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EFFECTS OF GLUTATHIONE AND PHENOBARBITAL ON THE TOXICITY OF CODEINONE

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Abstract—The ability of sulfhydryl compounds to provide protection against the acute toxicity of codeinone, a toxic metabolite of codeine, was investigated in mice. Subcutaneous administration of codeinone produced a slight reduction in hepatic glutathione concentration. Pretreatment of the mice with glutathione or cysteine significantly increased the survival rate for mice given a lethal dose of codeinone (10 mg/kg). The lethality of codeine was lowered by naloxone, whereas that of codeinone was not blocked by naloxone. The strychnine-like convulsant action of codeinone could be prevented by phenobarbital pretreatment. Glutathione pretreatment reduced the amounts of radioactivity in tissues of mice injected with [*N-methyl*-^{3}H]codeinone. A possible explanation for these observations is that glutathione reacts *in vivo* with codeinone and plays a role as a scavenger of this compound. This assumption is supported by the observation that codeinone reacts non-enzymatically with glutathione under physiological conditions.

Several reports have been published indicating a direct hepatotoxic effect of narcotics [1–4]. For example, morphine causes a rapid, dose-dependent decrease in hepatic glutathione (GSH) [5–7]. We recently reported that the morphine-induced depletion of hepatic GSH is due to the conversion of morphine to morphinone which then binds GSH by the Michael addition at C-8, and the injection of GSH into mice prior to the administration of morphinone provides protection against morphinone-induced lethality [5, 8]. We have also reported that codeine is metabolized to codeinone, and that this metabolite is about thirty times more toxic than codeine [9].

In this paper, we present the effects of sulfhydryl compounds and phenobarbital on the toxic action of codeinone.

MATERIALS AND METHODS

Chemicals. [*N-methyl*-^{3}H]Codeinone was synthesized from [*N-methyl*-^{3}H]codeine by the method of Rapoport and Reist [10]. Naloxone was a gift from the Endo Laboratory. Codeine phosphate, glutathione, cysteine and phenobarbital were obtained from commercial sources. All other reagents were the best grade commercially available.

Effects of glutathione, cysteine, phenobarbital and naloxone on the survival of mice given a lethal dose of codeinone and codeine. Codeinone or codeine was injected subcutaneously into each group of ten male mice (ddY, 20–25 g) 15–20 min after i.p. injection of glutathione, cysteine, phenobarbital or naloxone. Each compound was dissolved in saline and administered in 10 ml/kg quantities to produce the following doses: codeinone, 10 mg/kg; codeine, 200 mg/kg; glutathione, 500 mg/kg; cysteine, 500 mg/kg; phenobarbital, 50 mg/kg; and naloxone, 50 mg/kg. The control animals in each group received only an s.c. injection of saline. The surviving animals in each group were counted 6 hr after receiving codeine or codeinone.

Effect of codeine or codeinone on the hepatic glutathione content in mice. Five male mice in each group were given a single s.c. injection of codeine (100 mg/kg), codeinone (5 mg/kg) or naloxone (10 mg/kg). All animals were killed 3 hr after the injection, and their hepatic GSH (non-protein sulfhydryl compounds) was measured according to the method described by Ellman [11] with a slight modification. Liver homogenates (20%) in final 5% trichloroacetic acid containing 1 mM sodium ethylenediamine tetraacetic acid were centrifuged at 2000 *g* for 5 min. An aliquot of the supernatant fraction (400 μl) was transferred to a tube containing 4.55 ml of 0.1 M sodium phosphate buffer (pH 8.0), and 50 μl of 0.1 M 5,5-dithiobis-(2-nitrobenzoic acid) was added. After mixing, absorbance at 410 nm was measured against a reagent blank to determine the GSH concentration. Reduced GSH was used to establish a standard curve. GSH accounts for about 95% of acid-soluble thiols in the liver homogenate [12].

Effect of glutathione on the distribution of radioactivity following the s.c. administration of [N-methyl-^{3}H]*codeinone.* Five male mice (20–25 g) in each group were injected i.p. with GSH (100 mg/kg) or saline 10 min before the s.c. injection of [*N-methyl*-^{3}H]codeinone (5 mg/kg, sp. act. 12.6 mCi/mmole). The mice were killed by decapitation at various time intervals after the administration of [*N-methyl*-^{3}H]codeinone. Tissue and blood samples (about 100 mg) were placed in a paper cone cup, and

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the radioactivity was measured after oxidizing the samples with a sample oxidizer (Aloka ASC-111), using a liquid scintillation counter (Aloka LSC-651).

Reaction of codeinone and codeine with GSH. A mixture of 10 mM codeinone or codeine and 10 mM GSH in 0.1 M sodium phosphate buffer (pH 7.4) was incubated at 37°. The reaction rate was estimated by measuring the decrease in the amount of GSH in the reaction mixture at various time intervals according to the method of Ellman [11]. The reaction product was analyzed by high performance liquid chromatography (HPLC). The HPLC system was equipped with a Shimadzu LC-3A pump, a Rheodyne sample injector, a Waters Radial-pak C-8 column (8 mm × 10 cm), and a Shimadzu SPD-2A variable wavelength u.v. detector. The column was eluted with water–acetonitrile–triethylamine–acetic acid (170:30:1:1, by vol.) at a flow rate of 2 ml/min. The eluate was monitored at 280 nm.

RESULTS

Hepatic GSH content after codeine, codeinone or naloxone treatment. It has been shown that morphine elicits the maximal lowering of hepatic GSH content 3–4 hr after administration [5, 6]. Therefore, we investigated the effect of codeine or codeinone on hepatic GSH content 3 hr after administration of the drugs (Table 1). In the case of a high dose (8 mg/kg) of codeinone, the mice died within 2 hr after administration. From this result, the dose of codeinone was determined as 5 mg/kg. Treatment of mice with codeine (100 mg/kg) or codeinone (5 mg/kg) resulted in a 10% decrease in GSH content of the liver. Naloxone (10 mg/kg) did not alter hepatic GSH, and naloxone (10 mg/kg) administered 15 min before the codeine treatment completely blocked the depletion of hepatic GSH caused by codeine. In contrast, the depletion of GSH caused by codeinone was not blocked by naloxone pretreatment.

Effects of GSH, cysteine, phenobarbital and naloxone on the survival of mice given a lethal dose of codeinone or codeine. Injection of codeinone (10 mg/kg) into mice produced clonic convulsions which persisted for approximately 10 sec. Death followed tonic extensor seizures in all mice within 2 hr after the injection of codeinone. As shown in Table 2, mortality was 100% in the control mice given codeinone (10 mg/kg) alone, whereas 100% of the animals given GSH or cysteine (500 mg/kg, each) before the codeinone administration survived without any convulsions. When naloxone (50 mg/kg) was administered 15 min before the codeine treatment (200 mg/kg), the toxicity of codeine was blocked (data not shown). In contrast, the toxicity of codeinone was not blocked by naloxone pretreatment. Pretreatment with phenobarbital (50 mg/kg) blocked the convulsant action of codeinone and produced 100% survival.

Table 1. Effects of codeinone, codeine or naloxone on hepatic GSH content in mice

Treatment	Dose (mg/kg)	Hepatic GSH content (μmoles/g tissue)
None		6.42 ± 0.23
Codeinone	5	5.89 ± 0.15*
Codeine	100	5.87 ± 0.23*
Naloxone	10	6.90 ± 0.27
Naloxone + Codeinone	10 + 5	5.58 ± 0.15*
Naloxone + Codeine	10 + 100	6.35 ± 0.15

Data represent the mean ± S.E. of five mice.

* Significance of the difference compared with the control was calculated by using the one-way analysis of variance and Dunnett's test ($P < 0.05$).

Table 2. Effects of glutathione, cysteine, naloxone or phenobarbital on the survival of mice given a lethal dose of codeinone

Pretreatment	Dose (mg/kg)	Survival/Tested
None		0/10
Glutathione	500	10/10*
Cysteine	500	10/10*
Naloxone	50	0/10
Phenobarbital	50	10/10*

The surviving animals in each group were counted 6 hr after s.c. administration of codeinone (10 mg/kg).

* Significance of difference compared with the control was calculated by the chi-square test ($P < 0.05$).

Distribution of radioactivity following the administration of [N-methyl-^{3}*H*]*codeinone.* The distribution of codeinone in mouse tissues was measured over a period of 3 hr after an s.c. injection of [*N-methyl*-^{3}H]codeinone. To determine the effect of GSH on the distribution of [*N-methyl*-^{3}H]codeinone, GSH (100 mg/kg, i.p.) was injected 10 min before the [*N-methyl*-^{3}H]codeinone (5 mg/kg, s.c.) administration. As shown in Table 3, the radioactivity was most heavily concentrated in the liver and kidney. The pretreatment of GSH reduced the distribution of radioactivity of [*N-methyl*-^{3}H]codeinone in the mouse tissues.

Reaction of codeinone and codeine with GSH. Codeinone or codeine was incubated with GSH under the conditions described in Materials and Methods. The reaction was monitored by the decrease in GSH. Codeinone rapidly reacted with GSH, whereas codeine seemed to be inert to GSH (Fig. 1). The reaction of codeinone with GSH obeyed second-order kinetics with a rate constant of 68.2 $l \cdot mole^{-1}\ min^{-1}$. These results were confirmed by HPLC analysis of the reaction mixtures. Although the retention time of codeinone was 6.5 min under our elution conditions, analysis of the reaction mixture revealed a new peak increase with reaction time and retention time of 2.5 min (Fig. 2). The height of the new peak increased with the reaction time and that of codeinone decreased in inverse proportion to the increase in the new peak. Therefore, we concluded that the new peak was a product of the reaction of codeinone with GSH.

On the other hand, HPLC analysis of the incubation mixture of codeine and GSH did not show any new peak (data not shown). Therefore, we concluded that codeine was not reactive with GSH.

Table 3. Distribution of radioactivity following s.c. administration of [^{3}H]codeinone

Tissue	Pretreatment	Codeinone eq. (pmoles/g tissue) Time after administration (hr) 0.5	1	2	3
Blood	None	1292 ± 119	774 ± 89	957 ± 135	789 ± 177
	GSH	741 ± 94*	326 ± 34*	291 ± 54*	310 ± 49*
Brain	None	671 ± 46	626 ± 74	303 ± 9	470 ± 26
	GSH	400 ± 50*	191 ± 21*	167 ± 19*	174 ± 11*
Lung	None	1483 ± 152	1078 ± 155	878 ± 49	717 ± 61
	GSH	1029 ± 132*	485 ± 54*	402 ± 47*	439 ± 47*
Liver	None	4394 ± 344	2918 ± 450	1968 ± 101	1851 ± 96
	GSH	4283 ± 587	2656 ± 397	1606 ± 168	1370 ± 189*
Kidney	None	4546 ± 887	2960 ± 321	1759 ± 100	1686 ± 163
	GSH	2841 ± 379*	1841 ± 194*	1218 ± 132*	1133 ± 99*

Data represent the mean ± S.E. of five mice.
* Significance of difference compared with the control was calculated by using the one-way analysis of variance and Dunnett's test ($P < 0.05$).

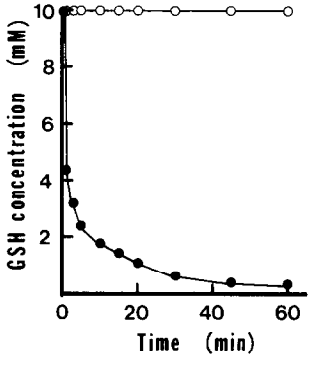

Fig. 1. Reactivity of codeinone and codeine with GSH. Data represent the mean of the two experiments. Key: (●) codeinone; and (○) codeine.

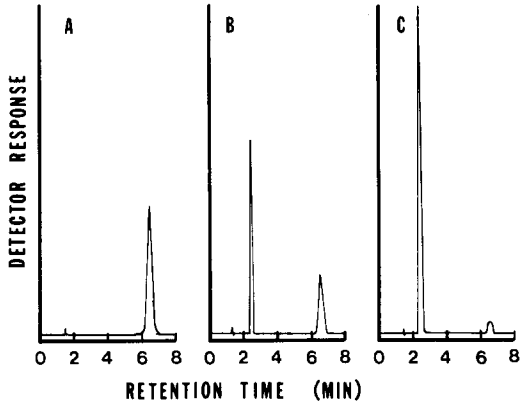

Fig. 2. Chromatogram of reaction product of codeinone with GSH: (A) codeinone standard; (B) reaction product for 1 min; and (C) reaction product for 10 min.

DISCUSSION

It has been shown that the administration of a large dose of morphine to mice significantly reduces the GSH level in the liver [5–7]. Similarly, in this study, administration of codeine caused a depletion of hepatic GSH in the mouse (Table 1). This depletion is possibly due to the formation of codeinone-GSH adduct from codeine. This assumption is supported by the fact that codeinone rapidly reacted *in vitro* with GSH, probably at the C-8 position by the Michael addition, whereas codeine was unreactive with GSH (Figs. 1 and 2). Moreover, codeine-induced GSH depletion can be demonstrated in isolated rat hepatocytes (Nagamatsu *et al.*, unpublished data). Codeine is actually converted in liver homogenates to codeinone [9]. However, we cannot rule out the possibilities that codeine acts centrally as does morphine to reduce hepatic GSH [13], and/or metabolic benzylic C-10 oxidation also results in the depletion of hepatic GSH by the formation of a C-10 GSH adduct [14].

Naloxone reduced the GSH-depleting effect of codeine, whereas it did not suppress the depletion caused by codeinone (Table 1). Naloxone is a specific inhibitor of morphine 6-dehydrogenase, which catalyzes the production of morphinone from morphine and codeinone from codeine [15]. Therefore, we assume that naloxone inhibited the formation of codeinone from codeine in the liver and caused the reduction in the GSH-depleting effect of codeine. However, another possibility is that naloxone acts centrally and reduces the GSH-depleting effect of codeine.

GSH provides protection against reactive metabolites generated in the liver by acting as a scavenger of electrophilic intermediates. Thus, GSH conjugation is an important detoxification pathway of many drugs. The GSH adducts are usually more polar than the original compounds, and this property facilitates their excretion. In addition, formation of

conjugates prevents the covalent binding of chemically reactive metabolites to tissue macromolecules such as nucleic acids and proteins. In fact, several reports have indicated the protective effect of sulfhydryl compounds against drug-induced acute liver injury [16–19] and lethality [20, 21]. Pretreatment with the two different sulfhydryl compounds, GSH and cysteine, provided dramatic protection against codeinone-induced convulsions and lethality in the mouse (Table 2). Since exogenously injected GSH is not taken up into liver cells [22], a possible explanation for this observation is that subcutaneously injected codeinone was inactivated primarily by reaction with the sulfhydryl compounds in the circulation and that some portion of codeinone which escaped from the circulation into the liver might be also inactivated by sulfhydryl compounds. This assumption was suggested by the observation that the GSH pretreatment lowered the codeinone concentration in the mouse tissues (Table 3).

Naloxone also prevented the codeine-induced lethality (Table 2). At present, we are not certain whether this was due to the antagonistic action of naloxone against codeine at some specific sites including opiate receptors, or if it was caused by the inhibition of some enzymes which convert codeine to toxic intermediates such as codeinone.

High doses of narcotics produce convulsions similar to those caused by strychnine. The convulsant action produced by strychnine is prevented by pretreatment with barbiturates [23]. Pretreatment of mice with phenobarbital prevented the convulsant effect and lethality produced by codeinone. This finding suggested that codeinone toxicity is due to its convulsant effect, and that the convulsion cannot be prevented by naloxone.

In conclusion, codeinone produced convulsions, which were followed by death. GSH and phenobarbital have different modes of action in preventing codeinone toxicity. GSH acts as a scavenger of codeinone; on the other hand phenobarbital acts against the convulsant effect produced by codeinone.

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