Table II. Inhibition of Seizures in Mice by **1e, 2a–e** and **3**

Compound	Anticonvulsant Activity[a]			
	MES[b]		ScMet[c]	
	0.5 h	4 h	0.5 h	4 h
1e	–	–	–	–
2a	–	–	–	–
2b	++	+	–	–
2c	–	–	–	–
2d	++	–	–	–
2e	–	–	–	–
3	+	–	–	–

[a]++ and + indicate activity at 300 and 600 mg/kg, respectively;
– signifies no activity observed at 600 mg/kg.
[b]Maximal electroshock seizure test.
[c]Subcutaneous pentylenetetrazol (Metrazol) seizure threshold test.

sic activity per se based on a comparison of the $\Sigma\sigma^*$ values of approximately 1.74[e] for stable **2b** with regard to the C=N and that of 2.01[f] for progabide. The inactivity displayed by **2c**, the dibromo derivative of active **2b**, and by **3**, the GABA derivative, is difficult to rationalize but this situation may be clarified by subsequent testing involving other *in vitro* and *in vivo* procedures. Compound **2b** may have an excessively high log P value, calculated as 4.40[g], for optimum effect as a centrally acting agent. While this present study constitutes an initial attempt to correlate electronic influences with prodrug activity of GABA and GABA analogue derivatives, there has been no investigation into the effect of hydrophilic-lipophilic balance on the absorption and distribution for these types of agents.

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[e]C_6H_4-2-OH [0.96 σ^* from C_6H_4 (0.75 σ^*) + 2-OH (1.34 σ^*) × 0.4^2] + 3-P (0) $(OCH_3)_2$ (0.42 σ^*) × 0.4^3].
[f]C_6H_4-4-Cl (0.87 σ^*) + C_6H_4-3-F (0.95 σ^*) + 2-OH [0.11 σ^* from OH (1.34 σ^*) × 0.4^2] + $(CH_2)_3C(O)NH_2$ [0.08 σ^* from $(CH_2)_2C(O)NH_2$ (0.19 σ^*) × 0.4].
[g]From salicylaldehyde (1.69), dimethyl phenylphosphonate, two aromatic Br (0.86 each) and given the equivalence of O and N.

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In vitro Metabolism of Bumetanide

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Abstract: A new metabolite of the diuretic drug bumetanide, the 4-[(4′-hydroxy)-phenoxy] analog (**7**), was identified in incubation mixtures of rat liver microsomes. Phenobarbital and clofibrate pretreatment to induce microsomal enzymes changed the relative amounts of the six metabolites formed. Compound **7** was the most prevalent metabolite after clofibrate pretreatment.

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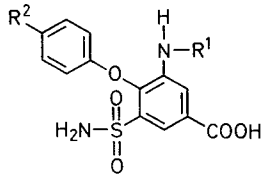
Bumetanide [3-(*n*-butylamino)-4-phenoxy-5-sulfamoylbenzoic acid] (**1**), is a potent high ceiling diuretic belonging to the class of polar water soluble sulfonamides. Its diuretic activity varies greatly in different animal species. The drug is most potent in man and dog, followed by the rabbit, mouse and rat; the decrease in activity is correlated with an increased rate of metabolism (1).

Previous extensive *in vivo* studies in different animal species (2) led to the identification of five metabolites (**2, 3, 4, 5, 6**) (Fig. 1); the rat was the species that shows the highest metabolic rate (3). All of these metabolites are produced by metabolic oxidation of the *n*-butyl chain. These findings and the increased diuretic response to bumetanide in rats treated with several microsomal enzyme inhibitors (4) suggest that this drug is mainly metabolized by the microsomal mixed-function oxygenase system. Metabolites resulting from aromatic hydroxylation, either in the benzoic acid ring or in the phenoxy substituent, have not yet been reported. It has been postulated that electron withdrawing groups (e.g. carboxyl and aminosulfonyl) might be responsible for the lack of metabolic hydroxylation in the benzoic acid ring (5).

However, no explanation exists for the apparent lack of hydroxylation in the phenoxy ring, particularly in the rat,



	R^1	R^2
1	$-CH_2CH_2CH_2CH_3$	H
2	$-CH_2CH_2CHOHCH_2OH$	H
3	$-H$	H
4	$-CH_2CH_2CH_2CH_2OH$	H
5	$-CH_2CH_2CHOHCH_3$	H
6	$-CH_2CH_2CH_2COOH$	H
7	$-CH_2CH_2CH_2CH_3$	OH

Fig. 1 Bumetanide and its *"in vitro"* metabolites.

which shows a high ability to form phenolic metabolites of various classes of compounds (6, 7). Moreover the presence of these hydroxylated metabolites was of interest since compounds of similar structure (i.e. the *N*-benzyl analog of **7** (8), show the same order of activity as bumetanide, whereas all the metabolites already described were devoid of activity (2).

We have studied the metabolic transformation of bumetanide in rat liver microsomes. We present here evidence for the formation of a new hydroxylated metabolite, the 3-(*n*-butylamino)-4-[(4'-hydroxy) phenoxy]-5-sulfamoylbenzoic acid (**7**).

Materials and Methods

Bumetanide was supplied by Sigma Tau s.p.a. (Pomezia-Italy). Microsomal preparations were obtained by a standard procedure from the livers of adult male rats (Wistar NOS) allowed free access to food and water. Microsomes were incubated with bumetanide for 1 hour at 37°C in a 0.1 M phosphate buffer (pH 7.4) containing 1.5 mM diethylenetriaminepentaacetic acid, in the presence of an NADPH generating system. The metabolites were extracted with ethyl acetate and examined by TLC and HPLC using ion-pair chromatography on a reverse phase C_{18} column and UV detection at 254 nm. The mobile phase was CH_3CN : H_2O (28:72) (pH 6.9) with tetrabutylammonium phosphate as the counter-ion.

Results and Discussion

In the rat liver microsomal preparations we detected the metabolites **2, 3, 4, 5, 6, 7** shown in Fig. 1. Metabolites **2, 3, 4, 5** and **6** were identified by direct comparison of their TLC R_f and HPLC R_t values (Figs. 2, 3) with those of authentic synthetic standards that were obtained by known literature methods (8, 9).

The structure of the new metabolite, **7**, was characterized by CIMS ($[MH]^+$ at *m/z* 381); ^{1}H-NMR: δ (270 MHz, CD_3OD) 6.76 (2H, d), 6.85 (2H, d), (a typical $A_2'B_2'$ pattern due to a *p*-disubstituted phenyl system) and confirmed by synthesis. The authentic sample of **7** was obtained through the catalytic reduction of 4-[(4'-benzyloxy) phenoxy]-3-nitro-5-sulfamoylbenzoic acid (8) with 10% Pd/C as a catalyst in ethyl alcohol, followed by reductive amination with butanal in absolute ethyl alcohol in the presence of a trace of *p*-toluenesulfonic acid and an appropriate amount of 10% Pd/C. The product was recrystallized from *n*-butyl alcohol (m.p. 247–249°C). The pertinent spectral data obtained for compound **7** were as follows: U.V. (EtOH): λ_{MAX}=222, 335 nm. I.R. (KBr): 3500, 3350, 2910, 1710, 1610, 1600, 1525, 1450, 1350, 1280, 835 cm^{-1}. ^{1}H-NMR: δ (270 MHz, CD_3OD) 7.95 (1H, s), 7.63 (1H, s), 6.85 (2H, d), 6.76 (2H, d), 3.13 (2H, t), 1.42 (2H, m), 1.17 (2H, m), 0.83 (3H, t).

The sum of the indivual peak area values of all the metabolites detected by HPLC was calculated and the amount of

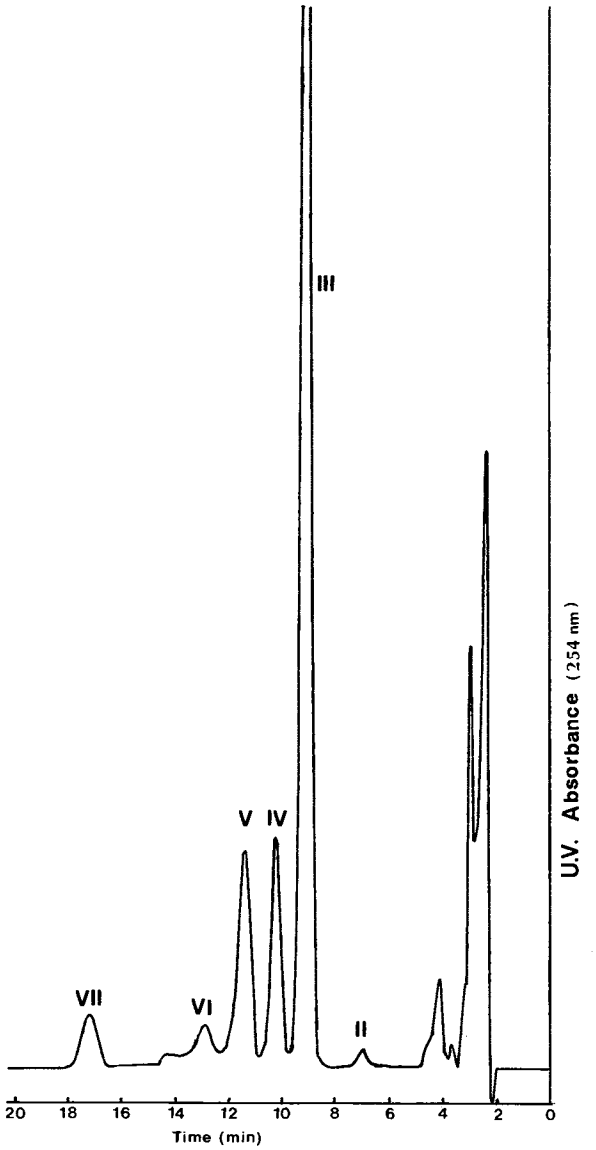


Fig. 2 HPLC chromatogram of bumetanide metabolites from a microsomal preparation (phenobarbital induction).

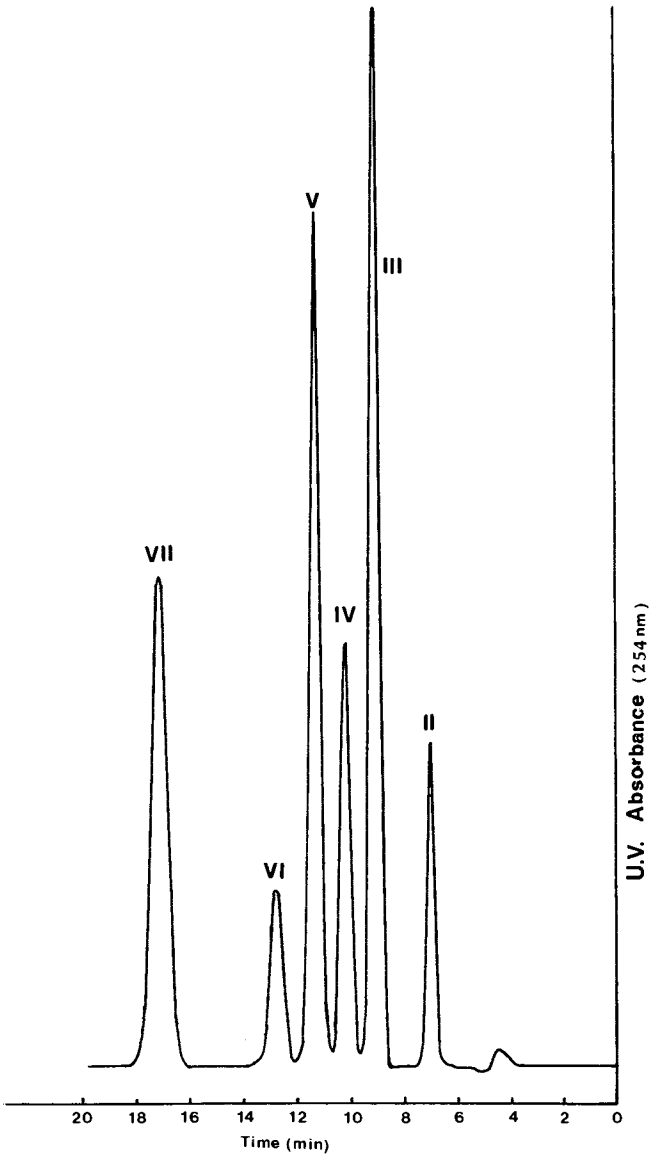


Fig. 3 HPLC chromatogram of bumetanide metabolite standards.

each metabolite expressed as per cent of the total area (average of three different experiments). The ability of different microsomal preparations to metabolize bumetanide was evaluated by comparing the total area of metabolites with the area of a standard (*N,N'*-diacetyl-3-amino-4-phenoxy-5-sulfamoylbenzoic acid) of known concentration.

The major metabolite was compound **4** (32 %) followed by, in order of decreasing amounts, **3** (22 %), **7** (22 %), **5** (16 %), **6** (5 %) and **2** (traces). No metabolites were found in control incubations carried out in the absence of the NADPH generating system.

Pretreatment of animals with sodium phenobarbital (80 mg/kg/day i.p. in saline for 3 days) greatly enhanced (40 %) the metabolism of bumetanide by hepatic microsomes. The relative amounts of the metabolites were also modified, as **3** (64 %) became the most abundant species, followed by **5** (14 %), **4** (12 %), **7** (6 %), **6** (2.8 %), **2** (traces). Pretreatment of animals for 3 days with 400 mg/kg/day i.p. of clofibrate, an inducer of a microsomal P-450 monooxygenase showing specificity for the ω-hydroxylation of lauric acid (10), did not significantly enhance the total metabolism of bumetanide. However the relative amounts of the metabolites were modified, with **7** (40 %) now being more abundant than **3** (27 %), **5** (18 %), **4** (12 %).

Different microsomal monooxygenase P-450 isozymes seem to be involved in the metabolism of bumetanide, since chemical substances, that are known as selective inducers of microsomal monooxygenase, are able to modify the metabolic pattern of bumetanide in rat liver microsomes.

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