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IN VITRO FORMATION OF CODEINONE FROM CODEINE BY RAT OR GUINEA PIG LIVER HOMOGENATE AND ITS ACUTE TOXICITY IN MICE

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Abstract—*In vitro* metabolism of codeine was investigated by using a 9000 *g* supernatant fraction of rat or guinea pig liver homogenate. When a mixture of [N-$^{14}CH_3$] and [C-6-^{3}H]codeine was incubated with the rat liver 9000 *g* supernatant fraction in the presence of NAD, formation of codeinone, morphine and norcodeine was detected. Replacement of NAD with NADP abolished only the formation of codeinone. On the other hand, when the guinea pig liver homogenate was used in the presence of NAD, codeinone was the main metabolite of codeine. NADP was also ineffective in forming codeinone with the guinea pig liver homogenate. The acute toxicity of codeinone was thirty times higher than that of codeine. The roles of codeinone as a metabolic intermediate and in the acute toxicity of codeine are discussed.

Codeine has been used extensively for relief of coughing and of pain. Although much information is available on the biotransformation of codeine in various experimental animals and in humans, the metabolic profile of this drug has not yet been completely established in the form of metabolites of known structure. The metabolic pathways of codeine in mammals have been found to be (a) conjugation to give codeine glucuronide, (b) O-demethylation to form morphine; and (c) N-demethylation to yield norcodeine [1–5]. Recently, minor metabolites of codeine were identified as hydrocodone, 6α-hydrocodol and 6β-hydrocodol [6, 7]. The relationship of these minor metabolites to the pharmacological activities of codeine remains in question.

The present paper provides evidence for the formation of codeinone *in vitro* in rat and guinea pig liver homogenates, as well as the pharmacological properties of codeinone.

MATERIALS AND METHODS

Chemicals. [N-$^{14}CH_3$] and [C-6-^{3}H]codeine were prepared by the method previously described [8]. Codeinone was synthesized by the method of Rapoport and Reist [9]. Norcodeine was synthesized by the method of Braun [10]. Hydrocodone was synthesized as described previously [11]. All other reagents were the best grade commercially available.

In vitro *metabolism.* After decapitation of male Sprague–Dawley rats (180–200 g) or male Hartley guinea pigs (250–300 g), their livers were removed immediately and washed with saline. The pooled livers from two rats or guinea pigs were homogenized with 3 vol. of ice-cold 1.15% KCl with a polytron homogenizer (Kinematica GmbH). The homogenate was centrifuged at 9000 *g* for 30 min at 4°. To 8 ml of supernatant fraction, corresponding to 2 g of the tissue, 4 μmoles of NAD or NADP was added, and the mixture was incubated at 37° for 1 hr with 2 μmoles of a mixture of [N-$^{14}CH_3$] and [C-6-^{3}H] codeine (1 μmole each: specific activity of both ^{14}C and ^{3}H was 0.75 mCi/mmole), in a total volume of 10 ml made up with 0.1 M glycine–NaCl–NaOH buffer, pH 8.5. The incubation mixture was rapidly cooled in an ice bath to stop the reaction, and extracted with 20 ml of chloroform–methanol (3:1, v/v). The extract was transferred to a tube containing 3 ml of 1 N hydrochloric acid and agitated with a Vortex mixer. The acidic aqueous layer was separated and adjusted to pH 10 with 2 N sodium hydroxide. One gram of sodium chloride was added, and the solution was extracted with chloroform–methanol (3:1, w/v). After evaporation of the extract to dryness under reduced pressure, the residue was dissolved in methanol, and thin-layer chromatography (TLC) was performed. Reference compounds were always run on the same plate. The metabolites were viewed under an ultraviolet lamp or by spraying with Dragendorff reagent.

Thin-layer chromatography. TLC was performed on a silica gel plate (Spotfilm Silicagel f, Tokyo Kasei Kogyo Co. Ltd.) with chloroform–methanol (9:2, v/v). For the detection of radioactivity, the TLC sheet was cut into 1-cm strips. These strips were then placed in counting vials and moistened with 0.5 ml of methanol–water (1:1, w/v), and the radioactivity was evaluated in a liquid scintillation spectrometer (Aloka, LSC-673) after addition of 10 ml of scintillation fluid (Aquasol-2, New England Nuclear).

Acute toxicity. The lethal dose of codeine and its metabolites (within 24 hr) was determined by subcutaneous injection into male mice (20–25 g). At least ten mice per group were used to determine the LD_{50}. The codeine derivatives were converted to

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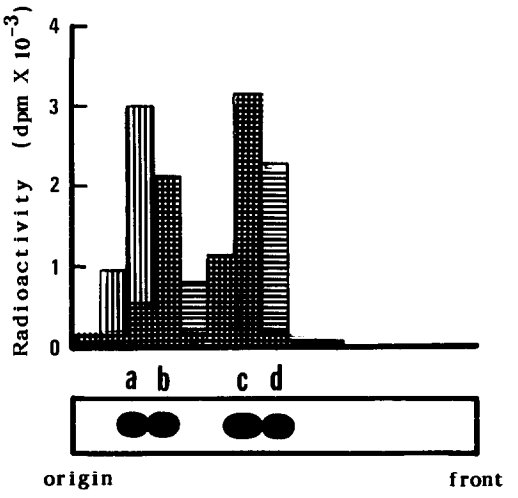

Fig. 1. Thin-layer radiochromatogram of *in vitro* metabolites of [N-$^{14}CH_3$] and [C-6-^{3}H]codeine. The chromatogram was developed with chloroform–methanol (9:2, v/v). Key: (▤) ^{14}C-radioactivity, and (▥) ^{3}H-radioactivity. Spot: authentic samples of norcodeine (a), morphine (b), codeine (c) and codeinone (d).

sulfates by the addition of an equivalent mole of 1 N sulfuric acid per mole of base, and made up with water in a volume of 10 ml of solution per kg. Doses of codeinone were 4, 5, 6, 7, 8, and 10 mg (base)/kg; those of codeine were 150, 200, 260, and 340 mg (base)/kg; those of norcodeine were 300, 400, 500 and 600 mg (base)/kg; those of morphine were 300, 400, 500, 600, and 700 mg (base)/kg; those of hydrocodone were 90, 100, 120, 160, and 200 mg (base)/kg; and those of hydrocodol were 100, 140, 180, 230 and 300 mg (base)/kg. The LD_{50} was estimated by the least squares fit to a log-probit analysis.

RESULTS

Identification and quantitation of in vitro *metabolites.* When a mixture of [^{14}C] and [^{3}H]codeine was incubated with the 9000 *g* supernatant fraction of rat or guinea pig liver homogenate, four major radioactive peaks were detected on the TLC plate (Fig. 1). Metabolite A gave mostly ^{3}H-radioactivity and lacked ^{14}C-radioactivity, suggesting that N-demethylation of $^{14}CH_3$ had occurred. Metabolite A was identified as norcodeine by co-chromatography with the authentic sample and by the negative phenolic group test (potassium ferricyanide–ferric chloride test). Metabolites B and C showed ^{14}C- and ^{3}H-radioactivity with the same ratio as the original mixture. Metabolite B gave a positive phenol group test, while metabolite C was negative. Metabolites B and C were identified as morphine and codeine by co-chromatography with authentic samples. Metabolite D had only ^{14}C-radioactivity, suggesting that oxidative elimination of ^{3}H at the C-6 position had occurred. Further, this metabolite gave a positive 2,4-dinitrophenylhydrazine solution test and a negative phenolic test. Metabolite D was separated by using a large TLC shee (20 × 20 cm) and eluted with chloroform. Metabolite D was identified as codeinone from the following evidence: (i) metabolite D treated with 2,4-dinitrophenylhydrazine gave codeinone-2,4-dinitrophenylhydrazone [12] which was identified by co-chromatography with TLC and the reverse isotope dilution method using authentic codeinone-2,4-dinitrophenylhydrazone; (ii) reduction of metabolite D in ethanol with sodium borohydride lead to the product identified as codeine; and (iii) the i.r. spectrum of metabolite D was identical to that of authentic codeinone (C=O, 1665 cm^{-1}).

Table 1 shows the amount of codeinone formed after 1 hr of incubation of codeine with the 9000 *g* supernatant fraction of rat or guinea pig liver. In the presence of NAD, a considerable amount of codeinone was formed with the 9000 *g* supernatants of both rat and guinea pig liver. However, NADP seems to be a poor cofactor for the transformation of codeine to morphine and norcodeine. However, there was a difference in the requirement for NAD and NADP for the formation of morphine and norcodeine between the liver homogenates of rats and guinea pigs. In the guinea pig liver homogenate, there was very little transformation of codeine to morphine and norcodeine with NAD. In contrast, the 9000 *g* supernatant fraction of rat liver utilized NAD fairly well for these reactions.

Acute toxicity. The LD_{50} values of codeine and its metabolites are presented in Table 2. Codeinone was about thirty times more toxic than codeine. The LD_{50}

Table 1. Metabolism of [^{14}C] and [^{3}H]codeine by 9000 *g* supernatants from rat and guinea pig liver*

	% of extracted amount			
	Rat liver 9000*g* supernatant with		Guinea pig liver 9000 *g* supernatant with	
	NAD	NADP	NAD	NADP
Codeine	32.0	35.1	89.2	68.2
Morphine	21.2	32.8	0.9	20.2
Codeinone	18.0	0.9	8.3	2.3
Norcodeine	24.9	31.2	1.6	8.8

* The reaction mixture contained 0.2 mM [^{14}C],[^{3}H]codeine, 0.4 mM NAD or NADP, and 9000 *g* supernatant fraction corresponding to 2 g liver in 10 ml of 0.1 M glycine–NaCl–NaOH buffer, pH 8.5. Data represent the mean of two experiments.

Table 2. Acute toxicity of codeine and its metabolites in mice

Compound	LD_{50} Values by s.c. injection (mg (base)/kg)
Codeine	225 (259–189)*
Codeinone	7.2 (8.3–6.1)
Hydrocodone	117 (138–80)
Hydrocodol	190 (219–155)
Norcodeine	482 (559–395)
Morphine	488 (570–340)

* Values in parentheses represent 95% confidence limits.

curves for all the compounds tested were parallel, and the signs of toxicity of codeinone were almost identical with codeine. The LD_{50} values of norcodeine and morphine agreed well with reported values [13].

DISCUSSION

Codeine is excreted mainly as codeine glucuronide and morphine glucuronide [4, 5]. However, several reduced types of codeine metabolites such as hydrocodone and 6α- and 6β-hydrocodol have also been reported recently [6, 7]. Biotransformation of dihydromorphinone to dihydromorphine has been observed [14]. Moreover, the reduction of naloxone and naltrexone to α- and β-naloxol and α- and β-naltrexol, respectively, has been reported [15–18]. Therefore, 6α- and 6β-hydrocodols are formed from hydrocodone by undergoing the same reaction of 6-keto-reduction. The biotransformation of codeine to hydrocodone remains in question.

In this paper, we showed the formation of codeinone from codeine in the 9000 *g* supernatant fraction of rat or guinea pig liver in the presence of NAD. Since the reduction of an α,β-unsaturated ketone to the corresponding saturated ketone has been shown in mammalian tissues [19], it is probable that codeinone is a precursor of hydrocodone in mammals. Figure 2 shows a possible metabolic pathway of codeine to 6α- and 6β-hydrocodol.

We could not demonstrate the formation of hydrocodone in the 9000 *g* supernatant fraction of rat and guinea pig liver. This suggests that the reduction of α,β-unsaturated ketone may be rate-limiting under our reaction conditions and that the extent of formation was very low. In fact, formation of hydrocodone and 6α- and 6β-hydrocodol is very low in human, guinea pig and dog, and essentially absent in the rat and rabbit [6].

Codeinone was about thirty times more toxic than codeine, and other typical metabolites of codeine were less or only a little more toxic than the parent compound. Therefore, the formation of codeinone might be, in part, responsible for the acute toxicity of codeine.

We have already shown that morphinone, a metabolite of morphine, is nine times more toxic than morphine for the mouse and that the acute toxicity might be due to its high lipid solubility, increasing brain concentration and its extreme reactivity with the sulfhydryl group of proteins and glutathione [8, 20]. Codeinone is chemically reactive and binds nonenzymatically to the sulfhydryl group of glutathione and various proteins (unpublished data); this may be the reason that codeinone has not been isolated from the urine of codeine-treated animals. It is therefore conceivable that the toxic action of codeine can be, in part, explained by the sulfhydryl blocking action of codeinone.

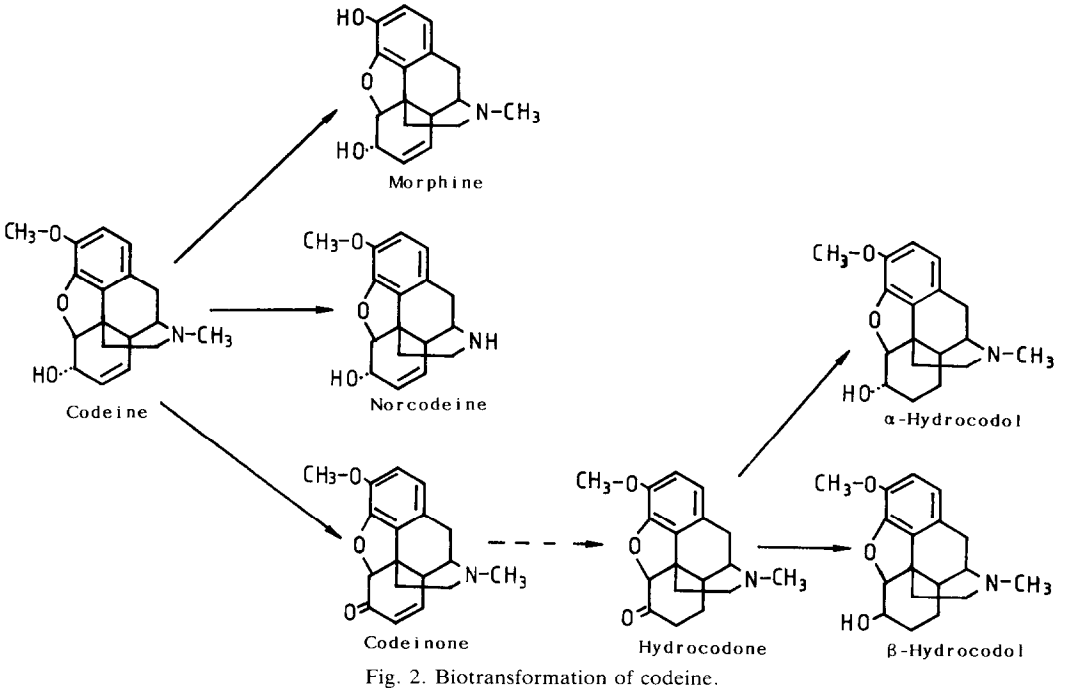

Fig. 2. Biotransformation of codeine.

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