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SPECIES DIFFERENCES IN THE BILIARY EXCRETION OF MORPHINE, MORPHINE-3-GLUCURONIDE AND MORPHINE-3-ETHEREAL SULFATE IN THE CAT AND RAT*†

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Abstract—Biliary excretion of sulfobromophthalein (BSP) and morphine, morphine ethereal sulfate (MES) and morphine glucuronide (MG) as *N*-^{14}C-methyl-labeled compounds was determined in renal-ligated rats and cats. After morphine administration the rat excreted MG and the cat MES. When administered to both species, MG and MES were excreted into bile unchanged. The rat excreted greater than 60 per cent of a morphine or MG dose into bile in 3 hr, but less than 30 per cent of a MES dose. The cat, in contrast, excreted less than 30 per cent of all three compounds. The quantitative similarity in excretion of MES between species and quantitative difference in excretion of MG between the same species suggest that MES and MG are not excreted into bile by the same pathway.

A QUANTITATIVE difference occurs between the rat and cat for the excretion of morphine into bile. March and Elliott[1] have shown that 63 per cent of a subcutaneous dose of morphine is excreted into the bile of the rat, while Yeh *et al.*[2] found that only 14 per cent of a morphine dose is excreted into the bile of the cat. In addition to this quantitative difference in morphine excretion, there is a qualitative difference in the morphine metabolites formed by the rat and cat. The rat forms morphine-3-glucuronide after morphine administration.[3] No conversion of morphine to morphine-3-ethereal sulfate has been detected in this species.[3] The cat forms primarily morphine-3-ethereal sulfate although some morphine-3-glucuronide is also formed.[2,4] Since metabolites of morphine, but little free morphine, are excreted into the bile, the possibility exists that this difference in the metabolites may account for the quantitative difference in morphine excretion.

To examine this hypothesis, we attempted to separate the process of the metabolism of morphine from the excretion of its metabolites by administering morphine metabolites as well as morphine to both species. This approach allowed us to compare the excretion of an exogenously administered metabolite of morphine to the excretion of

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that same metabolite formed endogenously from morphine. This type of approach has been fruitful in delineating morphine transport sites in the kidney.[5,6] In addition, we compared the biliary excretion of the major morphine metabolite native to each species to the excretion of a morphine metabolite relatively foreign to the species.

METHODS

Drugs. Morphine N-$^{14}CH_3$ hydrochloride (57 mCi/m-mole) was obtained from Amersham/Searle Corp. Sulfobromophthalein sodium (BSP) was provided by Hynson, Wescott & Dunning, Inc. Crystalline morphine N-$^{14}CH_3$-3-ethereal sulfate (MES), 3·45 μCi/mg, and morphine N-$^{14}CH_3$-3-glucuronide (MG), 0·45 μCi/mg, were prepared biosynthetically by techniques described by Fujimoto and Haarstad,[4] and Fujimoto.[7]

Animal preparation and experimental design. Male Sprague–Dawley rats (350–450 g) and male cats (2·2–5·3 kg) were fasted for 24 hr before use with water available *ad lib.* The animals were anesthetized with pentobarbitol sodium (rats, 50 mg/kg, i.p., and cats, 40 mg/kg, i.p.). The trachea and external jugular vein were cannulated. The bile duct was cannulated with PE 10 tubing in the rat and PE 190 tubing in the cat. The cystic duct in the cat and the renal pedicles in both species were ligated. Rectal temperature was maintained between 37 and 38° by incandescent lamps placed near the animals.

Twelve animals of each species were placed in three groups of four animals and administered morphine, MES or MG. The cats received 1·0 μCi of each compound while the rats received 0·5 μCi. Two animals in each group also received BSP (5 mg/kg) simultaneously with the labelled compound. All compounds were administered into the external jugular vein. Comparisons between drugs and species were tested for statistical significance using the Student's t-test and range test.[8]

Analytical procedures. Bile was collected in 15-min samples for 3 hr. Each 15-min bile sample was weighed, and distilled water was added to obtain a final sample weight of 0·5 g. BSP in each sample was determined according to the method described by Seligson *et al.*,[9] except that sodium-*p*-toluene sulfonate was not used. The amount of bile sample used for each BSP determination ranged from 1 μl to 0·1 ml depending on the BSP concentration of that sample. Standard curves for BSP in water and bile were similar and the experimental samples were compared against BSP standards in water. Total radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer. A portion of the diluted bile (0·1 ml) was added to 15 ml of a scintillation mixture prepared by adding 500 ml of Triton X-100 to 1000 ml of toluene containing 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-*bis*-[2-(5-phenoxazolyl)]-benzene (POPOP). An automatic external standard (AES) was used to correct for quenching. The AES was calibrated against a counting efficiency curve which was determined by adding ^{14}C-toluene to bile.

A 9 test tube, 8 transfer countercurrent distribution (CCD) was used to quantitate the relative amount of morphine, MES and MG present in the bile samples. The solvent system consisted of *n*-butanol–water–acetonitrile (25:25:4, by vol.). The water contained 1 M sodium chloride and 0·02 M sodium bicarbonate. The solvent system was adjusted with 1 M NaOH so that the pH of the aqueous phase was 10. Two ml of the aqueous and organic phases was used per tube. The aqueous phase of

tube zero, however, consisted of 1 ml of diluted bile and 1 ml of aqueous phase re-adjusted to pH 10 after the bile was added. After shaking each tube, the mobile, lower phase (aqueous) was transferred to the next tube with a syringe and long needle. Some of the tubes required brief centrifugation after shaking to separate the phases. After the transfers were completed, 0·5 ml of each aqueous and organic phase was assayed for radioactivity by liquid scintillation counting.

To quantitate the amount of morphine, MES and MG, each experimental CCD curve was fitted with a theoretical curve[10] to the dominant component present, which in all cases was either MG or MES. The difference between the experimental CCD curve and the fitted theoretical curves gave derived curves representing the minor components. The areas under the fitted theoretical curve and derived curves gave the per cent of morphine, MES and MG present in the bile.[11,12] The ability of the CCD system to separate and quantitate morphine, MES and MG was confirmed by adding known quantities of these compounds to bile and analyzing the quantities present by CCD.

RESULTS

Table 1 shows the total recovery of radioactivity or BSP equivalents as a per cent of the intravenously administered dose of morphine, MG, MES and BSP in rat and cat bile collected for 3 hr after drug administration. In the rat, recovery after administration of BSP, morphine, and MG was high (>60 per cent), while recovery after MES was low (29 per cent). In the cat, only after BSP administration was there high recovery (77 per cent). After morphine, MES and MG the recoveries were low (< 23 per cent). Comparing recovery between species, it can be seen that recovery after morphine or MG administration in the rat was significantly greater than recovery after these compounds were administered to the cat. Recovery after BSP administra-

TABLE 1. RECOVERY OF RADIOACTIVITY OR BSP EQUIVALENTS IN RAT AND CAT BILE AS PER CENT OF THE DOSE OF MORPHINE, MG, MES OR BSP ADMINISTERED

Drug administered	Recovery (%)		P value (rat vs. cat)
	Rat*	Cat†	
BSP	87 ± 4‡	77 ± 3	< 0·05
Morphine	64 ± 5	23 ± 1	< 0·001
MG	81 ± 4	17 ± 4	< 0·001
MES	29 ± 5	20 ± 3	> 0·2

* Within species comparisons in the rat: a student range test indicated that recovery after BSP was not significantly different from MG, though BSP recovery was significantly greater than morphine recovery ($P < 0.05$) and MES recovery ($P < 0.01$). Recovery after MG in the rat was not significantly different from recovery after morphine; MG recovery was significantly greater than MES recovery ($P < 0.01$). Recovery after morphine was significantly greater than recovery after MES ($P < 0.01$).

† Within species comparison in the cat: a student range test indicated that recovery after BSP was significantly greater than recovery after morphine, MG or MES ($P < 0.01$). The recoveries between morphine, MES or MG were not significantly different.

‡ Each value represents mean recovery ±S.E. in bile collected for 3 hr from six rats (BSP) or four rats (morphine, MG and MES).

tion was also greater in the rat than cat, though this difference in recovery was considerably less than the differences seen for morphine or MG. In contrast to these differences between species, the recovery of MES in the rat and cat was not different.

Biliary excretion for each 30-min time interval after BSP, morphine, MG and MES administration is shown in Fig. 1. It is evident that these compounds were excreted into bile of the rat at different rates. BSP was excreted at a fast rate as indicated by the steep initial slope; morphine and MG were excreted at intermediate rates and MES was excreted at a slow rate. The excretion process appeared to be first order. In the cat, however, at least two different rate-limiting processes appeared to be present, since more than a simple first-order excretion process was occurring. The most notable feature in this animal for the excretion of all four compounds was the apparent lag in attainment of peak excretion values. Because of these differences in the shape of the excretion curves between the rat and cat, interpretation regarding total recovery must be tempered.

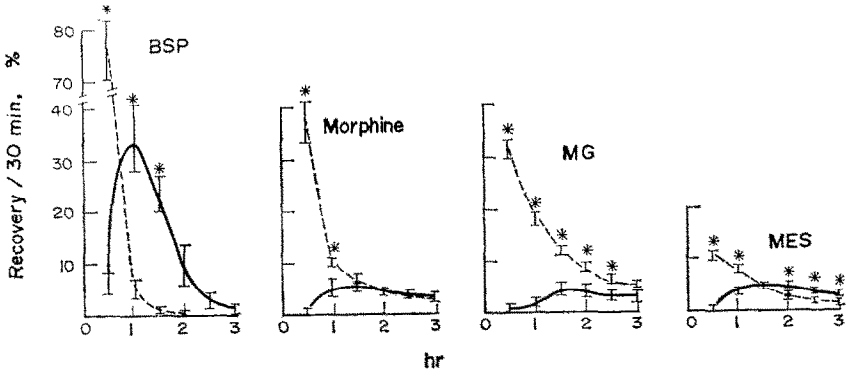

FIG. 1. Biliary excretion of radioactivity or BSP equivalents after morphine, MG, MES or BSP administration in the cat (solid line) and rat (dashed line) during 30-min intervals. Each value, expressed as per cent of the dose administered, is the mean ±S.E. of six animals (BSP) or four animals (morphine, MES and MG). The presence of an asterisk denotes a significant difference between species ($P < 0.05$) for biliary excretion during that collection period.

To see if the species differences in total recovery after administration of morphine, MG and MES might be due to differences in metabolism of these compounds, CCD analyses were done. First we did experiments to validate the ability of this method to quantitatively separate morphine, MES and MG as shown in Table 2. Four samples of rat bile containing known amounts of morphine, MES and MG were analyzed by CCD. The amounts as determined by this CCD procedure gave reliable results even though errors on the order of 5 per cent might occur. As further verification of this technique, a comparison of CCD analysis to planimetric analysis of a radiochromatogram scan[4] was done on bile obtained from a cat administered morphine. Both CCD and thin-layer chromatographic analysis gave similar results.

In Fig. 2, CCD curves for bile collected from the cat and rat after i.v. administration of morphine, MES and MG are shown. To facilitate visual comparisons, CCD curves for standards are also shown. MES administration to either species resulted in CCD curves which were similar to the curve for the MES standard. MG administration produced CCD curves in both species which closely resembled the MG standard.

TABLE 2. COUNTERCURRENT DISTRIBUTION ANALYSES OF RAT BILE TO WHICH WAS ADDED KNOWN AMOUNTS OF MORPHINE, MES AND MG

Sample*	Compounds	% Added	% Estimated by CCD†
1	Morphine	0	0
	MES	25	28 ± 1·7
	MG	75	72 ± 1·7
2	Morphine	0	3 ± 0·7
	MES	75	77 ± 0·9
	MG	25	20 ± 0·3
3	Morphine	15	20 ± 1·2
	MES	0	0
	MG	85	80 ± 1·2
4	Morphine	20	18 ± 0·7
	MES	65	68 ± 2·7
	MG	15	14 ± 2·0

* Bile volume was 0·5 ml and contained 0·07 μCi of radioactivity; solvent system was as given in Methods.

† Each value represents mean ±S.E. of three CCD analyses.

Analyses of these CCD curves by mathematically fitting theoretical curves showed that MES administered to the cat was excreted entirely as MES, while in the rat 82 per cent of the MES in bile was excreted as unchanged MES and 18 per cent was excreted as MG. After MG administration in the cat, 90 per cent was unchanged MG and 10 per cent was MES. The rat excreted a MG dose entirely as MG. CCD curves for bile after morphine administration were more complex. Analyses indicated that in the cat 77 per cent of the morphine in bile was MES and 24 per cent was MG. In the rat, 90 per cent of the excreted radioactivity was MG and 10 per cent was free morphine. Thus, the cat excreted administered morphine primarily as MES while the rat excreted administered morphine primarily as MG.

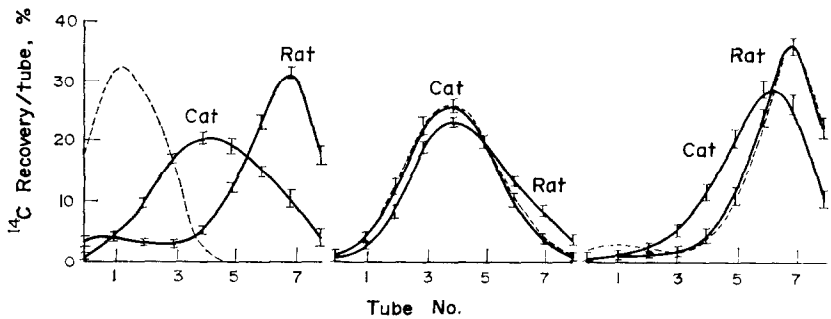

FIG. 2. CCD curves from bile of the cat and rat after i.v. administration of morphine (left panel), MES (middle panel) or MG (right panel) are shown along with standard CCD curves from bile to which each of these compounds has been added (thin dashed line). Each vertical bar represents the mean ±S. E. of at least four determinations except for the standard CCD in which only one determination is shown.

In addition to the above results, differences in bile flow between the rat and cat were found. The mean bile flow in the rat was 48 ± 4 (S. E.) mg of bile/kg body wt/min, while in the cat it was 8·0 ± 1·0 (S. E.) mg/kg/min.

DISCUSSION

The same quantitative difference in the biliary excretion of morphine in rats and cats with intact kidneys reported by others[1,2] persisted in the renal-ligated animals used here. In intact animals, 63 per cent of a morphine dose administered to the rat and 14 per cent of a morphine dose administered to the cat were excreted into bile. With renal ligation, rats still excreted 68 per cent of a morphine dose into bile while cats excreted 23 per cent. These results suggest that the quantitative difference in the biliary excretion of morphine in rats and cats occurs independently of renal function.

Since most of the morphine is metabolized before it is excreted into the bile, it is possible that a slow rate of morphine metabolism by the cat may account for the low total excretion after morphine administration. If the rate of morphine metabolism were a primary determinant in the cat for biliary excretion, the experiment in which MES was exogenously administered should bypass this rate-limiting step. Indeed, metabolism was bypassed in this experiment because all of the dose of MES excreted in bile was unchanged MES. With metabolism bypassed, 3-hr recoveries of exogenously administered MES were not significantly different from when morphine was administered (20 vs. 23 per cent, respectively). Also the shape of the two excretion curves was similar. Thus, metabolism does not appear to be the rate-limiting factor responsible for the low biliary excretion of morphine in the cat.

Another alternative to consider in attempting to explain the quantatative species difference in morphine excretion is related to the difference in the metabolites themselves. Perhaps MES is slowly excreted and MG is rapidly excreted regardless of the species. To test this possibility, MES was administered to the rat and MG to the cat. This alternative seemed to be well supported because the recovery of exogenously administered MES was the same in the rat and cat (29 vs. 20 per cent respectively). Unfortunately, the results with MG militate against this concept because recovery of administered MG although 81 per cent in the rat was only 17 per cent in the cat. Since MG when administered to the cat and MES when administered to the rat were both excreted primarily as the administered compound, our findings suggest that the species difference in the biliary excretion of morphine cannot be attributed to the fact that the rat metabolizes morphine to MG and the cat metabolizes morphine to MES.

Another alternative was suggested by the fact that the observed rate of bile flow in the rat was 4–5 times greater than in the cat. The difference between the shapes of the excretion curves for the four compounds in Fig. 1 might be correlated with this factor. However, the correlation is imperfect because even though total recovery after morphine, MES and MG excretion is consistently low in the cat (about 20 per cent), the recovery of BSP (Table 1) is high (77 per cent). Therefore, it remains to be seen whether this species difference in bile flow is responsible for the species difference in excretion of the morphine metabolites.

As an overall picture, our results may be visualized as follows:

$$\begin{aligned} &\text{Rat: morphine (as MG)} = \text{MG} > \text{MES} \\ &\qquad\qquad\qquad\qquad\qquad\qquad\quad \| \\ &\text{Cat: morphine (as MES)} = \text{MG} = \text{MES} \end{aligned}$$

These results suggest that the biliary excretion pathway of MG in the rat is different from that of MES. Also the biliary excretion pathway of MG in the rat is different from that of the cat. The suggestion that MG and MES are excreted into bile by different pathways at least in the rat has been confirmed by the further work of Peterson and Fujimoto.* They showed that phenobarbital pretreatment in the rat produced a significant increase in the biliary excretion of MES, but not in the biliary excretion of MG. Moreover, they found that the biliary excretion of exogenously administered MG was more sensitive to changes in body temperature than was MES. Their results indicate that the biliary excretion of MES can be affected independently of MG. This ability to differentially alter the biliary excretion of these metabolites would not be expected if both metabolites were excreted by the same pathway.

The hypothesis that biliary excretion of organic anions such as MES and MG may involve different excretory pathways was proposed by Alpert *et al.*[13] This was based on their finding that in mutant corriedale sheep the biliary excretion maximum of BSP was 7 per cent of that in normal sheep. In contrast, the biliary excretion maximum of taurocholate in mutant sheep was the same as normal sheep. Our results are in accord with this concept. Furthermore, the difference in morphine glucuronide excretion between the rat and cat is similar to the species difference found for other glucuronides by Abou-El-Makarem *et al.*[14] They showed that the recovery in bile of administered stilbestrol glucuronide in 3 hr was 99 per cent in the rat and 77 per cent in the cat. The 3-hr recovery of phenolphthalein glucuronide in the rat (54 per cent) was also greater than in the cat (34 per cent). Thus, the species difference in the biliary excretion of MG is not unique, but seems to apply to other glucuronides as well.

It is possible that the pathway for biliary excretion of exogenously administered morphine metabolites is different from that for excretion of the endogenously formed metabolites of morphine. If one assumes that after morphine administration appreciable amounts of the same morphine metabolites are present in the blood,[15] then at least part of the excretion of the endogenous metabolite in bile must have a final convergent pathway identical to the exogenously administered metabolite. Moreover, for one compound, epinephrine, there seems to exist a common pathway between exogenous and endogenous metabolite. Arias *et al.*[16] based their suggestion on the observation that in the corriedale sheep, not only the ability to form endogenous metanephrine glucuronide but also excreted administered metanephrine glucuronide was lost by a mutation.

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REFERENCES

1. C. H. March and H. W. Elliott, *Proc. Soc. exp. Biol. Med.* **86,** 494 (1954).
2. S. Y. Yeh, H. I. Chernov and L. A. Woods, *J. pharm. Sci.* **60,** 469 (1971).
3. K. Oguri, S. Ida, H. Yoshimura and H. Tsukamoto, *Chem. pharm. Bull., Tokyo* **18,** 2414 (1970).
4. J. M. Fujimoto and V. B. Haarstad, *J. Pharmac. exp. Ther.* **165,** 45 (1969).
5. W. M. Watrous, D. G. May and J. M. Fujimoto, *J. Pharmac. exp. Ther.* **172,** 224 (1970).
6. J. M. Fujimoto, in *Narcotic Drugs, Biochemical Pharmacology* (Ed. D. Clouet), p. 366. Plenum, New York (1971).
7. J. M. Fujimoto, *J. Pharmac. exp. Ther.* **168,** 180 (1969).
8. A. Goldstein, *Biostatistics, An Introductory Text*, p. 51. MacMillan, New York (1964).

* R. E. Peterson and J. M. Fujimoto, *J. Pharmac. exp. Ther.* manuscript accepted for publication.

9. D. SELIGSON, J. MARINO and E. DODSON, *Clin. Chem.* **3,** 638 (1957).
10. National Bureau of Standards, *Tables of the Binomial Probability Distribution*, U.S. Government Printing Office, Washington D.C. (1949).
11. E. L. WAY and B. M. BENNET, *J. biol. Chem.* **192,** 335 (1951).
12. L. C. CRAIG, *J. biol. Chem.* **155,** 519 (1944).
13. S. ALPERT, M. MOSHER, A. SHANSKE and I. M. ARIAS, *J. gen. Physiol.* **53,** 238 (1969).
14. M. M. ABOU-EL-MAKAREM, P. MILLBURN, R. L. SMITH and R. T. WILLIAMS, *Biochem. J.* **105,** 1289 (1967).
15. L. A. WOODS, *J. Pharmac. exp. Ther.* **112,** 158 (1954).
16. J. M. ARIAS, L. BERNSTEIN, R. TOFFLER and J. BEN-EZZER, *J. clin. Invest.* **44,** 1026 (1965).