THE MORPHINE 3-GLUCURONIDE DIRECTED ANTIBODY: ITS IMMUNOLOGICAL SPECIFICITY AND POSSIBLE USE FOR RADIOIMMUNOASSAY OF MORPHINE IN URINE

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Abstract—The major metabolite of morphine in human urine, morphine 3-glucuronide, was conjugated to bovine serum albumin by action of a water soluble carbodiimide. Immunization of rabbits with the conjugate produced the antibody which effectively bound $^{14}$C-labeled morphine. By comparing the potency of various morphine-related compounds to inhibit the binding, the specificity of the antibody was characterized. The ranking in the order of decreasing affinities to the antibody was codeine or morphine 3-glucuronide, morphine, codeine 6-glucuronide, dihydrocodeine, morphine 6-glucuronide and nalorphine. The results indicated that the hapten structure was correctly reflected in the specificity, except that recognition of the glucuronyl moiety by the antibody was not as sharp as had been expected. When the antibody was used for radioimmunoassay of urine sample, the urinary excretion patterns of morphine and codeine in rats could be followed easily for more than 5 days after the drug administration. In addition, experiments were carried out to estimate the excretion patterns of dextromethorphan and dihydrocodeine in humans. Based on the results, it is suggested that the morphine 3-glucuronide directed antibody can be utilized for radioimmunoassay of morphine and its 3-glucuronide in urine.

The radioimmunoassay which usually surpasses the other analytical techniques in sensitivity, specificity or simplicity, has other advantages also. There is a broadness in applicability of the method which could theoretically cover from the immunologically active macromolecules to the inert small compounds. One of the examples was recently provided by development of the radioimmunoassay method for morphine (1-2, 5, 7-9), which, at the same time, seems to have added a new dimension to either basic or clinical research of the opiate pharmacology.

In 1970, Spector and Parker (1) first reported preparation of morphine specific antibody using 3-O-carboxymethyl morphine as a haptenic group. Thereafter, the antibody directed to morphine 6-hemisuccinate (5, 7-9), 2-(p-aminophenylazo) morphine (5) or oxymorphone 6-(0-carboxymethyl) oxime (9) was introduced. Meanwhile, application of the antibody for immunological estimation of morphine in biological fluids, especially in serum, has been attempted, employing radioimmunoassay (2), electron spin resonance technique (6) or the serological techniques such as hemaggultination inhibition (3) and complement fixation inhibition (4).

On the other hand, more than half the morphine administered to humans is known to be excreted in urine as morphine 3-glucuronide (10). So far, only a few reports (11-12)
have appeared in which the morphine specific antibody was utilized for analysis of morphine in the urine sample. Since specificity and sensitivity of a radioimmunoassay are largely conditioned depending on how sharply the binding specificity of the antibody is directed to the compound to be assayed, one might expect that by directing the specificity of the antibody straight to the metabolite, a better urine test would be developed. From this point of view, we have prepared the antisera employing morphine 3-glucuronide as the haptenic group. In this report, the specificity of the antibody prepared as above and the scope of its usability in radioimmunoassay are described.

MATERIALS AND METHODS

Drugs: Morphine 3-glucuronide (M-3-G) and morphine 6-glucuronide (M-6-G) were biologically prepared by giving morphine to rabbits and isolated as described by Yoshimura et al. (13). Codeine 6-glucuronide (C-6-G) was synthesized according to the method of Yoshimura et al. (14). Elemental analysis and measurement of infra-red absorption spectra of these compounds gave results corresponding to those found in above references. Oxy morphine hydrochloride, naloxone hydrochloride and pentazocine base were kindly provided by Sankyo Pharmaceutical Co. Ltd. and dextromethorphan by Shionogi Pharmaceutical Co. Ltd. Other morphine analogs were purchased. Freund's complete adjuvant was obtained from Iatron Lab. (Tokyo), crystalline bovine serum albumin (BSA) from Sigma (U.S.A.), the water soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) from Protein Research Foundation (Osaka) and N-methyl-14C-labeled morphine (58 mc/m mole) from Radiochemical Centre (England).

Preparation of immunogen: For coupling to BSA, M-3-G (63 mg) was dissolved in 10 ml of distilled water containing 50 mg of the protein. The water soluble carbodiimide (100 mg) was added dropwise over a 4 hr period. During this time, the pH was maintained below 7 with 2N HCl. After standing overnight at 25°C, the reaction mixture was exhaustively dialyzed against distilled water at 4°C. The conjugate was purified by passing through a Sephadex G-25 column and obtained as lyophilized powder. The glucuronide content of the conjugate was spectrophotometrically analyzed to be about 3 moles per mole of BSA.

Immunization procedure: Immunization of the rabbit was primed by injecting 5 mg of the conjugate and thereafter, 3 mg given as a booster injection every 2 weeks. For injection, the conjugate was dissolved in 50 mM phosphate buffered saline (pH 7.0, PBS) and emulsified with an equal volume of Freund's complete adjuvant. During the immunization period, increase of morphine binding activity in serum was monitored. Ten days after each injection, the blood sample was taken and the amount of labeled morphine bound by 1.0 μl of the serum estimated as described below.

Radioimmunoassay: The reaction mixture in a centrifuge tube contained 0.1 ml of a 1:100 dilution of the antisera, 4 mg as protein of normal rabbit sera and 64 picomoles of labeled morphine (7.4×10^4 c.p.m.) in 2 ml of PBS. For testing the specificity of the antibody in the antisera, morphine or its analogue was added in various concentrations
(0.010~10 nanomoles), while for analysis of urine, an aliquot (0.1 ml) of the sample was added. In any case, the final volume of the reaction mixture was adjusted to 2.5 ml by adding PBS. After overnight incubation at 4°C, 2.5 ml of saturated ammonium sulfate was added to the reaction mixture. One hr later, the formed precipitate was collected by centrifugation at 7,000 r.p.m. at 4°C for 20 min, washed with half saturated ammonium sulfate and dissolved in 0.2 ml of PBS to apply on a glass fibre disc. The disc was placed in a vial containing 5 ml of toluene scintillator (PPO 0.4%, POPOP 0.01% in toluene) and the radioactivity was counted in a liquid scintillation counter with an efficiency of 90%.

Measurement of urinary excretion patterns of morphine or its analogs in rats and human:
To 2 Wistar strain female rats weighing about 200 g each, 10 mg/kg of morphine or codeine was injected intraperitoneally. The urine was pooled for 0 to 8, 8 to 24 hr and thereafter for every 12 hr, and an aliquot (0.1 ml) subjected to radioimmunoassay. To be noted was the observation that, without any pretreatment of the animals with morphine-type drugs, the urine contained some factor(s) which slightly inhibited binding of morphine to the antibody. The quantity of the drug in the urine sample was then read off a standard curve run in the presence of 0.1 ml of normal urine. Since the standard curve was made using morphine or codeine itself, the quantity of the drug was expressed as the value corresponding to each original compound.

In the case of humans, three types of cold remedy tablets already on the market and which contain dihydrocodeine, dextromethorphan or not any of the morphine-type of drug, were orally administered to nine healthy male adults, one type of tablets to three persons each. The dose per person was 5 mg of dihydrocodeine or 15 mg of dextromethorphan. Urine samples were collected 4, 8, 24, and 48 hr after tablet intake and an aliquot (0.1 ml) of each sample was analyzed.

RESULTS

Binding specificity of anti-M-3-G antibody

Morphine binding activity which appeared in serum after the priming injection, was consistently elevated by each booster injection. Radioactivity of morphine bound by

![Graph](image-url)

**Fig. 1.** Increase of anti-M-3-G titer during the course of immunization by M-3-G-BSA conjugate. The curve represents the mean of two determinations.
1.0 μl of the antiserum increased from 25 to $2.8 \times 10^3$ c.p.m. and when plotted in logarithmic scale to time, a straight line was obtained until before the fifth injection (Fig. 1). Based on this observation, the priming and four booster injections were employed as the standard immunization procedure and the antisera collected 10 days after the fifth injection were used for this experiment. In radioimmunoassay developed using the antisera, the minimum detectable amount of morphine was found to be about 5 ng.

The specificity of the antibody in the antisera was characterized by comparing the relative potencies of various compounds to inhibit formation of $^{14}$C-morphine-antibody complex. The structures and inhibition curves of the compounds are given in Figs. 2 and 3, respectively. Table 1 shows a comparison of the amounts of the compounds (the 150 value) which were required to induce the 50% inhibition. M-3-G or codeine was the most effective inhibitor and the 150 values for both were about 20 ng. Morphine followed next. For oxymorphone and naloxone, the values could be estimated but in far higher dose ranges as compared to the above three compounds. Some morphine surrogates such as methadone, pethidine or pentazocine, which are analgesically active but lack the phenanthrene ring structure, failed to induce the 50% inhibition in the dose ranges utilized.
Urinary excretion patterns of morphine and its analogs

In rats, a single dose of morphine was excreted in the pattern depicted in Fig. 4. The pattern seemed to be multiphasic. With codeine, a similar pattern was obtained. In either case, the drug was detectable in urine for more than 108 hr after the drug injection.
**DISCUSSION**

In this study, the major metabolite of morphine in man (10), M-3-G, was employed as the haptenic group. It was assumed that the glucuronyl moiety which serves as the bridge for coupling morphine with the carrier protein, would also function as one of the antigenic determinants in the hapten structure. Analysis of the specificity of the antibody prepared as such, however, revealed that the specificity could not be directed so sharply to the carbohydrate moiety. As shown in Fig. 2, the ranking in the order of decreasing affinities to the antibody was M-3-G or codeine and morphine, indicating that the 3-substituted structure of M-3-G rather than the carbohydrate moiety itself would have functioned as one of the determinants. Spector (5) has described that the antibody elicited by the morphine 6-hemisuccinate-BSA bound M-3-G almost as effectively as morphine on a molar basis, though the metabolite carries a bulky substituent on the phenolic function. The specificity test of the antibody prepared by employing the same haptenic group in this laboratory gave similar results (Fig. 6). These results suggest that the glucuronyl
moiety of M-3-G would not necessarily function as a decisive determinant either in directing the antibody specificity or in the antibody-antigen binding.

Besides this rather exceptional finding, the specificity test of the M-3-G directed antibody provided results which appear to meet the general rule that the specificity of a given antibody strictly reflects the structural characteristics of the hapten used for immunization. When the antibody with such specificity was applied for radioimmunoassay of morphine and M-3-G in urine, the absolute sensitivity was 5 ng for both compounds in a final assay mixture. The sensitivity was lower than that reported by Spector (5) for these compounds using the morphine 6-hemisuccinate directed antibody. In Spector's work, 3H-labeled dihydromorphine was used as the tracer as this compound would be less effectively bound by the antibody than morphine (compare the affinities of codeine and dihydrocodeine in Fig. 3). Since the use of a tracer which has a moderately lower affinity to the antibody than morphine would theoretically raise the sensitivity of the radioimmunoassay method, the relatively lower sensitivity of our assay method seems to be partly due to employment of morphine as the tracer.

In spite of such a drawback in the employed tracer, the radioimmunoassay method developed here was capable of following the urinary excretion patterns of morphine and codeine for more than 108 hr after the drug administration. The results in Fig. 4 indicate that the pattern would be multiphasic for both drugs and also imply that morphine remains in the animal body for a long time even after a single dose. Recently, it was reported (15–16) that one analgesic dose of morphine would be enough to induce a significant level of analgesic tolerance in the same animal species. It is, therefore, highly probable that the existence of the alkaloid in the target tissue or in the milieu surrounding the tissue for a long time may play a role in the mechanism of developing tolerance.

In the case of humans, two types of codeine analogs replaced the morphine administration. The urinary excretion patterns of the compounds could be followed easily, although highly diluted excretion of the drugs in urine or low binding affinities of the com-
pounds to the antibody curtailed the period during which the drug was detectable in urine.

In Japan, there are strict regulations on the arbitrary use or import of opiates, except codeine analogs such as dihydrocodeine employed in this experiment which have been marketed quite freely. In order to develop a clinical test for detection of the opiate abuser, the problem would be differentiation of the type of drug taken, i.e. the morphine type or the codeine type. In this context, the M-3-G directed antibody would be more suitable for analysis of urine than other types of antibodies prepared so far, because in humans, morphine is found mainly as M-3-G in urine while codeine as the 6-glucuronide and the latter metabolite was less effectively recognized by the antibody than was M-3-G.

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