CONJUGATE OF BOVINE SERUM ALBUMIN WITH NICOTINE

Hajime Matsushita, M. Noguchi and E. Tamaki
Central Research Institute, The Japan Tobacco & Salt Public Corporation, 6-2 Umegaoka, Midori-ku, Yokohama, Kanagawa 227, Japan

Received March 4,1974

Summary: The preparation and characterization of a nicotine-albumin conjugate are reported. The nicotine derivative, 6-(p-aminobenzamido)nicotine, which was coupled to bovine serum albumin(BSA), was synthesized by the following sequence; l-nicotine→6-aminonicotine→6-(p-nitrobenzamido)nicotine→ 6-(p-aminobenzamido)nicotine. Thirty molecules of conjugated nicotine per molecule of BSA were distinguished by the determination of 6-aminonicotine after hydrolysis. The distribution of the conjugated nicotine on BSA was examined by the stoichiometry of the composed amino acids before and after conjugation, and also by the electrophoretic behavior of the conjugated BSA on a polyacrylamide gel.

The conventional assay methods of nicotine in biological fluids are usually time-consuming and laborious procedures and often cannot be applied to small samples(1). The radioimmunological methods which have been developed in the assay of morphine(2-4), lysergide(5-6) or steroids(7-8) seem to be useful for the simple and sensitive nicotine analysis. The present investigation was initiated to develop an immunological assay system for the detection of minute quantities of nicotine. To obtain an antibody specific for nicotine, the use of macromolecules conjugated with nicotine as antigens is thought to be necessary, because it has been observed that nicotine has very low antigenisity by itself. As nicotine has no functional group bindable to macromolecules, it is necessary to introduce some functional groups into it. To preserve the antigenic site specific for nicotine, the modification of the pyrrolidine ring moiety is not recommended, since the structural character of nicotine which differs from most of nicotine metabolites is the N-methyl pyrrolidine ring(9). In addition, it may be also desirable that the combined nicotine molecules are not buried in the three dimensional structure of the conjugate, otherwise the conjugate would have less effectiveness. Thus, the linkage between nicotine and a macromolecule should be fairly rigid and appropriately long. On the view of these considerations, 6-(p-aminobenzamido)-nicotine was synthesized and coupled to BSA. This paper deals with the preparation and the characterization of the nicotine-albumin conjugate.

A preliminary experiment carried out at the Kyoto Hospital of our corporation showed that antibodies highly sensitive to nicotine were produced in rabbits by the immunization with the conjugate. For example, as low as

0.0006 nmole nicotine could be detected by the antiserum radioimmunologically. Advanced results of the immunization and the radioimmunoassay will be presented in another communication(10).

SYNTHETIC PROCEDURE

The nicotine-BSA conjugate was prepared as shown in Fig. 1.

6-(p-Nitrobenzamido)nicotine. 6-Aminonicotine was prepared according to the direction of Tschitschibabin and Kirssanow(11). To a solution of 6-aminonicotine(1.77 g) in ether(30 ml) was added sodium carbonate(4.15 g) suspended in water(10 ml). To the mixture, p-nitrobenzoyl chloride(4.64 g) in ether(15 ml) was added dropwise under vigorous stirring at 0^0C. The precipitate was collected and washed with cold water. The yield was 1.48 g (45.4 %) after drying in vacuo. Recrystallization from ethanol gave yellow flat plates: mp, 176-177^{0}C; nmr($CDCl_3$) δ1.70-2.77, 3.15-3.84(m,7H), 2.41(s, 3H), 7.94(d,1H,J9.0), 8.25(d,2H, J8.0), 8.34(s,1H), 8.48(d,2H,J8.0), 8.56(d, 1H,J9.0), 10.46(s,1H); mass m/e 326(M^+), 325, 297, 176, 150, 84; ir(nujole mull) 3350, 3050, 2750, 1658, 1660, 1520, 1405, 1345, 1310, 832 cm^{-1}. Anal. Calcd for $C_{17}H_{18}N_4O_3$: C,62.56; H,5.56; N,17.17. Found: C,62.58; H,5.91; N,17.31.

6-(p-Aminobenzamido)nicotine. 6-(p-Nitrobenzamido)nicotine(326.4 mg) in ethanol(5 ml) was reduced catalytically with Raney Nickel at room temperature. Recrystallization from acetone-ether gave 275.7 mg(93.0 %) of white needles: mp, 150-152^{0}C; nmr($CDCl_3$) δ1.80-2.80, 2.97-3.70(m,7H), 2.48(s,3H), 4.43(s, 2H), 6.90(d,2H,J8.0), 7.94(d,1H,J9.0), 7.98(d,2H,J8.0), 8.38(s,1H), 8.55(d, 1H,J9.0), 9.00(s,1H); mass m/e 296(M^+), 295, 268, 176, 120, 84; ir(nujole mull) 3370, 3180, 2780, 1660, 1600, 1565, 1408, 1305, 838 cm^{-1}. Anal. Calcd for $C_{17}H_{20}N_4O_1$: C,68.89; H,6.80; N,18.91. Found: C,68.91; H,6.80; N,18.94.

Conjugation of BSA with 6-(p-aminobenzamido)nicotine. Diazotized 6-(p-aminobenzamido)nicotine was coupled to BSA according to the method of Tabachnick et al.(12) for the preparation of the azoproteins. Sodium nitrite (6.9 mg) was added to a solution of 6-(p-aminobenzamido)nicotine(29.6 mg in 5 ml of 0.1N HCl) with continuous stirring in an ice-salt bath. After an hour, the diazotized 6-(p-aminobenzamido)nicotine solution was added to a solution of BSA(68.0 mg in 5 ml of 0.2N borate buffer, pH 9.3) over a period of 30 minutes with continuous stirring in an ice bath. During the coupling reaction, the pH was kept between 9.0-9.5 with 0.5N NaOH. After being kept at 0^0C overnight, the reaction mixture was adjusted to pH 7 and was dialyzed against 0.1M NaCl for 5 days with daily changes of the dialysis fluid. The dialysis was continued for additional two days against distilled water. The solution was concentrated with a collodion bag and the concentrate was lyophilized (yield, 79.6 mg).

Table 1. Amino Acid Compositions of BSA and the Conjugate expressed as relative ratios to alanine (mole/mole)

Amino Acid	B S A	Conjugate
Lysine	0.8 (40.3)*	0.4 (21.2)
Histidine	0.25 (12.7)	0.1 (7.2)
Arginine	0.4 (20.8)	0.4 (19.7)
Aspartic Acid	1.2 (62.0)	1.2 (61.3)
Threonine	0.7 (36.6)	0.7 (36.0)
Serine	0.6 (29.7)	0.6 (30.9)
Glutamic Acid	2.1 (106.9)	2.0 (105.5)
Proline	0.6 (32.0)	0.5 (28.0)
Glycine	0.4 (18.1)	0.5 (24.2)
Alanine	1.0 (51.7)	1.0 (51.7)
Valine	0.7 (36.9)	0.7 (38.0)
Isoleucine	0.3 (14.2)	0.3 (14.1)
Leucine	1.3 (67.7)	1.3 (67.2)
Tyrosine	0.4 (20.5)	0.2 (11.9)
Phenylalanine	0.6 (29.4)	0.6 (28.5)

* The figures in blackets show the numbers of respective amino acid residue per molecule of each peptide.

THE EXTENT OF CONJUGATION

The conjugate was hydrolyzed with 6N HCl at 110°C for 24 hours and the hydrolyzate was evaporated *in vacuo*. After dissolving the residue in 0.2N citrate buffer(pH 2.2), an aliquot of the solution was subjected to a high pressure liquid chromatography (resin, Beckman 150-A; column size, 3x300 mm; flow rate, 70.0 ml/hr; eluant, 0.2N borate buffer, pH 10.5). 6-Aminonicotine, detected by monitoring at 294 nm, was eluted out after 19 minutes. On the contrary, BSA hydrolyzate gave no peak corresponding to 6-aminonicotine. From 5.273 mg of the conjugate, 0.515 mg of 6-aminonicotine·2HCl was recovered. No destruction of the authentic 6-aminonicotine was observed during the hydrolysis procedure. Calculated from the molecular weight of BSA (68,000), it was clarified that 30 moles of nicotine was conjugated to a mole BSA showing the molecular weight of the conjugate to be 77,200.

AMINO ACID ANALYSIS

Amino acids in peptide moiety were determined after the same hydrolysis procedure as mentioned above by a Beckman Amino Acid Analyzer Model 120C.

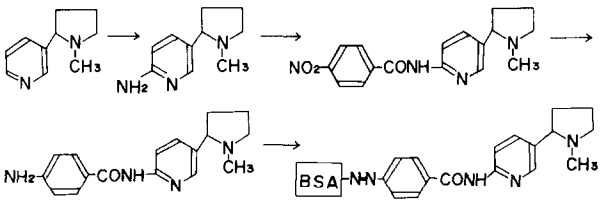

Fig. 1

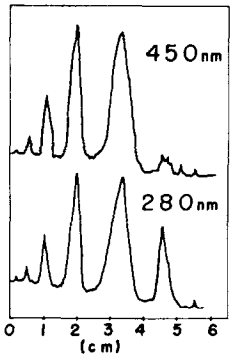

Fig. 2. Densitometric tracing of electrophoretic patterns

Amino acid compositions of both peptides are shown in Table 1, where the numbers are calculated as the relative molar ratio to alanine. As both peptides were suspected to contain identical amount of alanine per mole, the molecular weight of the conjugate was estimated by a quantitative analysis. In the experiment, 0.181 μmole alanine was derived from 0.238 mg BSA, whereas the same amount of alanine from 0.273 mg of the conjugate. Since the molecular weight of BSA is 68,000, the average molecular weight of the conjugate was calculated to be 78,000.

The probable numbers of individual amino acids in BSA and conjugate are listed in the blackets of Table 1. It has been known that lysine, histidine and tyrosine are the amino acids which combine with diazo compounds easily(13). As expected, these amino acids were greately reduced during the conjugation procedure, accounting for 33.2 moles reduction in total. The average molecular weight of the conjugate was calculated as 78,200 by summation of 68,000(BSA) and 10,200(33.2 equivalents of azobenzamido nicotine residue), suspecting that all of the reduced amino acid moieties were replaced by the respective nicotine-amino acid conjugate. The molecular weight, 78,200, was closely coincident with that calculated from the weight base(78,000).Thus, it was proved that the conjugated nicotine was almost exclusively located on the three amino acids. In addition to the three amino acids, glycine and proline contents were also changed during the procedure. These changes might be introduced by the hydrolysis. The glycine increase was demonstrated to be due to the overlapping of the degradation products of azo compounds of lysine and histidine, since they were also derived from the authentic azo compounds. The reason of proline decrease was still unknown.

The molecular weight of the conjugate and the extent of the conjugation obtained by amino acid analysis were closely similar to those calculated from the results of the high pressure liquid chromatography. Thus, the average number of the conjugated nicotine per mole of the conjugate was decided to be 30-33.

ELECTROPHORESIS

Electrophoresis was performed according to Davis(14) using 7.5 % polyacrylamide gel. In the case of the conjugate, five reddish yellow bands were observed on the gel. As shown in Fig. 2, both patterns obtained by scanning at 280 nm and 450 nm respectively were identical. The result suggested that the nicotine derivative was covalently bound to BSA.

ACKNOWLEDGMENTS

We wish to thank Drs. N. Kawashima, S. Mizusaki and M. Noma for many helpful discussions and suggestions during this work.

REFERENCES

1. Willits, C.O., Swain, M.L., Connelly, J.A., and Brice, B.A. (1950) Anal. Chem. 22, 430-433.
2. Spector, S., and Parker, C.W. (1970) Science 168, 1347-1348.
3. Adler, F.L., and Liu, C.T. (1971) J. Immunol. 106, 1684-1685.
4. Wainer, B.H., Fitch, F.W., Rothberg, R.M., and Fried, J. (1972) Science 176, 1143-1145.
5. Rigby, A.T., Sher, S.E., and Kelley, P.R. (1972) Science 181, 165-166.
6. Loeffer, L.J., and Pierce, J.V. (1973) J. Pharm. Soc. 62, 1817-1820.
7. Erlanger, B.F., Borek, F., Beiser, S.M., and Lieberman, S. (1957) J. Biol. Chem. 228, 713-727.
8. Erlanger, B.F., Borek, F., Beiser, S.M., and Lieberman, S. (1959) J. Biol. Chem. 234, 1090-1098.
9. McKennis, J.H.(1965) Tobacco Alkaloids and Related Compounds, pp53-74, Oxford, Pergamon Press.
10. Imura, H., Matsuyama, H., Ishiguro, T., and Muranaka, H., in preparation.
11. Tschitschibabin, H.E., and Kirssanow, A.W. (1924) Chem. Ber. 57, 1163-1168.
12. Tabachnick, M., and Sobotka, H. (1961) J. Biol. Chem. 236, 2250-2253.
13. Tabachnick, M., and Sobotka, H. (1959) J. Biol. Chem. 234, 1726-1730.
14. Davis, B.J. (1964) Ann. N. Y. Acad. Sci. 121, 404-427.