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# CONJUGATE OF NICOTINE AND COTININE TO BOVINE SERUM ALBUMIN

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Summary: A new simple method for the conjugation of nicotine and cotinine to protein has been developed. The pyridine alkaloids were coupled with ethyleneimine to form their aminoethylpyridinium derivatives, which were conjugated to bovine serum albumin (BSA) in the presence of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate. About forty molecules of S-nicotine and thirty-five of S-cotinine per molecule of starting BSA were respectively recognized to be present in the conjugates by determining the liberated aminoethylpyridinium compounds after hydrolysis.

Radioimmunoassays for nicotine and one of its major mammalian metabolites, cotinine, have been reported from three laboratories (1-3). The results have shown that as low as a few ng of nicotine or cotinine in physiological fluid can be determined radioimmunologically by using their antibodies. Some changes in nicotine and cotinine content in human urine and blood after smoking were estimated by the method (1,4). In these cases, however, the preparation of the antigens, the conjugates between protein and either nicotine or cotinine, seems to be rather troublesome for the immunologists unfamiliar to organic synthesis, requiring several synthetic steps. To make radioimmunoassays of nicotine and its metabolites easily available in any physiological laboratories, a new simple method to conjugate nicotine and cotinine to BSA via aminoethylpyridinium derivatives was developed and this paper deals with the results. The reaction schemes for each conjugate (I and II) are illustrated in Fig. 1.

A preliminary experiment carried out at the Kyoto Hospital of our corporation showed that anti-nicotine and anti-cotinine sera which were highly specific and sensitive to nicotine and cotinine respectively were produced in rabbits by the immunization with the conjugates. For example, 1 ng of cotinine could be detected radioimmunologically by the anti-cotinine sera at a final dilution of 1 : 10000. Advanced results of the immunization and radioimmunoassay will be presented later.

## SYNTHETIC PROCEDURE

S-1-(β-Aminoethyl)-nicotinium chloride dihydrochloride. S-Nicotine (8.11 g; 0.05 mol) was dissolved in 20 ml of 6N-HCl (f=1.03) in an ice bath. To the solution was added 2.15 g (0.05 mol) of ethyleneimine in 10 ml of

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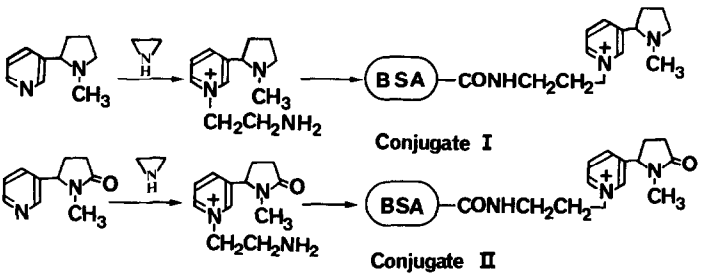


Fig. 1

distilled water dropwise under stirring and the solution was left for three days at room temperature. The reaction mixture was evaporated in vacuo to a syruppy mass, which was dissolved in 20 ml of the upper layer of a solvent system of n-butanol - acetic acid - water (4 : 1 : 5). The solution was placed onto the top of a cellulose powder column (5$\phi$ x 35 cm), which was previously equilibrated with the upper layer of the same solvent system. Elution was carried out using the same solvent system and the eluate was collected in 17 g fractions. Fractions containing uv absorptive and ninhydrin positive matter (Fr 98-190) were combined and concentrated in vacuo. To the residue was added 100 ml of N-HCl and the solution was concentrated in vacuo to dryness. The residue was freed of remaining HCl by redissolution in distilled water, and subsequent drying, and finally dried over KOH in vacuum desiccator, giving 11.6 g of hygroscopic amorphous solid: nmr ($D_2O$) $\delta$2.2-2.7 (m, 3H), 2.7-2.9 (m, 1H), 2.98 (s, 3H), 3.4-3.7 (m, 1H), 3.82 (t, 2H), 3.8-4.2 (m, 1H), 4.7-5.0 (m, 1H), 5.13 (t, 2H), 8.38 (m, 1H), 8.92 (d, 1H), 9.14 (d, 1H), 9.33 (s, 1H); $[\alpha]_D^{23}$ +11.1° (c=6.23 in water); uv in 0.2 M borate buffer (pH 10.5) $\lambda_{max}$ 265 nm ($\varepsilon$=3910); mp 200-201 °C (picrate). Anal. Calcd for $C_{30}H_{28}N_{12}O_{21}$ (picrate): C,40.37; H,3.16; N,18.83 %. Found: C,40.50; H,3.14; N,18.72 %.

Conjugation of BSA with S-1-($\beta$-Aminoethyl)-nicotinium chloride dihydrochloride. S-1-($\beta$-Aminoethyl)-nicotinium chloride dihydrochloride (950 mg, 3 mmol) was dissolved in 20 ml of water and pH of the solution was adjusted to 5.0 with 0.5 N NaOH. To the solution was added 680 mg of BSA, and pH of the solution was adjusted to 4.8. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate, 6.34 g, was added to the solution in three portions under stirring. The solution was adjusted to pH 4.8 after each addition. After being left overnight at room temperature, the solution was filtered through sintered glass disk and the filtrate was exhaustively dialyzed successively against 0.1 M NaCl, and distilled water, and then lyophilized (yield 910 mg).

S-1-($\beta$-Aminoethyl)-cotininium chloride hydrochloride. S-Cotinine (3.52 g, 0.02 mol), prepared by the method of Pinner (5), was dissolved in 40 ml of N-HCl (f=1.003). To the solution was added dropwise a solution of ethyleneimine in water (0.861 g, 0.02 mol in 10 ml). After being left for three days at room temperature, the reaction mixture was concentrated in vacuo to a viscous syrup, which was then suspended in 10 ml of the upper layer of n-butanol - acetic acid - water (4 : 1 : 5). The suspension was placed onto the top of a cellulose powder column (5$\phi$ x 40 cm) which was previously equilibrated with the upper layer of n-butanol - acetic acid - water (4 : 1 : 5). Chromatography was carried out using the same solvent mixture as the eluant and the eluate was collected in 15 ml fractions.

Fractions 104-200 which contains only the main reaction product (uv absorption and ninhydrin positive) were combined. After the addition of 60 ml of N-HCl, the combined solution was evaporated in vacuo to dryness. The residue was freed of HCl and acetic acid by redissolution in distilled water and subsequent drying, and finally dried over KOH in vacuo, giving 4.8 g of hygroscopic amorphous solid: nmr ($D_2O$) δ2.4-3.02 (m, 5H), 2.76 (s, 3H), 3.79 (t, J6.5Hz, 2H), 5.80 (t, J6.5, 2H), 8.24 (m, 1H), 8.62 (d, 1H), 9.00 (d, 1H), 9.03 (s, 1H); $[\alpha]_D^{25}$ -27.6° (c=10.1 in water); uv in 0.2 M borate buffer (pH 10.5) $\lambda_{max}$ 266 nm (ε=4530); mp 92-95 °C (picrate). Anal. Calcd for $C_{24}H_{23}N_9O_{15}$ (picrate): C,42.55; H,3.42; N,18.61 %. Found: C,43.28; H,3.65; N,18.11 %.

Conjugation of BSA with S-1-(β-Aminoethyl)-cotininium chloride hydrochloride. S-1-(β-Aminoethyl)-cotininium chloride hydrochloride (880 mg, 3 mmol) was dissolved in 10 ml of distilled water and pH of the solution was adjusted to 5.3 with N-NaOH. To the solution was added 680 mg of BSA, and pH of the solution was adjusted to 4.75. By the same procedure as described in the case of the nicotinium compound, the conjugation of BSA and the cotininium compound and isolation of the resultant conjugate were carried out (yield 718 mg).

## CELLULOSE POLYACETATE ELECTROPHORESIS

On cellulose polyacetate electrophoresis in Tris - barbital - sodium barbital buffer pH 8.8 at 80 V for 30 min., both conjugates migrated toward anode, whereas BSA migrated toward cathode. At pH 8.8, it is known that BSA is negatively charged, but the nicotine and cotinine derivatives are positively charged. This shows that the total charge of these conjugates are positive. This result indicates that the nicotine and cotinine derivatives were covalently bound to BSA.

## THE EXTENT OF CONJUGATION

An estimation of the content of aminoethylpyridinium derivative in each conjugate was carried out as follows; each conjugate was hydrolyzed with 6N-HCl at 110 °C for 24 hours and the corresponding hydrolyzate was evaporated in vacuo. After dissolving the residue in 0.2 M borate buffer pH 10.5, an aliquot of the solution was subjected to a high pressure liquid chromatography (resin, Beckman 150A; column size, 3ϕ x 300 mm; flow rate, 75 ml / hr; eluant, 0.2 M borate buffer pH 10.5). S-1-(β-Aminoethyl)-cotininium hydroxide or S-1-(β-aminoethyl)-nicotinium hydroxide was detected by monitoring at 265 nm.

On the other hand, amino acid compositions of both conjugates were determined after the same hydrolysis procedure as mentioned above by a Beckman Amino Acid Analyzer Model 120 C. In these experiments, 0.421 μmol of the S-nicotine derivative and 0.449 μmol alanine were derived from one mg

conjugate I, whereas 0.326 μmol of the S-cotinine derivative and 0.433 μmol alanine from one mg conjugate II. Since a mole of BSA contains 46 mol of alanine, the extent of conjugation of each conjugate was calculated as follows.

Conjugate I

$$46 \times \frac{0.421}{0.449} = 43.2 \text{ mol of nicotine deriv. / one mol of BSA}$$

Conjugate II

$$46 \times \frac{0.326}{0.433} = 34.6 \text{ mol of cotinine deriv. / one mol of BSA}$$

References

1) J.J. Langone, H.B. Bjika and H.V. Vunakis, Biochemistry, 12, 5025 (1973).
2) H. Matsushita, M. Noguchi and E. Tamaki, Biochem. Biophys. Res. Commun., 57, 1006 (1974).
3) A. Castro and I. Prieto, Biochem. Biophys. Res. Commun., 67, 583 (1975).
4) A. Matsukura, N. Sakamoto, H. Imura, H. Matsuyama, T. Tamada, T. Ishiguro and H. Muranaka, Biochem. Biophys. Res. Commun., 64, 574 (1975).
5) A. Pinner, Chem. Ber., 26, 292 (1893).