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Carboxymethyl-Chitin as a Drug Carrier of Sustained Release

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ABSTRACT

Methamphetamine-bound carboxymethyl-chitin (CM-chitin) was found to induce hapten-specific antibodies through subcutaneous injection only in the presence of Freund's complete adjuvant in spite of its highly biodegradable property. CM-Chitin was employed as a sustained-release drug carrier for the subcutaneous injection. The induced specific antibody was applied to the titration of methamphetamine secreted into the blood of rabbits. The methamphetamine concentration in blood serum was maintained for 120 h at fairly high levels. Methamphetamine was also excreted into urine at high levels with a similar time course to that in blood serum.

INTRODUCTION

Chitin, a supporting mucopolysaccharide of crustaceans, is known to be digested slowly by lysozyme in an animal body. The rate of lysozymic hydrolysis was found to be enhanced remarkably by the carboxymethylation of chitin even at a low degree of substitution (Tokura *et al.*, 1983*a*). The peritoneal injection of carboxymethyl-chitin (CM-chitin) has also been reported to induce the activation of short-acting peritoneal macrophages in mice, and the degree of macrophage activation was obviously dependent on the degree of carboxymethylation of the C-6 hydroxyl groups on the *N*-acetylglucosamine (GlcNAc) residues (Nishimura *et al.*, 1984). When the hapten-bound CM-chitin was injected subcutaneously in the form of an emulsion of Freund's complete adjuvant into rabbit, the hapten-specific antibody of a higher titration level was produced by the use of a diaminobutane spacer but a

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somewhat lower titration level by the use of a diaminoethane spacer. However, in the absence of the immunoadjuvant, there was little antibody production with the injection of hapten-CM-chitin conjugate having a diaminoethane spacer, although a trace of antibody formation was observed with the diaminobutane spacer (Tokura *et al.*, 1987). The specificity of the induced antibody was limited only to several hapten analogues, such that a sensitive titration of hapten was obtained successfully by the enzyme-linked immunosorbent assay (ELISA) (Takatori *et al.*, 1985).

In this study, the sustained release of methamphetamine was investigated by using CM-chitin as a drug carrier, and the methamphetamine released into serum was titrated by the ELISA method by using a methamphetamine-specific antibody. The methamphetamine derivative that was released into the serum maintained a significant level in serum for 120 h after a single subcutaneous injection of polymeric prodrug. The amount of methamphetamine derivative in serum can be regulated by the volume or the concentration of polymeric prodrug to be injected.

EXPERIMENTAL

Materials

Chitin was prepared from Queen Crab shells according to the method of Hackman (1954) and powdered to less than 60 mesh before use.

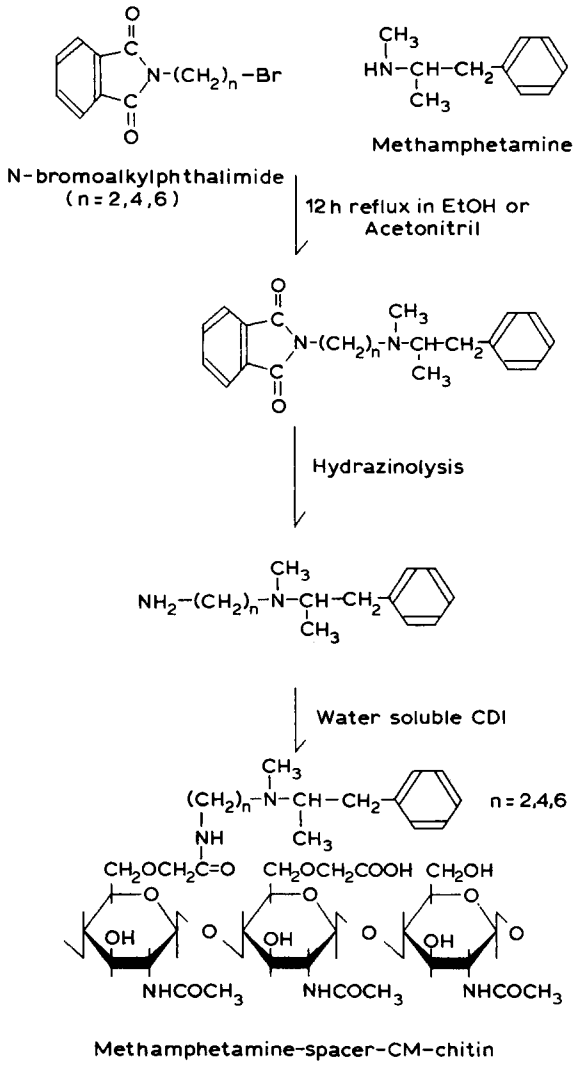
Preparation of CM-chitin

CM-chitin was prepared according to the improved method of Trujillo as reported previously (Tokura *et al.*, 1983*b*). The degree of carboxymethylation was estimated by potentiometric titration and elemental analysis to be around 75% of substitution of the C-6 hydroxyl groups on the GlcNAc residues. The molecular weight of CM-chitin was estimated to be around $6{\cdot}3 \times 10^4$ by applying the viscosity formula reported previously (Inoue *et al.*, 1982).

Preparation of methamphetamine-bound CM-chitin

Methamphetamine (MA) was purchased from Dainippon Pharmaceutical Co. Ltd and used without further purification. Methamphetamine was bound to CM-chitin through diaminoethane or -butane as

reported previously (Takatori *et al.*, 1985). The synthetic route of MAEA-CM-chitin is shown in Scheme 1. In brief, *N*-bromoethyl phthalimide and MA were refluxed for 12 h in acetonitrile to prepare D-*N*-(2-aminoethyl)methamphetamine (MAEA). The purity of MAEA was analyzed by elemental analysis and IR spectra. Mass spectrometry was also applied to confirm the purity after trifluoroacetylation of MAEA. The optical rotation of MAEA was measured with a Horiba SEPA-200 spectropolarimeter in H_2O ($c = 0{\cdot}1$) at room temperature to be $[\alpha]_D = +18{\cdot}2°$. D-*N*-(2-Aminoethyl)methamphetamine-CM-chitin



Scheme 1. Outline of synthetic route for MA-bound CM-chitin through alkyl spacer.

(MAEA-CM-chitin) was prepared by the coupling of MAEA and sodium-free CM-chitin with the use of morpho-CDI[1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-*p*-toluene sulfonate] in deionized water at room temperature. After 24 h of the coupling reaction, the reaction mixture was dialyzed against deionized water repeatedly to remove impurities and then lyophilized. The amount of bound MA was analyzed spectrophotometrically by using a Hitachi automatic UV spectrophotometer (U-3200/U-3400). The methamphetamine-specific antibody was prepared by the method reported previously (Takatori *et al.*, 1985) by using MABA-CM-chitin and immunoadjuvant. The enzyme-linked immunosorbent assay (ELISA), applying horseradish peroxidase and chromogenic substrate, was employed to titrate the methamphetamine presented in the serum.

Subcutaneous injection

Male rabbits (2–3 kg) were employed to investigate the sustained release from methamphetamine-bound CM-chitin after subcutaneous injection in the back. MAEA-CM-chitin solution in saline (2 ml), containing 4·2 mg of MA, was injected subcutaneously into a 3 kg rabbit's back; a solution containing 2·8 mg of MA was used for a 2 kg rabbit (1·4 mg of MA/kg of rabbit body weight). MAEA solution (2 ml) in saline (3·7 mg) was also injected subcutaneously as a control. Blood (3 ml) was drawn from the ear vein at regular time intervals after the subcutaneous injection. The serum was fractionated by the method used previously (Takatori *et al.*, 1985) and stored in 0·05% sodium azide solution at −20°C after the inactivation of the complement by the heat treatment. A serum sample of 50 μl was applied in the ELISA method to estimate the MA derivative (oligomeric prodrug) content as reported previously (Takatori *et al.*, 1985). The titration of oligomeric prodrug in urine was also achieved by the ELISA method after extraction with hexane.

RESULTS AND DISCUSSION

Purity of MAEA

The purity of MAEA was confirmed by mass spectrometry by using the direct-injection (DI-MS) method after trifluoroacetylation of MAEA. Three major peaks were observed by the CI method. They were at 288 QM^+ m/z, the ion peak of the aggregated molecule, and at 197 and 91, resulting from β-cleavage. Four major peaks were observed by the EI

method; at 197 and 91 resulting from β-cleavage; and at 140 and 58 QM^+ m/z by tertiary-amine cleavage. TLC in a 30:7:7:1 (v/v) chloroform:methanol:acetic acid:water system showed a single spot of MAEA (R_f, 0·22).

Preparation of MAEA-CM-chitin

The coupling reaction between MAEA and CM-chitin was achieved under similar conditions to those used for the MABA-CM-chitin (antigen) preparation. The amount of MA in the MAEA-CM-chitin molecule was estimated spectrophotometrically to be 1 mol of MA per 5 CM-GlcNAc residues. Since the behavior of lysozymic hydrolysis for MAEA-CM-chitin in aqueous solution (shown in Fig. 1) was almost identical with that for CM-chitin, the similar susceptibility of MAEA-CM-chitin toward lysozyme as CM-chitin was suggested.

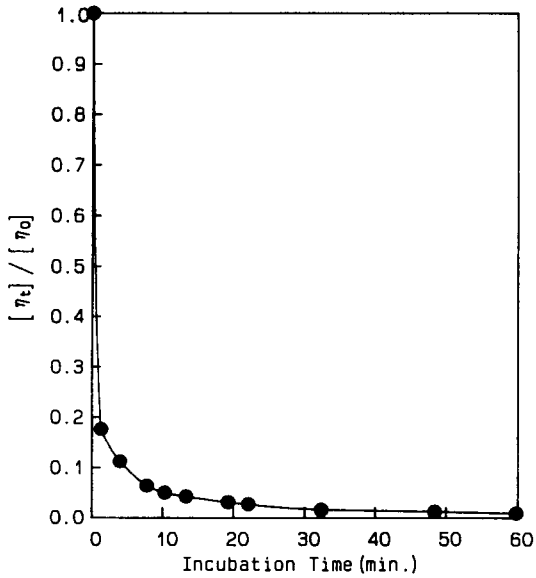


Fig. 1. Progress with time of lysozymic hydrolysis of drug-bound CM-chitin.

Antibody productions by MABA- and MAEA-CM-chitins

The induction of antibody production by MAEA-CM-chitin was significantly lower than that by MABA-CM-chitin in the presence of immunoadjuvant as shown in Fig. 2. It was also found that MAEA-CM-chitin induced very little antibody in the absence of immunoadjuvant. No

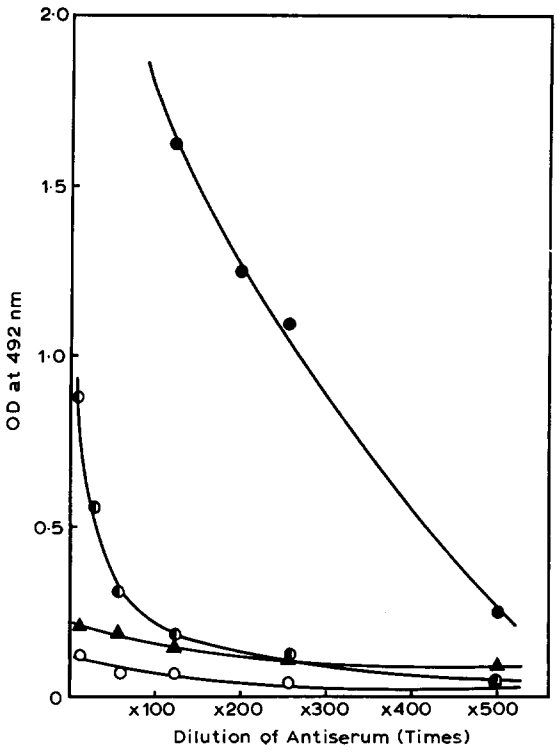


Fig. 2. Immunoadjuvant effect and dependence of chain length of spacer on the induction of antibody for methamphetamine. (3 months after first immunization): —●—, in the presence of Freund's complete adjuvant (chain length of spacer; C_4); —○—, in the presence of Freund's complete adjuvant (chain length of spacer; C_2); —▲—, in the absence of Freund's complete adjuvant (chain length of spacer; C_4); —○—, control.

antibody production was observed in the immunization of CM-chitin under the present experimental conditions. These results suggest that CM-chitin is expected to have an advantage as a sustained-release drug carrier. On the other hand, the antibody induced by the immunization of MABA-CM-chitin in the presence of immunoadjuvant is expected to be a highly sensitive detector of MA derivative released into blood. Thus the working curve was prepared to detect secreted MAEA-CM-GlcNAc residue after the hydrolysis with glycosidase as shown in Fig. 3 by applying the ELISA method. As shown in the working curve, the minimum sensitivity for MA detection was 0·5 ng/50 μl of blood.

Sustained release of MA into serum

A sample of MAEA-CM-chitin was dissolved in 2 ml of saline and then injected subcutaneously into rabbits of 2–3 kg body weight (1·4 mg of

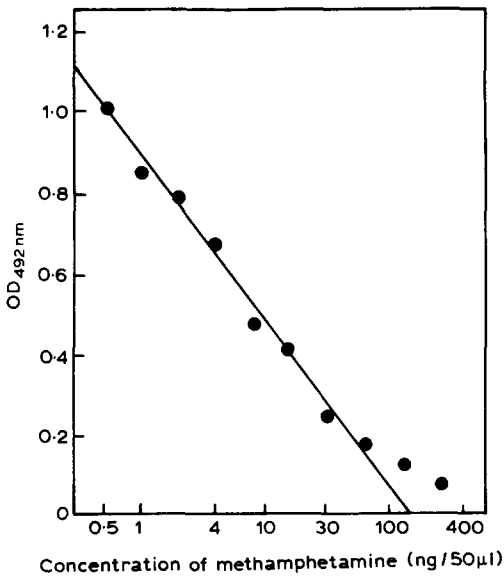


Fig. 3. Working curve for the detection of methamphetamine by applying the ELISA method.

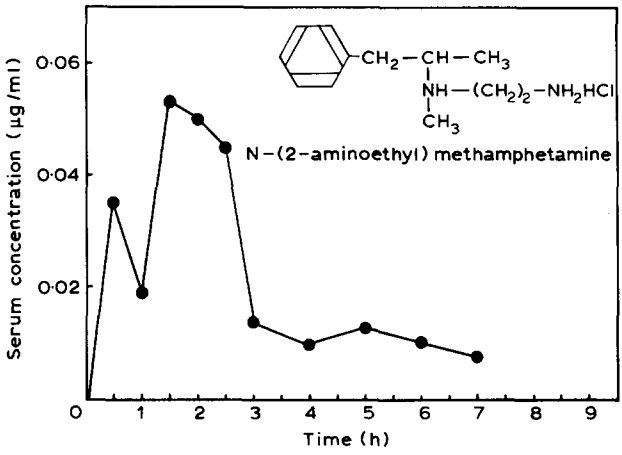


Fig. 4. Progress with time of release of MAEA into serum after subcutaneous injection of MAEA saline solution; 3·7 mg of MAEA was injected.

MA/kg of rabbit body weight). MAEA hydrochloride (3·7 mg) in 2 ml of saline solution was also injected as a standard (1·4 mg of MA/kg). A 3-ml sample of blood was drawn from the ear vein at each time interval, and the serum was separated to estimate the concentration of MA derivative by the ELISA method. The results are shown in Fig. 4 for MAEA

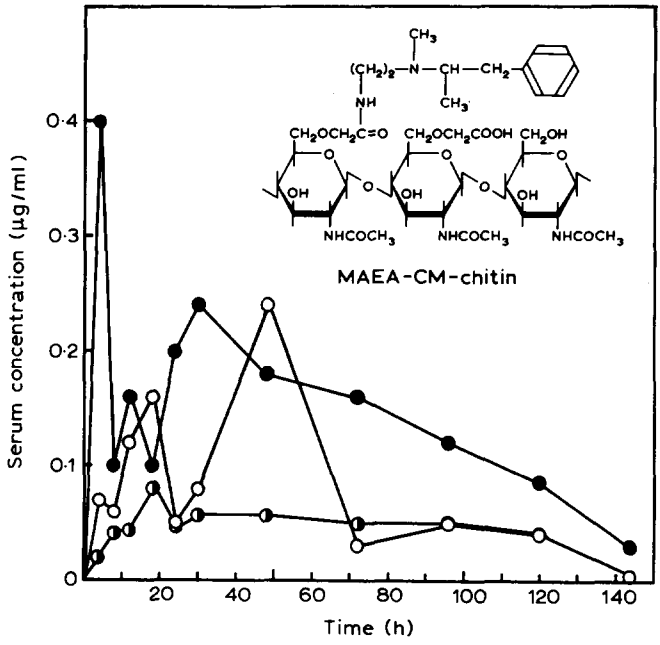


Fig. 5. Progress with time of release of methamphetamine derivative into serum after subcutaneous injection of MAEA-CM-chitin saline solution; —●—, 4·2 mg of MA containing polymer was injected; —○—, —◐—, 2·8 mg of MA containing polymer was injected.

hydrochloride and Fig. 5 for MAEA-CM-chitin, respectively. The MAEA oligosaccharide was released slowly after the biodegradation of MAEA-CM-chitin and maintained at a significant level in serum for more than 120 h in this experiment. As the blood level of MAEA (model of hydrolysate) was out of the range of detection within 7 h after the injection, probably owing to rapid metabolism, 6-*O*-CM-chitin was revealed to be a suitable drug carrier for controlled release. Since subcutaneous injection of an equivalent amount of MA was fatal to the rabbit within 1 h, MAEA oligosaccharide and MAEA are considered to be ineffective prodrugs. Thus, MAEA oligosaccharide in hydrolysate should be designed to release MA by enzymatic hydrolysis *in vivo* in future.

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