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METABOLISM OF DRUGS—LXV

STUDIES ON THE URINARY CONJUGATED METABOLITES OF CODEINE

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Abstract—The detection and estimation of urinary metabolites of codeine in rabbits, guinea pigs, rats, and mice were carried out by thin-layer and gas chromotography. It was found that the conjugated metabolites in all species examined consisted of codeine glucuronide and morphine-3-glucuronide. Other conjugated metabolites could not be demonstrated. The amounts of codeine, morphine, codeine glucuronide and morphine-3-glucuronide which were excreted per 24-hr urine were estimated by gas chromatography to be about 3·5, 5·0, 11 and 29 per cent, respectively, for rabbits; 9, 2, 13 and 4·5 per cent for guinea pigs; and 6, 7, 1 and 19 per cent for rats.

CODEINE has been widely used in therapeutics for many years, and a number of reports have appeared in recent years with respect to its biological disposition. The pathways of codeine metabolism in man and various animals have been established to be conjugation, *O*-demethylation, and *N*-demethylation.[1–3] These reactions result in the formation of the respective metabolites, conjugated or free bases of codeine, morphine, and norcodeine. Among these metabolites, conjugated codeine was first suggested to be an important metabolite of codeine in man by Oberst,[4] and later it was shown that a major part of each demethylated metabolite also appears as a conjugated base in man by Adler *et al.*[5] More recently, Axelrod and Inscoe[6] have suggested that one of the conjugated codeines may be codeine glucuronide since the amount of free codeine increases after treatment of the urine sample with β-glucuronidase. In these studies, however, none of the conjugated bases excreted after administration of codeine has been isolated and identified as a chemical entity. In their previous studies,[7–9] the present authors have succeeded in the chemical synthesis of codeine glucuronide together with morphine-3-and 6-glucuronides and have elucidated the structure of urinary conjugated metabolites of morphine utilizing these synthetic samples as reference standards.

In the present investigation, the method has been applied to identify the conjugated metabolites of codeine in the urine of various animal species. The quantitative determination of urinary metabolites (free and conjugates) will be also described.

METHODS

Materials. Reference standards of codeine, morphine-3- and 6-glucuronides were prepared by the method previously described.[7,8,10]

Administration of codeine. Codeine phosphate was dissolved in water and injected

subcutaneously into male rabbits, male guinea pigs, male rats (Donryu strain) and female mice (DDD strain) at a dose of 28·36 mg/kg (equivalent to 20 mg of free base/kg), or 14·18 mg/kg (equivalent to 10 mg of free base/kg) for identification or determination of the metabolites respectively. The urine samples excreted within 24 hr after injection were collected.

Thin-layer chromatography. Thin-layer chromatography was carried out by use of silica gel plates (silica gel G, Merck), 0·25 mm thick, activated at 105° for 30 min. The solvent systems employed were: A, *n*-butanol–acetone–acetic acid–5% ammonium hydroxide–water (45:15:10:10:20, v/v); and B, ethanol–dioxane–benzene–concentrated ammonium hydroxide (5:40:50:5, v/v, upper layer). The organic solvent extracts of urine samples or column eluates were dissolved in a small quantity of MeOH or H_2O, respectively, and spotted on the plates along with authentic samples. The metabolites were detected by spraying the plates with Dragendorff reagent. The R_f values of authentic samples are listed in Table 1.

TABLE 1. R_f VALUES FOR THIN-LAYER CHROMATOGRAMS OF AUTHENTIC SAMPLES

Compounds	System A*	System B*
Codeine		0·41
Morphine		0·23
Norcodeine		0·17
Codeine glucuronide	0·29	
Morphine-3-glucuronide	0·15	
Morphine-6-glucuronide	0·25	

* System A: *n*-butanol–acetone–acetic acid–5% ammonium hydroxide–water (45:15:10:10:20, v/v), System B: ethanol–dioxane–benzene–concentrated ammonium hydroxide(5:40:50:5, v/v, upper layer).

Sample preparation for gas chromatographic analysis. Since the retention times of norcodeine and morphine by the gas chromatography described below were almost the same, the following separation procedure was necessary for the gas chromatographic analysis of the metabolites. The urine samples excreted in 24 hr after each injection were collected and extracted continuously with $CHCl_3$ for 3 hr. This $CHCl_3$ extract was shaken three times with 5 ml of 0·01 N NaOH solution for 3 min each. The $CHCl_3$ extract was dried over anhydrous Na_2SO_4, evaporated to dryness and set aside for estimation of codeine and norcodeine. For the estimation of morphine, the aqueous NaOH layer was made ammonia-alkaline by addition of 11 mg of NH_4Cl and extracted three times with 20 ml of $CHCl_3$–isopropanol (3:1) by shaking for 5 min. The extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated to dryness. Both residues for estimation of codeine, norcodeine, and morphine were further dried under reduced pressure and dissolved in 0·3 to 1·0 ml of methanol for gas chromatographic analysis.

Gas chromatography. The instrument used was a Shimadzu Model GC-1C Gas Chromatograph equipped with hydrogen flame ionization detector (dual column and differential flame type). The column was a glass U-shaped tube (4 mm × 2·625 m). The column packing was 1·5 per cent OV-17 on Shimalite W (80–100 mesh), which

was pretreated with hexamethyldisilazane. The column temperature was maintained at 230°, the sample chamber temperature at 250°, and the detector cell temperature at 240°. Nitrogen was used as the carrier gas with a flow rate of 30 ml/min. A methanol solution of the urine extract was injected with a Hamilton microsyringe. The retention times of codeine, norcodeine, and morphine were 7·8, 8·6, and 8·5 min respectively. The amount of codeine and morphine in the sample was calculated from the standard curve by measuring the peak height. The standard curve was made by running through the same extraction and separation procedure using authentic codeine, norcodeine,

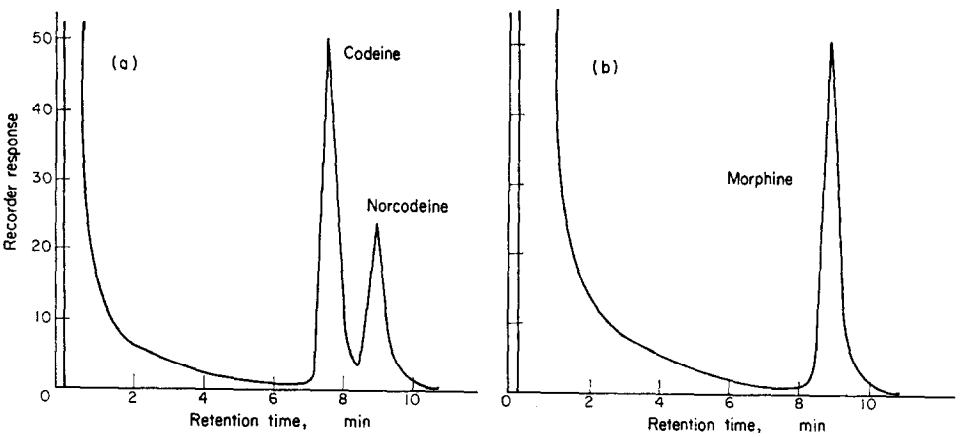

FIG. 1. Gas chromatograms of codeine and norcodeine, and of morphine separated from a standard mixture. a: A fraction containing codeine and norcodeine. b: A fraction containing morphine. Column: 1·5% OV-17 on Shimalite W (80–100 mesh) 2·625 m × 4 mm. Temperature: column, 230°, sample chamber, 250°, detector cell, 240°. Carrier gas: N_2 30 ml/min. Sens. 100, Range; 1·6 mV (morphine), 3·2 mV (codeine, norcodeine).

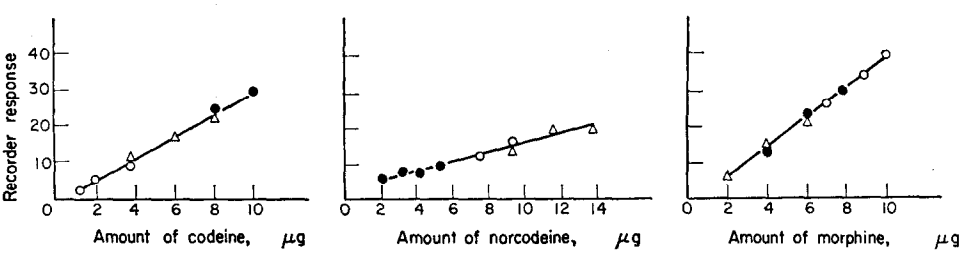

FIG. 2. Separation of codeine, norcodeine, and morphine. Sample A: ●. Sample B: ○. Sample C: ▲.

and morphine. The recovery of codeine, norcodeine, and morphine by this procedure was about 92, 82, and 75 per cent, respectively, and variability was within ± 2 per cent. A typical gas chromatogram is shown in Fig. 1. By this method including extraction and separation proceedures, three representative samples of A, B and C which contained codeine, morphine, and norcodeine in the amounts of 5·0, 3·0, 1·0; 1·0, 5·0, 3·0; and 3·0, 1·0, 5·0 mg, respectively, were analyzed. The results indicated very good separation of the three components, each of which possessed a peak height proportional to the amount added (see Fig. 2).

RESULTS

Direct detection of conjugated metabolites by thin-layer chromatography. An aliquot (30 ml) of each urine sample, collected as described above, was purified according to the previously reported method[9] which consisted of three steps: adsorption on charcoal, chromatographic separation on columns of cationic and anionic exchange resins. The extract obtained after the first step, adsorption on charcoal, was diluted to 30 ml with water and submitted to the next step, chromatographic separation on a column of Dowex 50 W- × 8 (H-form, 40 ml). The column was washed successively with 1 l. of water, 500 ml of ethanol, and 1 l. of water. The metabolites which were retained on the resin were eluted with 0·15 N ammonium hydroxide and collected in 20ml-fractions. By thin-layer chromatographic examination (system A) of each fraction, it was found that fraction 7 and neighbouring flasks contained codeine glucuronide (R_f, 0·29) and morphine-3-glucuronide (R_f, 0·15), but not morphine-6-glucuronide nor any other conjugated metabolites. Codeine, morphine, and norcodeine were also eluted in these fractions, but these metabolites were not separated from each other by thin-layer chromatography with this solvent system. For the identification of these non-conjugated metabolites, solvent system B could be used effectively, as described below.

The residues obtained after evaporation of the ammonium hydroxide from fractions 3 to 12 were combined, diluted to 30 ml with water, and then passed through a column of 30 ml of Dowex 1- × 2 (formate form). The column was washed with 1 l. of water and the metabolites were eluted with 0·01 N formic acid and collected in 20 ml-fractions. A representative thin-layer-chromatogram of fraction 5 and neighbouring fractions is shown in Fig. 3. From this chromatogram it is evident that each of the

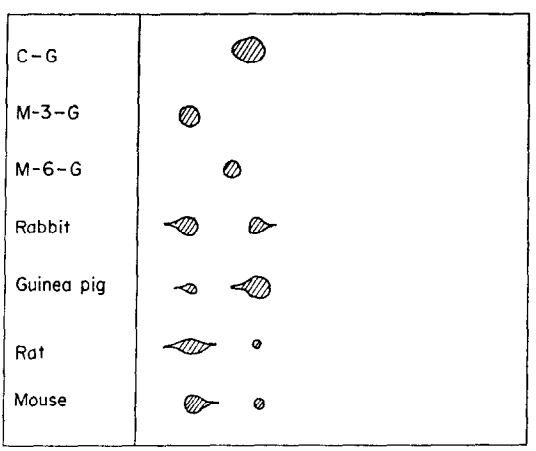

FIG. 3. Diagram of the thin-layer chromatograms of fraction 5 obtained by column chromatography on anionic exchange resin. C-G, codeine glucuronide; M-3-G, morphine-3-glucuronide; M-6-G, morphine-6-glucuronide; solvent system A was used.

species examined excreted not only codeine glucuronide but also morphine-3-glucuronide in considerable amount as conjugated metabolites after the administration of codeine. Other possible conjugated metabolites such as morphine-6-glucuronide, morphine-3- or 6-sulfate, or normorphine conjugates could not be detected.

Direct detection of unconjugated metabolites and indirect detection of conjugated metabolites. A portion (50 ml) of the 24-hr urine samples was adjusted to pH 9·0 with 30% NaOH solution and diluted with 50 ml of 3·0 *M* phosphate buffer, pH 9·0. To this solution was added 1 ml of 40% $NaHSO_3$ solution, and the mixture was extracted with $CHCl_3$ for 3 hr in a continuous extractor. The $CHCl_3$ layer was dried over anhydrous Na_2SO_4, and the solvent was evaporated to dryness. The residue obtained was submitted to thin-layer chromatography for the detection of nonconjugated metabolites (nonhydrolyzed sample). The remaining aqueous layer was brought to an acid concentration of 20 per cent by addition of 74·6 ml of concentrated HCl (see below, *Acid hydrolysis of codeine glucuronide*). After further addition of 1 ml of 40% $NaHSO_3$ solution, the mixture was heated on a boiling water bath for 1 hr. The resulting hydrolysate was extracted continuously with $CHCl_3$ for 3 hr, and the $CHCl_3$ layer was dried over anhydrous Na_2SO_4. The residue obtained was submitted to thin-layer chromatography for indirect detection of conjugated metabolites (acid hydrolyzed sample). As seen in Fig. 4, the metabolites which gave R_f values equivalent to authentic codeine and morphine were detected in both hydrolyzed and nonhydrolyzed samples from all the species examined; however, norcodeine was detected only in non-hydrolyzed samples from guinea pigs and mice.

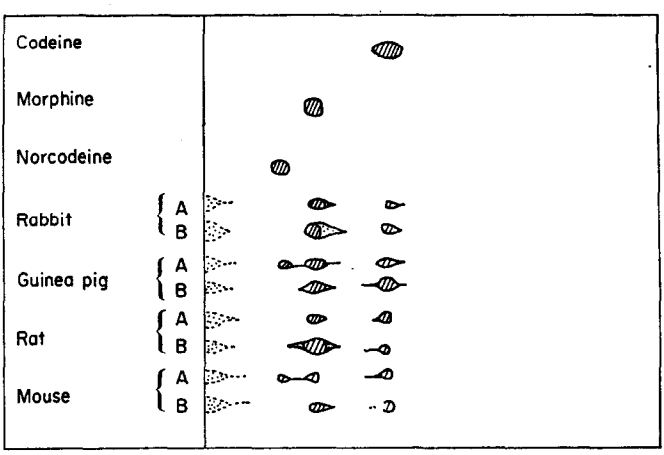

FIG. 4. Diagram of the thin-layer chromatograms of non-hydrolyzed and hydrolyzed samples. Solvent system B was used. A: Non-hydrolyzed sample. B: Acid hydrolyzed sample.

Acid hydrolysis of codeine glucuronide. Since the detection and estimation of codeine glucuronide were usually based on the codeine liberated by acid hydrolysis, it was important to establish the rate of acid hydrolysis of codeine glucuronide. Therefore, the optimal condition for hydrolysis was sought using various concentrations of hydrochloric acid and different heating times as follows: to 50 ml of control urine was added codeine glucuronide (3·0 mg), 1 ml of 40% $NaHSO_3$ solution, and appropriate volumes of concentrated HCl (18·7, 28·0, and 37·3 ml) to bring the acid concentration to 10, 15, and 20 per cent. The mixture was then heated on a boiling water bath for 30 min or 60 min. To this hydrolysate was again added 1 ml of 40% $NaHSO_3$ solution. The mixture was adjusted to pH 9·0 with 30%NaOH solution, diluted with 50 ml of

3·0 M phosphate buffer, pH 9·0, and extracted with $CHCl_3$ for 3 hr in a continuous extractor. The extract was analyzed for the liberated codeine. That rather drastic conditions are necessary to liberate codeine completely from its glucuronide is seen in Table 2. On the other hand, morphine-3-glucuronide is more easily hydrolyzable than codeine glucuronide.[9]

Estimation of codeine metabolites. Representative results of gas chromatographic estimation of codeine metabolites excreted into 24-hr urine of rabbits, guinea pigs and rats given codeine are summarized in Table 3. The amounts of bound metabolites

TABLE 2. ACID HYDROLYSIS OF CODEINE GLUCURONIDE

Concentration of HCl (%)	Per cent hydrolysis	
	30 min	60 min
10	50·5	54·5
15	81·3	88·3
20	93·5	99·5

TABLE 3. URINARY EXCRETION OF CODEINE AND ITS METABOLITES, EXPRESSED AS PERCENTAGE OF ADMINISTERED DOSE*

No. of and animals used	Dose (mg/kg)	Codeine		Morphine		Total (%)
		Free (%)	Bound (%)	Free (%)	Bound (%)	
Rabbit (4)	20	2·8	10·8	5·2	28·8	47·6
	20	3·4	12·0	6·0	32·9	54·3
	10	3·5	9·8	4·0	25·8	43·1
	10	4·5	11·0	6·5	29·7	51·7
Guinea Pig (6)	20	9·1	13·2	1·4	5·0	28·7
	10	7·8	12·2	1·4	4·1	25·5
	10	9·5	14·6	2·0	4·5	30·6
Rat (9)	15	7·0	0·8	7·0	21·1	35·9
	10	6·2	1·0	6·3	20·0	33·5
	10	5·2	0·5	7·0	17·1	29·8

*The individual values in this table were obtained from experiments using a single rabbit, two guinea pigs, or three rats.

were calculated from the amounts of free metabolites in nonhydrolyzed samples and those of free plus bound metabolites in hydrolyzed samples. Since our gas chromatographic procedure was not sensitive enough to detect low levels of norcodeine, and also since the amount of norcodeine excreted in the urine was not as great as that of codeine and morphine, the quantitative estimation of norcodeine was omitted in the present study.

Although the recovery in 24-hr urine was only about a half or less of the dose, the major fraction was accounted for as codeine and morphine conjugates in all animal species examined. By thin-layer chromatography, as shown in Fig. 4, these conjugates were found to consist only of codeine glucuronide and morphine-3-glucuronide. Therefore, the excretion percentages of bound codeine and morphine in this table

actually indicate those of each glucuronide. It is very interesting to note that rabbits and rats excreted more morphine glucuronide than codeine glucuronide, while the results in guinea pigs were the reverse.

DISCUSSION

Codeine has been shown to be excreted mainly as conjugated metabolites in man,[5] monkeys,[2] dogs,[2,3,11] guinea pigs[6] and rats,[12] but the structures of these conjugates have not been elucidated. In the light of the present results, the major metabolites of codeine in the rabbit, guinea pig, rat, and mouse were characterized as codeine glucuronide and morphine-3-glucuronide by comparison with authentic samples. The possible presence of other conjugates was also looked for carefully on the samples obtained in the various steps of purification of urinary metabolites, but without success.

Axelrod and Inscoe[6] reported that free and conjugated codeine excreted in 24-hr urine of guinea pigs after injection of codeine accounted for 8 and 18 per cent of the dose respectively. Quite recently, Yeh and Woods[12] demonstrated that when administered 2 mg/kg of ^{14}C-labelled codeine, rats excreted free codeine, and free and conjugated morphine, with recoveries of 7·8, 7·6, and 23·8 per cent of the dose, respectively, during the first 24 hr. These results are in good agreement with our present findings.

Norcodeine and/or its conjugates were recognized as urinary metabolites of codeine in man,[5] monkeys,[2] rats[12,13] and guinea pigs,[13] but in the present study only a free form was detected in the urine of guinea pigs and mice; no evidence was obtained concerning the formation of conjugated norcodeine. In rabbits which metabolized codeine mainly to codeine glucuronide, morphine and its glucuronide, *N*-demthylation to form norcodeine seems to be a minor metabolic pathway. Kuhn and Friebel[13] also found traces of normorphine in urine of rats treated with codeine; however, the present authors could not detect this metabolite in all of the urine samples examined.

Previously, Yoshimura *et al.*[9] demonstrated that morphine-3-glucuronide was a major metabolite of morphine in rabbits, guinea pigs, rats, mice, and man.* Considering this and the present results, the metabolic pathways of codeine could be illustrated as follows (Fig. 5).

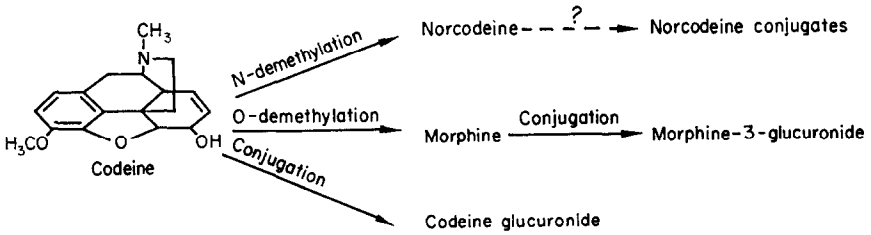

FIG. 5. The metabolic pathways of codeine.

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*H. YOSHIMURA, S. IDA, K. OGURI and H. TSUKAMOTO, unpublished data.

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