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Metabolism of ethyl 2-carbamoyloxybenzoate (4003/2), a prodrug of salicylic acid, carsalam and salicylamide

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Ethyl 2-carbamoyloxybenzoate (4003/2, Fig. 1) is a new anti-inflammatory, analgesic [1] and platelet aggregation inhibitor.* This compound apart from possessing the above useful effects is safe in terms of ulcerogenicity† and toxicity. It seems to be a potentially safer alternative to other salicylates [2] and is presently undergoing clinical evaluation in patients with rheumatoid arthritis.

During preliminary pharmacokinetic studies in rats, rabbits and dogs, a rapid first pass metabolism of 4003/2 was observed,‡ and the presence of three metabolites in plasma was disclosed by the HPLC method.

The present communication describes the production of these three metabolites by incubation of 4003/2 with post-mitochondrial supernatant from rat liver, their isolation, and their identification by comparison of UV, i.r., NMR and MS properties with synthetic samples.

These metabolites were also formed by incubation with post-mitochondrial supernatants from liver of rabbit and dog. An interesting feature observed in this study was that the addition of potassium fluoride to this incubation, inhibited the formation of metabolite *Y*.

* A. Kamal and C. S. Rao, unpublished results.

† Drug Data Report XI: 84, 1989.

‡ A. Kamal, M. V. Rao, A. B. Rao and P. B. Sattur, unpublished results.

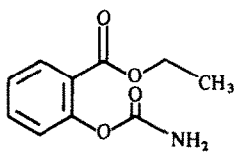
Materials and Methods

Chemicals. 4003/2 was synthesized by the reported procedure [1]. All reagents and biochemicals used were commercially available. Solvents were HPLC or spectroscopy grade.

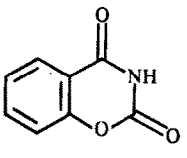
High pressure lipid chromatography. HPLC instrumentation consisted of a Shimadzu liquid chromatograph LC-6A equipped with a SCL-6A controller, SPD-6A Fixed Wavelength UV Monitor as detector, FCV-100B Fraction Collector and a Chromatopac C-R4A Data Processor as recording integrator. The column was 4.6 × 250 mm Ultropac TSK ODS-120A, 5 μm (LKB) for analytical studies and for semi-preparative work the column employed was 20 × 250 mm Shim-pack PREP-ODS, 15 μm (Shimadzu). The eluents were water–methanol (80:20) with 1% acetic acid (v/v). Flow rates were 1.2 mL/min (analytical) and 9.9 mL/min (Semi-preparative). Column effluents were monitored at 254 nm wavelength.

UV and i.r. spectrophotometry. UV spectra were obtained on an LKB Ultrospec K spectrophotometer using methanol as solvent. i.r. spectra were recorded on a Perkin–Elmer 283B spectrophotometer and the samples were taken in KBr pellet form.

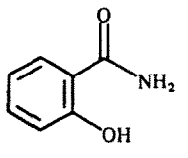
NMR and mass spectrometry. ^{1}H-NMR spectra were taken in $CDCl_3$ + d_6-DMSO on a Jeol FX90Q FT NMR spectrometer. TMS was used as an internal reference. EI-MS (electron energy 70 eV, trap current 200 μA) at source



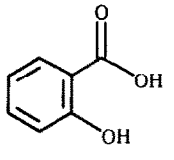
4003 / 2



Carsalam (*X*)



Salicylamide (*Y*)



Salicylic acid (*Z*)

Fig. 1.

temperature 200° and CI-MS electron energy 50 eV, trap current 500 μA) using methanol as reagent at source temperature 140° were obtained on a VG Micro mass 7070H spectrometer equipped with a VG 2015 data system.

In vitro *metabolism*. Hepatic post-mitochondrial supernatants were prepared from six male Wistar rats (weighing 180–200 g). The livers were homogenized in four volumes ice-cold 50 mM Tris–HCl buffer, pH 7.5 containing 5 mM $MgCl_2$ and 0.25 M sucrose. The homogenate was centrifuged at 4° at 15,000 *g* for 20 min. The supernatant was collected and the protein concentration was determined by the method of Lowry *et al.* [3] and was found as 21.6 mg/mL employing bovine serum albumin as a standard. The overall pattern of the drug metabolites formed by post-mitochondrial suspension was obtained by incubating 4003/2 (50 μM) for 60 min. All incubations were performed at 37° in 0.1 M phosphate buffer (pH 7.4) at a protein concentration of 2 mg/mL. Another set of incubations was also carried out by the addition of 500 μL of a 25 μM solution of $KF \cdot 2H_2O$ to the post-mitochondial supernatant along with the drug. Reactions were stopped by the addition of 1.5 mL of acetonitrile. After removal of protein precipitate by centrifugation the supernatant was diluted with 2 mL of water and collected on a Sep-PaK C18 cartridge employing water–methanol (80:20) as eluent and injected into the HPLC system.

Isolation of metabolite fractions. The HPLC injection (semi-preparative) was up to 500 μL and fractions were collected on the Fraction Collector by manual mode programme. As soon as the peak appeared on the CRT of the data processor the fraction was collected. When the peak was about to touch the base line the collection was diverted to the drain. In this way all the metabolites (*X*, *Y* and *Z*) were collected as different fractions. These fractions were stored below 0°. Each fraction of metabolite was checked for its purity on HPLC employing analytical column. The fractions were extracted with ethyl acetate and evaporated to dryness with a stream of nitrogen.

Identification of metabolites. After isolation metabolite *X* was identified as 1,3-benzoxazine-2,4-(3*H*)dione (carsalam) [4], *Y* was found to be salicylamide and *Z* as salicylic acid (Fig. 1). The physical characteristics and spectroscopic data for metabolite *X* were comparable to the carsalam sample prepared synthetically and characterized unambiguously [5] whereas metabolites *Y* and *Z* compared with the commercially available samples of salicylamide and salicylic acid. The results depicting the significant data are illustrated in Table 1.

Results and Discussion

Incubation of 4003/2 with rat liver supernatant resulted in formation of metabolites *X*, *Y* and *Z* as shown in Fig. 2a. The metabolites were compared for retention times on HPLC with synthetic reference compounds. The metabolism of 4003/2 involves its cyclization to 1,3-benzoxazindione system i.e., metabolite *X*, its further hydrolysis to salicylamide i.e., metabolite *Y* and in another route by the direct hydrolysis of the ester and carbamoyl functions of 4003/2 to afford salicylic acid i.e., metabolite *Z*. The hydrolytic cleavages are usually on account of the high concentration of esterases present in the post-mitochondrial supernatant. It has been observed in the metabolism studies of aspirin [6, 7], that immediate addition of sodium fluoride or potassium fluoride stops the rapid hydrolysis of aspirin with esterases. The drug 4003/2 has a close similarity to aspirin in having a carbamoyl group instead of an acetyl group. Therefore, in an attempt to reduce the hydrolysis of 4003/2 the effect of potassium fluoride in the incubation was investigated. In the *in vitro* metabolic pathway of 4003/2 in the presence of potassium fluoride, it was interesting to observe the non-formation of salicylamide (Fig. 2b). This can be explained by the inhibition of some specific esterases which are responsible for the hydrolysis of the benzoxazine ring. This feature has been further investigated by direct incubation of 1,3-benzoxazindione with post-mitochondrial supernatant which did give rise to salicylamide,

Table 1. Selected spectroscopic data of the metabolites *X*, *Y* and *Z*

Principal peaks/ bands	Carsalam (*X*)	Salicylamide (*Y*)	Salicylic acid (*Z*)
^{1}H-NMR δ	7.16–7.93 (4H), 11.7 (1H)	6.84–8.70 (6H), 12.96 (1H)	6.71–7.83 (4H), 11.50 (1H)
EI-MS *m/e*	163 ($M^{+\cdot}$), 120 (M-HNCO)	137 ($M^{+\cdot}$), 120 (M-NH_3)	138 ($M^{+\cdot}$)
i.r. cm^{-1}	3050, 1760, 1680, 1600	3500, 3200, 3000–2800, 1660, 1605	3250–2800, 1670 1610

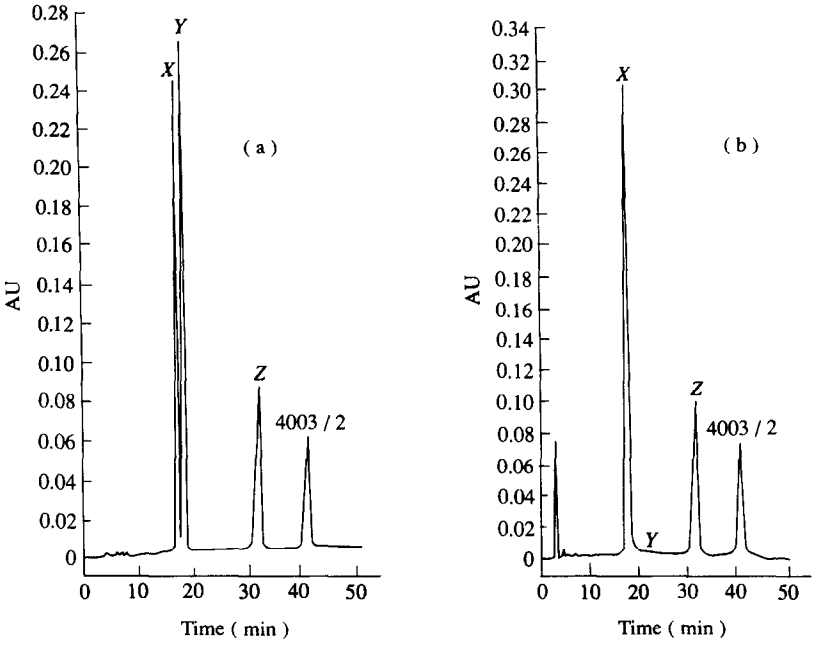


Fig. 2. Chromatograms from incubations of 4003/2 with post-mitochondrial supernatants: (a) rat liver (10 μg 4003/2, 60 min); (b) rat liver with $KF \cdot 2H_2O$ (10 μg 4003/2, 60 min). The disappearance of metabolite *Y* is indicated by an arrow.

while its formation was inhibited by the addition of potassium fluoride. Further, there was no difference in the concentration of salicyclic acid by the addition of potassium fluoride. The results from the *in vivo* metabolism studies of 4003/2 in rat and dog will be published later.

In summary, the metabolites of 4003/2 observed *in vivo* have been produced by incubation *in vitro* of 4003/2 with liver post-mitochondrial supernatants from rat, rabbit and dog. The metabolites were characterized by UV, i.r., NMR, MS and HPLC. All the metabolites detected have been or are in use as drugs for the relief of pain and inflammation. Hence, the new drug 4003/2 is a pro-drug of salicyclic acid, carsalam and salicylamide.

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