Biochemical Pharmacology, Vol. 27, pp. 809–810. Pergamon Press, 1978. Printed in Great Britain.

The metabolism of bromazepam in the rat—identification of mercapturic acid and its conversion *in vitro* to methylthio-bromazepam

(*Received* 13 *June* 1977; *accepted* 1 *September* 1977)

During the course of investigating the metabolism of a minor tranquilizer, bromazepam (7-bromo-1,3-dihydro-5-(2′-pyridyl)-2H-1,4-benzodiazepin-2-one), we isolated in rat urine a methylthio-containing metabolite, the structure of which was identified as 7-bromo-1,3-dihydro-5-[2′-(6′-methylthio)-pyridyl]-2H-1,4-benzodiazepin-2-one (methylthio-bromazepam) [1]. Biotransformation of several xenobiotic compounds to respective methylthio (CH_3S)-containing metabolites has been reported recently. Working at the biotransformation of 2-acetamidothiazole, Chatfield and Hunter [2] demonstrated the *in vivo* conversion of the mercapturic acid conjugate to methylthio-acetamidothiazole and suggested that the conjugate might be a precursor of the methylthio-containing metabolites.

Another pathway for the formation of CH_3S-containing metabolites has been proposed by Calder *et al.* [3], and Miller and his colleagues [4–7]. According to their assumption, methionine serves as the donor of CH_3S grouping as a result of nucleophilic attack to the active intermediate, hydroxylamine ester, which can be formed by enzymic *N*-hydroxylation of certain drugs. Since the methionine donor theory was very unlikely in the case of the formation of methylthio-bromazepam as we discussed previously [1], we tried to isolate the mercapturic acid conjugate of bromazepam, which was now isolated in the bile of rat given the drug. This communication will describe the identification of bromazepam mercapturate and subsequent conversion of the mercapturic acid to methylthio-bromazepam in rat liver preparation: an *in vitro* confirmation of the hypothetical mechanism proposed by Chatfield and Hunter [2] *in vivo*.

Non-radioactive bromazepam, [5-^{14}C]bromazepam and bromazepam *N*′-oxide (7-bromo-1,3-dihydro-5-[2′-(1′-oxo)-pyridyl]-2H-1,4-benzodiazepin-2-one) were synthesized at Hoffmann-La Roche, Basle, and were kindly supplied to us. 7-Bromo-1,3-dihydro-5-[2′-(6′-methyl,*N*-acetyl-L-cysteinate-*S*-yl)-pyridyl]-2H-1,4-benzodiazepin-2-one (bromazepam mercapturate methyl ester) was synthesized from bromazepam *N*′-oxide and methyl *N*-acetyl-L-cysteinate in acetic anhydride according to the pyridine-*N*-oxide nucleophilic substitution described by Bauer and Dickerhofe [8]. The desired product was finally crystallized from ethanol, m.p. 228°, u.v. absorption maximum in ethanol, 235 nm. The purity and identity of the synthesized compound were confirmed from nuclear magnetic resonance and high resolution mass spectra.

Identification of bromazepam-mercapturic acid in rat bile. Male rats (Sprague–Dawley strain, 8-week-old, 180 g) were cannulated each with a polyethylene tube to the bile duct and then received orally [5-^{14}C]bromazepam (0.63 μCi/mg) at a single dose of 80 mg/kg. The initial 24 hr bile specimen (about 40 ml) was evaporated under reduced pressure at 37° to give a brownish residue, which was subsequently dissolved in 10 ml of a mixture of methanol-water (9:1, v/v). The solution was quantitatively applied on t.l.c. plates (Kiesel Gel 60F_{254}, 0.25 mm thick, Merck), followed by development with a solvent system, *n*-butanol-acetic acid-H_2O (3:1:1, v/v, system No. 1). A u.v. absorbing substance migrated to R_f 0.70 was extracted with methanol, followed by further purification on t.l.c. with a second solvent system, ethylacetate–ethanol–acetic acid (2:1:1, v/v, system No. 2). The major u.v. absorbing substance was migrated to R_f 0.78, and was extracted again with methanol. Subsequent evaporation of the methanol under nitrogen stream yielded a white residue (about 0.5 mg).

In order to achieve the identification of ^{14}C-labeled bromazepam-mercapturic acid, a portion of the isolated metabolite was derivatized with diazomethane to its methyl ester and the chemical characteristics of the ester was compared with those of synthesized bromazepam-mercapturate methyl ester. In the mass spectra of both the isolated and synthesized substances the parent and base peaks were recognized at m/e 490 ($C_{20}H_{19}N_4O_4SBr$, relative intensity 32 per cent) and at m/e 360 ($C_{15}H_{11}N_3OSBr$), respectively. The latter peak resulted from the cleavage between C_α and C_β of the cysteinyl moiety. Side chain cleavages accounted for other main peaks at m/e 431 (41 per cent), m/e 389 (27 per cent), m/e 372 (32 per cent), m/e 347 (73 per cent) and m/e 268 (98 per cent), which corresponded to M-CO_2CH_3, M + H-CO_2CH_3-$CHCO_3$, M-H-H_3CCO_2-NHCOCH$_3$, M-H_3CCO_2—C(=CH$_2$)—NHCOCH$_3$, M-H_3CCO_2—C(=CH)—NHCOCH$_3$-Br, respectively. In order to further verify the identification, about 0.7 μmole of the synthesized methyl ester was mixed with 5 mμmoles (2200 dpm) of the derivatized metabolite and the specific radioactivity was determined after each step of consecutive t.l.c. developed with solvent system No. 1, and then *n*-heptane–chloroform–ethanol–conc. ammonia (50:50:20:1, v/v, system No. 3) and finally with chloroform–ethanol (9:1, v/v, system No. 4). The sp. act. was reasonably constant throughout each step of the chromatography, i.e. 1.87 ~ 1.88 ~ 1.87 ($\times 10^3$ dpm/Emax). Based on these observations, it was concluded that bromazepam was metabolized in the rat to bromazepam-6′-mercapturic acid (7-bromo-1,3-dihydro-5-[2′-(6′-*N*-acetyl-L-cystein-*S*-yl)-pyridyl]-2H-1,4-benzodiazepin-2-one.

In vitro formation of methylthio-bromazepam. The liver was excised from male rats (Sprague–Dawley strain) and was homogenized in 2 vols of ice-chilled 1.15% KCl solution adjusted to pH 7.4 with 10 mM Na^+/K^+ phosphate buffer. The homogenate was centrifuged at 9000 *g* for 20 min (0 ~ 4°). The supernatant fluid was collected and used as the enzyme preparation.

Two *in vitro* experiments were carried out with the enzyme preparation. In one experiment, the reaction mixture contained in a total 0.5 ml medium: [5-^{14}C]bromazepam-mercapturic acid or its methyl ester (0.1 μmole for both), $MgCl_2$ (12.5 μmols) and 0.4 ml of the 9000 *g* supernatant fluid (18 mg protein). The incubation was carried out for 30 min at 37° in air, and was terminated by adding 0.05 ml of 0.1 N NaOH. Carrier non-radioactive methylthio-bromazepam (0.13 μmole) was then added to each reaction mixture, and the product was extracted with 10 ml of chloroform–ethanol (9:1, v/v), followed by purification on t.l.c. using three different solvent systems, chloroform–ethylacetate–ethanol–conc. ammonia (200:200:30:3, v/v, system No. 5), benzene–acetone–ethanol–conc. ammonia (1000:500:30:3, v/v, system No. 6), and No. 3, successively.

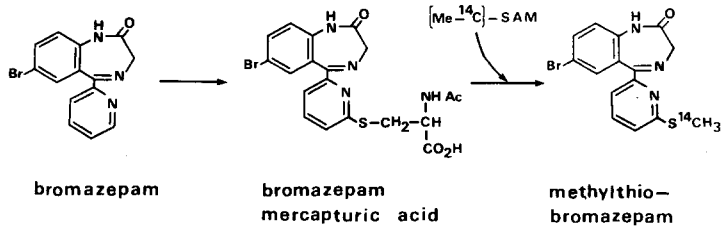

Fig. 1. A proposed pathway for the biotransformation of bromazepam to methylthio-bromazepam in the rat.

The radioactive product mixed with the carrier was located under u.v. lamp and extracted from the zone of silica gel by a mixture of chloroform–ethanol (9:1, v/v). The extract was completely dried under reduced pressure and then the residue was dissolved in 3.0 ml of ethanol. A 2.5 ml portion of the extract was used for counting ^{14}C radioactivity in a liquid scintillation spectrometer and another 0.5 ml portion for monitoring the recovery of added non-radioactive methylthio-bromazepam by measuring u.v. absorbance at 233 nm, the absorption mixumum of the metabolite.

During the incubation of [5-^{14}C]bromazepam-mercapturic acid (100 nmoles) with the 9000 *g* supernatant fluid, 190 ~ 230 pmoles of methylthio-[5-^{14}C]bromazepam were formed. When the methyl ester was used as the substrate instead of the mercapturic acid itself, the rate of formation of methylthio-[5^{14}C]bromazepam was 2–3 times higher. In control samples run simultaneously with heat denatured enzyme (100°, 5 min), the formation of methylthio-[5-^{14}C]bromazepam was less than 0.01 pmole/mg protein/min. The identification of methylthio-[^{14}C]bromazepam formed in the reaction mixture was confirmed by the constant specific activity method. Namely, during repeated t.l.c. with solvent systems No. 3, No. 1 and No. 4, the specific radioactivity remained in a reasonably constant range: 0.77 ~ 0.77 ~ 0.76 ($\times 10^3$ dpm/E). Thus, conversion *in vitro* of the mercapturate to methylthio-bromazepam was demonstrated.

Since *S*-adenosyl methionine was considered *a priori* as a possible methyl donor of the methyl moiety of methylthio-bromazepam, another *in vitro* experiment was carried out to see whether the methyl moiety of *S*-adenosyl-[Me-^{14}C]-methionine could be incorporated to form [Me-^{14}C]methylthio-bromazepam during incubation of the mercapturate methyl ester with the liver preparation. In this experiment the substrates used were non-labeled bromazepam-mercapturate methyl ester (0.5 μmole) and *S*-adenosyl-[Me-^{14}C]-methionine (0.25 μmole, 0.6 mCi/mmole), the latter being purchased from the Radiochemical Center (Amersham). $MgCl_2$ and the enzyme concentrations were the same as those in the first experiment. In the presence of freshly prepared liver preparation, [Me-^{14}C]methylthio-bromazepam was indeed formed at rates 3.66 ± 0.12 pmoles/mg protein/min (the average of five determinations, ±S.E.). The heat denatured enzyme did not form the product at rates higher than 0.02 pmole/mg protein/min in several control experiments. Therefore, the direct donor of the methyl moiety of methylthio-bromazepam is likely to be *S*-adenosyl methionine.

The result presented in this communication is the first demonstration of an *in vitro* formation of methylthio-containing metabolite from the corresponding mercapturic acid, an *in vivo* example having been shown for 2-acetamidothiazole [2]. The pathway of this biotransformation is currently unknown. It is very likely, however, that a cleavage reaction occurs at the *S-C* bond of the *N*-acetyl cysteinyl moiety in the mercapturic acid, thereby the methyl group of *S*-adenosyl methionine transferring to the *S*-atom of the mercapturate before or after the cleavage. The present results also suggested that reaction(s) involved in the pathway might be catalyzed by at least one enzyme existing in the supernatant fluid of rat liver, because neither of the present enzyme reactions did proceed when the supernatant fluid was denatured by heating. Together with the finding that the mercapturic acid was isolated from the bile of rats, we can now depict the metabolic pathway for the formation of methylthio-bromazepam as shown in Fig. 1. Purification and characterization of pertinent enzyme(s) are now in progress in our laboratory.

A mercapturic acid conjugate of bromazepam was isolated from the bile of rats which received a 80 mg/kg dose of bromazepam orally. After incubation of the mercapturic acid with a rat liver preparation (9000 *g* supernatant fluid) at 37°, 6′-methylthio-bromazepam was formed. In another *in vitro* experiment with the same liver preparation, the methyl moiety of methylthio-bromazepam was found to be derived from S-adenosyl methionine. The results indicated that 6′-methylthio-bromazepam isolated previously in the rat urine is formed at least in part via the mercapturic acid and that rat liver contains enzyme(s) capable of catalyzing the conversion from the mercapturic acid to methylthio-bromazepam.

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