1% Triton X-100. After centrifugation, 0·5 mL of the supernatant was transferred to another tube for use as the HPLC sample. The HPLC sample was supplemented with 3 mL of ethyl acetate and shaken. After centrifugation, the ethyl acetate layer was transferred to another tube and dried under reduced pressure. The dried sample

was supplemented with the mobile phase for analysis of BOF-4272 and shaken. The dissolved sample was then filtered through a membrane filter (pore size, 0·2 μm). A sample (500 μL) of the filtrate was injected into the HPLC system for analysis of BOF-4272.

Preparation of plasma samples for HPLC
Each plasma sample was prepared by the successive addition of 2 volumes of acetonitrile at 4°C with shaking. After centrifugation, the supernatant was transferred to another tube, 2 volumes of 10 mM NH$_4$H$_2$PO$_4$ (pH 3·0) were added, and the mixture was shaken again. The mixture was then filtered through a membrane filter (pore size, 0·2 μm). A sample (500 μL) of the filtrate was injected into the HPLC system.

Preparation of liver samples for HPLC
Each frozen liver sample was prepared by the successive addition of 9 volumes of PCA at 4°C with shaking. After centrifugation, the supernatant was transferred to another tube, 3 mL of ethyl acetate was added, and the mixture was shaken again. After centrifugation, the ethyl acetate layer was transferred to two tubes and dried under reduced pressure. The two dried samples were dissolved in each mobile phase for analysis of uric acid and BOF-4272 and shaken. The dissolved samples were then filtered through a membrane filter (pore size, 0·2 μm). Samples (50 μL) of each filtrate were injected into the HPLC system for analysis of uric acid and BOF-4272, respectively.

Preparation of urine samples for HPLC
Each urine sample (0·5 mL) was mixed with 1 N HCl (0·1 mL) and ethyl acetate (4 mL) and shaken. After centrifugation, 1 mL of the ethyl acetate layer was transferred to another tube and dried under reduced pressure. The dried sample was redissolved in 3 mL of 10 mM NH$_4$H$_2$PO$_4$ (pH 3·0) containing 20% acetonitrile. The sample was then filtered through a membrane filter (pore size, 0·2 μm). A sample (500 μL) of the filtrate was injected into the HPLC system.

Preparation of faeces samples for HPLC
Each faeces sample was prepared by the successive addition of 10 volumes of acetonitrile at 4°C with shaking. After centrifugation, the supernatant was transferred to another tube, 10 volumes of 10 mM NH$_4$H$_2$PO$_4$ (pH 3·0) were added, and the mixture

was shaken again. The mixture was then filtered through a membrane filter (pore size, $0.2\,\mu$m). A sample ($500\,\mu$L) of the filtrate was injected into the HPLC system.

Data analysis

The concentration at time 0 (C_0) for intravenous administration was estimated by the residual method. The maximum concentration (C_{max}) and the time to reach the maximum concentration (t_{max}) after oral administration were read directly from the mean plasma concentration data. The elimination half-life ($t_{1/2}$) was calculated using the equation $t_{1/2} = \ln 2/K$, where K is the slope of the terminal portion of the natural logarithm of the concentration–time curve obtained by linear regression. The area under the concentration–time curve from time 0 to the measurement point (AUC_{0-t}), the area under the plasma concentration–time curve from time 0 to infinity ($AUC_{0-\infty}$) and the mean residence time (MRT) were calculated according to the trapezoidal rule (Yamaoka et al 1978). Total systemic clearance (CL) and volume of distribution (Vdss) were calculated according to the following equations:

$$CL = D/AUC \qquad (1)$$

$$Vdss = D{\cdot}MRT/AUC_{0-\infty} \qquad (2)$$

where D is the administered dose. The bioavailability (F) was assessed by comparing AUC_{0-8h} values after oral and intravenous administration.

Results

Figure 1 shows the plasma concentrations of BOF-4272 and its sulphide metabolite (BOF-4269) after intravenous and oral administration of $5\,$mg$\,$kg^{-1} of BOF-4272 to mice. BOF-4269 appeared in plasma 4 h after the intravenous injection of BOF-4272 and was detected as the only metabolite up to 8 h after administration. BOF-4269 was detected after a lag time following the oral administration of BOF-4272. Table 1 shows the pharmacokinetic parameters for BOF-4272 in plasma after the intravenous or oral administration of BOF-4272 to mice. The Vdss of BOF-4272 was calculated to be $1553\,$mL$\,$kg^{-1}, indicating a larger volume of distribution. The bioavailability (F), based on the AUC_{0-8h} values, was 12.1%.

Figure 2 shows the liver concentrations and ratio of liver to plasma concentrations (L/P ratio) of BOF-4272 after the oral administration of $5\,$mg$\,$kg^{-1} of BOF-4272 to mice. The L/P ratio of
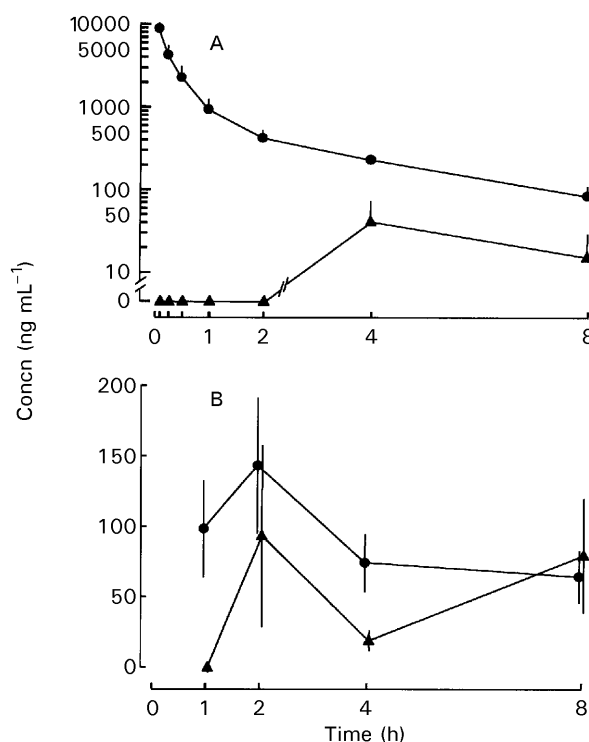

Figure 1. Plasma concentrations of BOF-4272 (●) and its metabolite BOF-4269 (▲) after the intravenous (A) or oral (B) administration of $5\,$mg$\,$kg^{-1} of BOF-4272 to mice. Each point for intravenous and oral administration represents mean $\pm$ s.d. for 4 and 5 mice, respectively.

Table 1. Pharmacokinetic parameters for BOF-4272 in plasma after the intravenous or oral administration of $5\,$mg$\,$kg^{-1} of BOF-4272 to mice.

Parameter	BOF-4272
Intravenous administration	
C_0 (ng$\,$mL^{-1})	10753
$t_{1/2}$ (h)	2.6
AUC_{0-8h} (ng$\,$h$\,$mL^{-1})	5469
$AUC_{0-\infty}$ (ng$\,$h$\,$mL^{-1})	5775
MRT (h)	1.8
CL (mL$\,$h$^{-1}\,$kg^{-1})	914
CL* (mL$\,$h$^{-1}\,$kg^{-1})	866
Vdss (mL$\,$kg^{-1})	1553
Oral administration	
C_{max} (ng$\,$mL^{-1})	143
t_{max} (h)	2
$t_{1/2}$ (h)	5.8
AUC_{0-8h} (ng$\,$h$\,$mL^{-1})	662
F (%)	12.1

C_0, plasma concentration at time 0; C_{max}, maximum plasma concentration; t_{max}, time to maximum plasma concentration; $t_{1/2}$, elimination half-life; AUC_{0-8h}, area under the plasma concentration–time curve from time 0 to 8 h; $AUC_{0-\infty}$, area under the plasma concentration–time curve from time 0 to infinity; MRT, mean residence time; CL, clearance $=$ dose/AUC_{0-8h}; CL*, dose/$AUC_{0-\infty}$; Vdss, volume of distribution; F, bioavailability based on AUC_{0-8h} values.

BOF-4272 increased slightly over time up to 8 h after oral administration. Table 2 shows the pharmacokinetic parameters for BOF-4272 in the liver after the oral administration of BOF-4272 to mice. Regarding BOF-4272, both the liver $t_{1/2}$ and the liver AUC_{0-8h} were 4−5 times those in plasma.

Figure 3 shows the liver concentrations of uric acid in mice with and without the oral administration of BOF-4272. The liver concentration of uric acid was $5.03 \pm 1.16 \mu g$ (g tissue)$^{-1}$ before dosing.
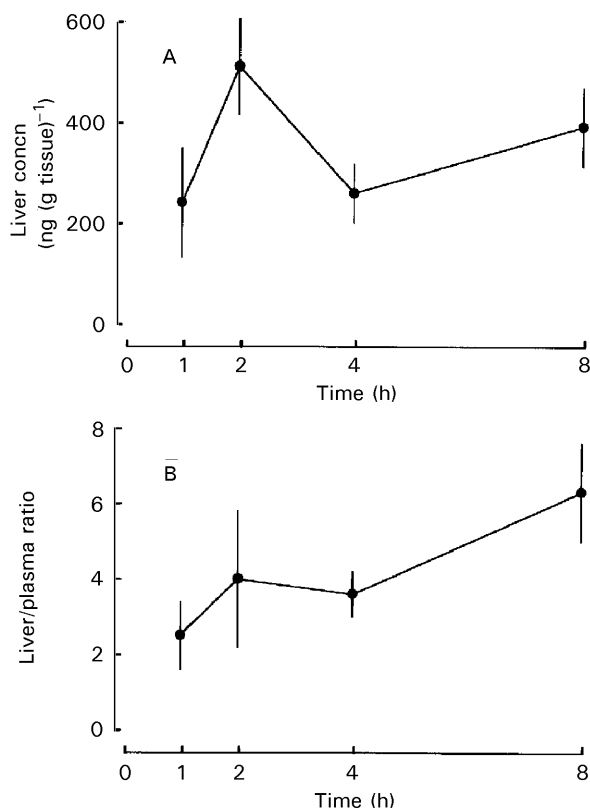

Figure 2. Liver concentrations (A) and ratio of liver to plasma concentrations (L/P ratio) (B) of BOF-4272 after the oral administration of 5 mg kg^{-1} of BOF-4272 to mice. Each point represents mean ± s.d. for 5 mice.

Table 2. Pharmacokinetic parameters for BOF-4272 in the liver after the oral administration of 5 mg kg^{-1} of BOF-4272 to mice.

Parameter	BOF-4272
C_{max} (ng (g tissue)$^{-1}$)	512
t_{max} (h)	2
$t_{1/2}$ (h)	29.5
AUC_{0-8h} (ng h (g tissue)$^{-1}$)	2574
L/P ratio of $t_{1/2}$	5.1
L/P ratio of AUC	3.9

C_{max}, maximum liver concentration; t_{max}, time to maximum liver concentration; $t_{1/2}$, elimination half-life; AUC_{0-8h}, area under the liver concentration–time curve from time 0 to 8 h; L/P ratio of $t_{1/2}$, ratio of liver $t_{1/2}$ to plasma $t_{1/2}$; L/P ratio of AUC, ratio of liver AUC_{0-8h} to plasma AUC_{0-8h}.

The uric acid levels 1−8 h after the oral administration of BOF-4272 were $0.34-0.75 \mu g$ (g tissue)$^{-1}$, which were significantly lower than those in control mice (without the oral administration of BOF-4272).

Table 3 shows the cumulative urinary and faecal excretion of BOF-4272 and its metabolites after the intravenous or oral administration of BOF-4272 to mice. Following intravenous administration, 35.3% of the administered BOF-4272 was excreted in the urine and 4.51% was excreted in the faeces. The corresponding values following oral administration were 13.1% and 3.11%, respectively. Metabolites were not detected in the urine, and BOF-4269 and M-1 (see Figure 4) were detected in the faeces.

In the in-vitro study, $1 \mu g$ of BOF-4272 was incubated for 15 min at 37°C or 4°C with mouse hepatocytes. The hepatic uptake of BOF-4272 was 359 ng per 1×10^6 viable cells at 37°C, and 37 ng per 1×10^6 viable cells at 4°C (values are means, n = 2). These results show the effect of temperature on the hepatic uptake of BOF-4272. The concentration of BOF-4272 in the hepatocytes at 37°C was 10 times that at 4°C.

Discussion

Elion et al (1966, 1968) showed that allopurinol is converted rapidly to oxipurinol, which is also an inhibitor of xanthine oxidase. Oxipurinol has a long $t_{1/2}$ of 14−26 h in patients with normal renal function (Elion et al 1966; Hande et al 1978; Matsunaga et al 1982). On the other hand, BOF-4272 has a short $t_{1/2}$ of 1.65−1.92 h in healthy volunteers (Uematsu & Nakashima 1994). The serum concentration of uric acid was reduced in a dose-dependent manner to about 80% of the pre-dose value by the administration of BOF-4272 at doses of 200 mg and 400 mg
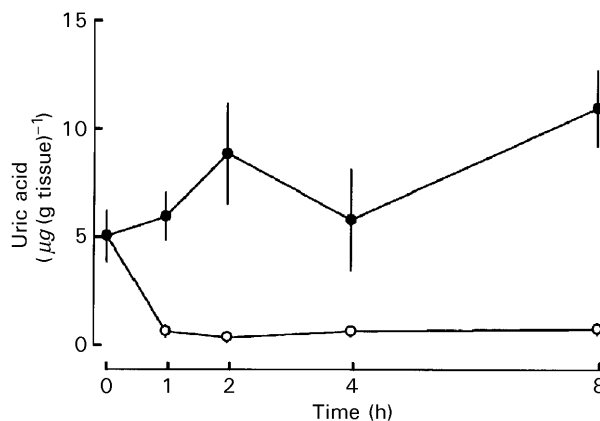

Figure 3. Liver concentrations of uric acid in mice with (○) and without (●) the oral administration of 5 mg kg^{-1} of BOF-4272. Each point represents mean ± s.d. for 5 mice.

Table 3. Cumulative urinary and faecal excretion of BOF-4272 and its metabolites after the intravenous or oral administration of $5 \, mg \, kg^{-1}$ of BOF-4272 to mice.

	Time (h)	BOF-4272	BOF-4269	M-1
Intravenous administration				
Urine	24	34·9 ± 3·8	ND	ND
	96	35·3 ± 4·2	ND	ND
Faeces	24	4·51 ± 1·55	23·8 ± 5·4	5·07 ± 1·52
	96	4·51 ± 1·55	23·8 ± 5·4	5·07 ± 1·52
Oral administration				
Urine	24	13·1 ± 4·2	ND	ND
	96	13·1 ± 4·2	ND	ND
Faeces	24	3·11 ± 3·69	35·1 ± 14·3	14·2 ± 5·4
	96	3·11 ± 3·69	35·2 ± 14·2	14·2 ± 5·4

Values represent mean ± s.d. (% of dose). n = 6 for both oral and intravenous administration. ND, not detected.
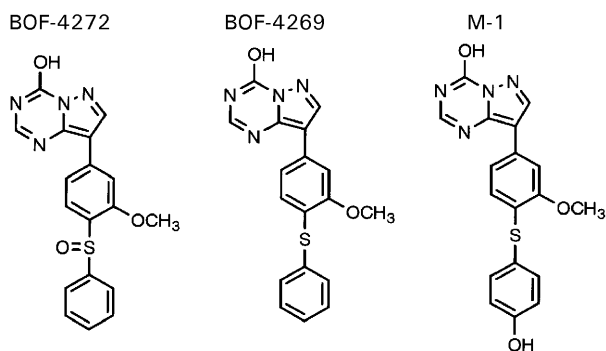

Figure 4. Chemical structures of BOF-4272 and its metabolites.

to healthy volunteers, and a significant decrease was observed up to 24 h after administration (Uematsu & Nakashima 1994). We previously demonstrated that the mouse is a suitable animal species for evaluating the clinical pharmacokinetics of BOF-4272 (Naito et al 1999a).

The results of the present study showed that the liver/plasma BOF-4272 concentration ratio increased over time up to 8 h after oral administration. The $t_{\frac{1}{2}}$ of BOF-4272 in the liver was longer than that in the plasma, and high concentrations of BOF-4272 (242–512 ng (g tissue)$^{-1}$) were observed in the liver up to 8 h after oral administration. Thus, the elimination of BOF-4272 from the liver, a target organ, was slower than its uptake from plasma to the liver. Furthermore, the in-vitro experiment using hepatocytes supported the high accumulation of BOF-4272 into liver cells. Significant uptake of BOF-4272 into the hepatocytes was observed at 4°C, and uptake was markedly increased at 37°C. Therefore, the influx of BOF-4272 into hepatocytes may be attributable not only to passive diffusion but also to temperature-dependent active uptake.

When the pharmacological effect of an anti-hyperuricaemic drug is examined in terms of the reduction in serum uric acid levels, little effect is observed in rodents, but a significant effect is seen in humans (Hosoya et al 1991). This remarkable species difference between rodents and humans is thought to be due to differences in uric acid metabolism, including the tissue distribution of XO/XDH and the presence of blood uricase. In the present study, a change in uric acid concentration in the liver was identified as a pharmacological action of BOF-4272 in the mouse. The levels of uric acid in the liver after the oral administration of BOF-4272 were significantly lower than those in control mice that did not receive BOF-4272. Furthermore, the reduction in liver uric acid levels was a long-lasting effect that persisted after the plasma concentrations of BOF-4272 had declined. These results indicate that the level of liver uric acid should be determined in the mouse to assess the direct pharmacological action of BOF-4272. The observation in a clinical study involving healthy volunteers, that a reduction in serum uric acid concentrations was maintained for up to 24 h after the administration of BOF-4272 (Uematsu & Nakashima 1994), may be related to the prolonged high concentration of BOF-4272 in the liver.

The bioavailability (F) based on AUC_{0-8h} values was 12·1%, which is in good agreement with the findings in fasted mice in a previous study (Naito et al 1999a). The chemical structures of BOF-4272 and its metabolites in mice are shown in Figure 4. BOF-4269 was detected after a lag time following the intravenous or oral administration of BOF-4272 in mice. It has been shown that BOF-4272 is metabolized to BOF-4269 by the intestinal flora and is absorbed from the intestinal tract in rats (Naito & Nishimura 1999). BOF-4269 is metabolized to M-1 sulphate via M-1 in the liver and is then excreted into the bile in rats (Naito & Nishi-

mura 1999). The metabolic pathways of BOF-4272 in the mouse after oral administration are thought to be similar to those in the rat. Specifically, some of the orally administered BOF-4272 may be metabolized by the intestinal flora to BOF-4269, which may be further biotransformed to other metabolites in the liver. Some BOF-4272 may also undergo enterohepatic recycling in the unchanged form.

In conclusion, the results of the present study indicate that the decrease in uric acid levels in the liver should be monitored to evaluate the pharmacological action of BOF-4272 in rodents. The present results also clarify the detailed metabolism and excretion of BOF-4272 after intravenous or oral administration to male mice.

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