Chlorella, either reduce the amount of enzyme or increase the number of nonfunctioning enzyme molecules caused by misreading errors.[3,11]

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Biliary excretion of methadone by the rat: identification of a para-hydroxylated major metabolite

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THE EXTENSIVE biotransformation of methadone in both man and the rat has been shown to result in production of the *N*-demethylated cyclic metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP),[1,2] which is further *N*-demethylated to 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP).[2] Hydroxylated derivatives of EDDP and EMDP were found in human urine and rat urine and bile.[3] Baselt and Casarett[4] described the biliary secretion by the rat of a conjugated methadone metabolite, representing nearly one-third of an administered methadone dose, and assumed it to be a hydroxylated EDDP derivative. In the present study we identified this metabolite as conjugated *p*-hydroxy-EMDP.

MATERIALS AND METHODS

Male Sprague–Dawley rats (200–300 g) were used.

dl-Methadone hydrochloride was purchased from Merck Chemical Co. EDDP perchlorate and EMDP hydrochloride were gifts of Eli Lilly & Co. The *p*-methoxy analogue of methadone (*p*-OCH_3-methadone) was synthesized by the method of Shapiro[5] and was crystallized as its perchlorate salt (m.p. 163–164°).

Under ether anesthesia, the common bile ducts of the rats were cannulated using AWG-30 teflon tubing. Drugs were administered for metabolite collection by subcutaneous injection at a dose of 20 mg/kg and bile was collected for a period of 20 hr. For the excretion rate study, the drugs were administered by injection into the femoral vein at a dose of 1 mg/kg and bile collected for 4 hr.

The 20 hr bile samples (11–15 ml) were diluted with an equal volume of water, adjusted to pH 1 with conc. HCl and centrifuged. The supernatant was adjusted to pH 7 with NaOH and extracted twice with 3 volumes of $CHCl_3$ to remove all non-conjugated drug or metabolites. The $CHCl_3$ extract was reserved for analysis by thin-layer and gas–liquid chromatography. The aqueous phase was then centrifuged and the supernatant lyophilized, after which the residue was extracted with 5 ml of methanol. The methanol extract was centrifuged and the supernatant evaporated to dryness under a stream of nitrogen. This residue was dissolved in 5 ml of phosphate buffer pH 5·2 and incubated at 37° for 24 hr with 0·2 ml (1·0 IU) of beta-glucuronidase solution (Boehringer, Mannheim, Germany). The solution was then extracted with 25 ml of $CHCl_3$ and the solvent evaporated to a small volume for application to thin-layer plates.

Thin-layer chromatography (TLC) was accomplished on Kieselgel G using methanol/conc. ammonia, 100/1·5, as solvent. Spots were visualized with iodoplatinate spray. Unsprayed portions were scraped off and extracted with $CHCl_3$ for analysis by gas–liquid chromatography.

Gas–liquid chromatography (GLC) was performed on a Perkin–Elmer model 900 gas chromatograph with a flame ionization detector, using a stainless steel column packed with 1% SE-30 on Chromosorb G (80/100 mesh) and operated at 225°.

The *O*-methyl derivative of the new metabolite was prepared by dissolving the compound in 50 μl of tetramethylammonium hydroxide solution and injecting 1 μl of the solution into the gas chromatograph while the injection port was maintained at 350°.

Mass spectrometry (MS) was performed on a Varian MAT CH5 double-focussing mass spectrometer with a Varian 1400 gas chromatograph inlet. The GLC column was packed with 3% OV-17 on Gas Chrom Q (80/100 mesh) and was operated at 230°.

Heptane/water partition coefficients and pK_a values were determined according to Cohn[6] using a Unicam SP-800 ultraviolet spectrophotometer.

The 4 hr bile samples (3–4 ml) were analyzed for unchanged drug by a previously described GLC method.[7]

RESULTS AND DISCUSSION

The extraction of bile from three rats administered methadone yielded unchanged methadone, EDDP, EMDP and, after enzymatic hydrolysis, the new metabolite; two rats given EDDP yielded unchanged EDDP, EMDP and the new metabolite; while the two rats given EMDP showed only unchanged EMDP and the new metabolite in the bile. The new metabolite produced the usual reddish color with iodoplatinate spray on TLC, and in addition gave a positive reaction to Pauly's phenol reagent.[8] These results, together with chromatographic properties indicative of a polarity greater than that of EMDP (Table 1) as well as the fact that the new metabolite exists in the bile as a conjugate, suggest a phenolic derivative of EMDP.

TABLE 1. PHYSICO-CHEMICAL PROPERTIES OF METHADONE AND RELATED COMPOUNDS

Compound	Origin	Mol. wt by MS	R_f*	Retention time (min)†
dl-Methadone	Synthetic	309	0·26	1·7
EDDP	Synthetic	277	0·09	1·1
EMDP	Synthetic	263	0·70	0·9
New metabolite	Biological (from methadone)	279	0·64	2·5
p-OCH_3-methadone	Synthetic	339	0·26	3·1
p-OCH_3-EMDP	Biological (from *p*-OCH_3-methadone)	293	0·70	1·5

* TLC conditions are given in Materials and Methods.
† GLC conditions are given in Materials and Methods.

This was further substantiated by the MS results, shown in Fig. 1. The molecular ion peak of the new metabolite (*m/e* 279) was of very low intensity, while the peak of maximum intensity (*m/e* 224) corresponds to the elimination of the $—N{=}C—CH_2CH_3$ fragment from the molecular ion. This pattern is very similar to that of EMDP, the difference in molecular weight corresponding to the mass of a single oxygen atom.

In order to ascertain the location of the hydroxyl group in the new metabolite, *p*-OCH_3-methadone was administered to a biliary fistula rat. After $CHCl_3$ extraction of the unhydrolized bile and appli-

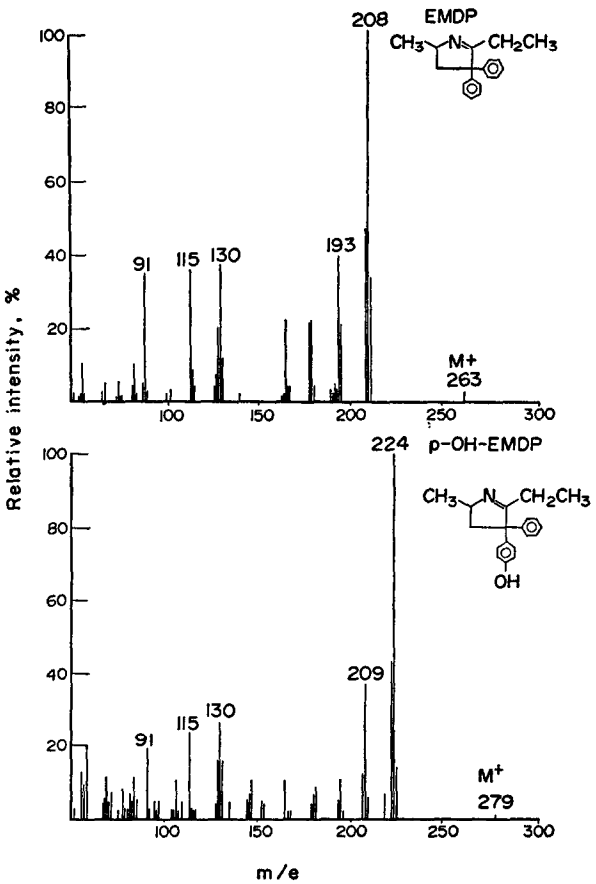

FIG. 1. Mass spectra of reference EMDP and the new metabolite (identified as *p*-OH-EMDP) obtained from the bile of rats given methadone.

cation of the $CHCl_3$ extract to TLC, a metabolite at the same R_f as EMDP was found to have a GLC retention time distinct from that of EMDP and a molecular weight by MS corresponding to *p*-OCH_3-EMDP (Table 1). A sample of the new metabolite was then *O*-methylated using tetramethylammonium hydroxide and the retention time of the methylated derivative was found to be equal to that of the biologically produced *p*-OCH_3-EMDP, thus confirming the structure of the new metabolite as *p*-OH-EMDP (2-ethyl-5-methyl-3-phenyl-3-*p*-hydroxyphenyl-1-pyrroline).

Without the necessary reference standards we cannot state unequivocally that the GLC conditions used will distinguish between *p*-OH-EMDP and its *m*-hydroxylated isomer, the enzymatic formation of which is a possibility. However, by comparison with previous work, such as that involving the GLC assay of *p*- and *m*-hydroxylated diphenylhydantoin metabolites,[9] the isomers of OH-EMDP should be easily separable with the *p*-isomer having the greater retention time.

The biotransformation of approximately one-third of an administered methadone dose by rats to conjugated *p*-OH-EMDP is surprising in view of the fact that its immediate precursor, EMDP, was considered to be produced to only a minor extent.[2] Our results indicate, however, that EMDP may be a major intermediary methadone metabolite, but one which is very poorly excreted. This can be explained by the results shown in Table 2 which demonstrate that, while EDDP is much more polar than methadone, EMDP is in the unusual position of being a metabolite considerably less polar than its parent, methadone. Furthermore, the degree of polarity for each compound is in direct proportion to the extent of its biliary excretion in unchanged form, following the intravenous administration of that compound. This suggests that the rat has difficulty in eliminating EMDP and that further biotransformation resulting in greater water solubility is necessary.

TABLE 2. LIPID SOLUBILITY VS BILIARY EXCRETION OF METHADONE AND TWO OF ITS METABOLITES

Compound	pK_a	Partition coefficient (heptane/water)*	Biliary excretion (%)†
dl-Methadone	8·62	5·0	8·8
EDDP	10·42	0·04	36·0
EMDP	5·88	13·4	0·2

* pH 7·4 phosphate buffer at 23°.

† Percentage of the dose excreted unchanged in 4 hr after a 1 mg/kg i.v. injection of the compound, average of two experiments.

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Effects of benziodarone on the biliary excretion of bromosulfophthalein and iodipamide

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SOME authors assume that the limiting step of the excretion of bromosulfophthalein (BSP) is the conjugation with glutathione.[1-3] This reaction is catalyzed by *S*-arylglutathione transferase and strongly inhibited by benziodarone.[4*]

Priestley found that benziodarone markedly decreases the biliary excretion of BSP in the rat.[5] This author believes that a direct action of benziodarone on the conjugation of BSP is not sufficient to explain this effect and, therefore, he suggests that this drug may modify both the conjugation and the active transport of BSP.

* Benziodarone: 2-ethyl,3-(4-hydroxy, 3 diiodobenzyl)-benzofurane.