RADIOIMMUNOASSAY OF NICOTINE

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Summary : A sensitive and specific radioimmunoassay of nicotine was developed using antisera raised against 6-(p-aminobenzamido) nicotine coupled to bovine serum albumin. Inhibition studies with various nicotine analogues revealed that the antisera are highly specific for both the N-methylpyrrolidine ring and the pyridine ring of nicotine, and especially for the structural changes of the former. The use of these antisera in an assay of nicotine in biological fluids is desirable, since the pyrrolidine ring of nicotine is first metabolized in vivo and antibodies must, therefore, discriminate nicotine from other nicotine metabolites.

To study the pharmacological effects of smoking on the metabolic and hormonal milieu of man, it is desirable to use a method that is capable of measuring the nicotine level in various biological fluids with great sensitivity and specificity. Although several methods (1-2) to measure nicotine have been developed, they are not sensitive and simple enough to follow changes in blood levels of nicotine. Gas-liquid chromatography (3-4) has been reported to be quite sensitive, but this assay technique is still complicated and relatively time-consuming, since it requires the extraction procedures of nicotine from blood prior to analysis. Langone et al. (5) recently reported a radioimmunoassay of nicotine. The present communication describes the production of highly specific antisera to nicotine in rabbits and their use in a radioimmunoassay of this compound.

Materials and methods

L-nicotine and other analogues of nicotine used in the present study were supplied by the Central Research Institute, Japan Tobacco & Salt Public Corporation. [^{3}H]-nicotine-d-bitartrate with specific activity of 520 mCi/mmol was purchased from the Radiochemical Centre, Amershm, England.

Anti-nicotine antisera were prepared in rabbits by immunizing with 6-(p-aminobenzamido) nicotine conjugated to bovine serum albumin (BSA) (Fig. 1). Details of the procedure for preparing the conjugate were previously described by Matsushita et al. (6). Two mg of the nicotine-BSA conjugate were dissolved in 0.5 ml of saline and emulsified with an equal volume of complete Freund's adjuvant. The animals were given weekly intradermal injections of 0.25 ml of the emulsion into each toepad for the first 4 weeks and once every 2 to 4 weeks thereafter. They were bled from the central ear artery 10 days after the last immunization.

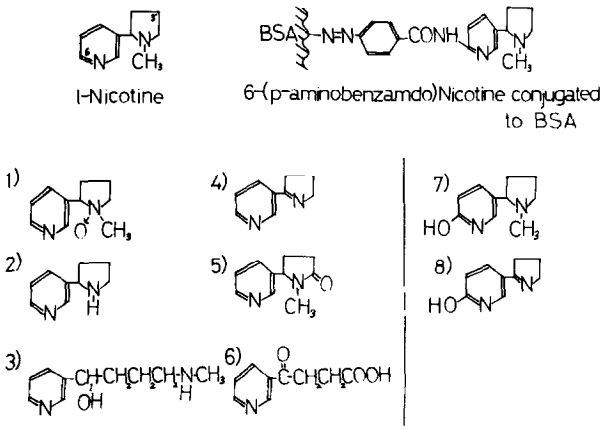

Fig. 1. Nicotine, nicotine-BSA conjugate and analogues of nicotine used. 1) oxynicotine 2) nornicotine 3) 4-metylamine-1-(3-pyridyl)-1-butanol 4) myosmine 5) l-cotinine 6) γ-(3-pyridyl)-γ-oxobutyric acid 7) 6-hydoxynicotine 8) 6-hydoxymyosmine

For the inhibition experiments, 0.1 ml of saline containing different amounts of nicotine or analogues of nicotine and 0.4 ml of buffer (0.1 % bovine serum albumin and 0.05 % bovine γ-globulin -0.05M phophate-pH 7.4) containing approximately 5000 cpm of [^{3}H]-nicotine and 2.5 µl of an antiserum (a 1:200 final dilution) were incubated at 4 C overnight. After incubation, separation of bound from free was accomplished by the addition of 0.5 ml saturated ammonium sulfate solution. After centrifugation at 3000 rpm for 20 min, an aliquot of the supernatant (free fraction) was transferred to a vial, to which was added scintilation cocktail containing toluene (1000 ml), PPO (5 g) and methanol (20 ml). The radioactivity was counted in a Packard liquid scintilation spectrometer. The percent free fraction (%F) was calculated.

For the recovery experiments, 0.1 ml of pooled plasma with different amounts of nicotine added was mixed with 0.4 ml of the buffer containing [^{3}H]-nicotine and antiserum. To construct a standard curve, 0.1 ml of nicotine-free plasma, prepared by charcoal treatment, was added to the incubation tubes to correct the error caused by the non-specific effect of plasma.

Results and discussion

The lower limit of sensitivity of our assay is 0.5 ng. Addition of 5 ng nicotine causes a 50 % reduction of the initial binding (Fig. 2). The sensitivity of our assay is similar to that of Langone et al. (5) who reported that the lower limit of detection was about 350 pg while 3.2 ng of nicotine was required for 50 % inhibition. Displacement curves for several analogues of nicotine tested at different concentrations are shown in figures 3a and 3b. Data on cross-reactivity of these compounds with nicotine are presented in table 1. The antiserum used seem to be directed toward both the N-methylpyrrolidine and the pyridine ring

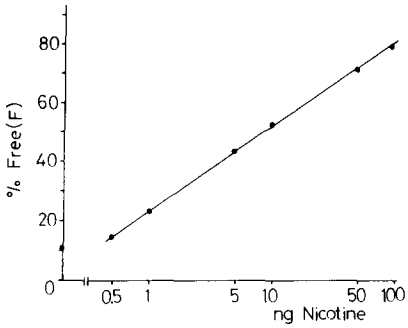

Fig. 2. A typical standard curve of radioimmunoassay of nicotine. Per cent free fraction (%F) of [^{3}H]-nicotine was plotted on the ordinate and ng of unlabeled nicotine added on the abscissa.

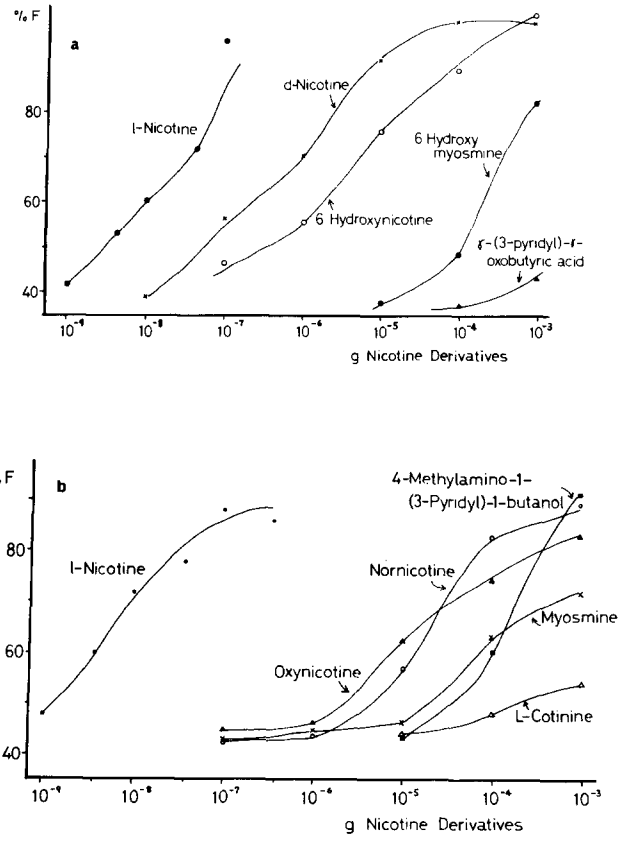

Fig 3a and 3b. Cross-reactivity of various analogues of nicotine in the radioimmunoassay of nicotine.

Table 1. Cross-reactivity of various derivatives of nicotine

Derivatives	% Reactivity*	
l-nicotine	100	
d-nicotine	5.9	
6-hydroxynicotine	1.7	(0.02)**
6-hydroxymyosmine	0.48	
oxynicotine	0.04	(0.54)
nornicotine	0.03	(0.90)
4-metylamine-1-(3-pyridyl)-1-butanol	0.006	
myosmine	0.004	
l-cotinine***	0.0006	(<0.4)
γ-(3-pyridyl)-γ-oxobutyric acid***	0.0001	(0.01)

*Amount of analogues of nicotine giving 50 % inhibition in the nicotine-antinicotine reaction was compared to that of nicotine taken as 100 %.
**The figures in parentheses were calculated on the basis of data presented on the report of Langone et al. (5).
***Cross-reactivity was compared at the lower inhibition in the nicotine-antinicotine reaction as shown in Fig. 3a and 3b.

structure of nicotine, since the structural modifications of both rings of nicotine molecule rendered the derivatives less reactive to antiserum (Table 1). We found our antiserum to be more specific for the pyrrolidine ring than the antisera used by Langone et al. (5). In our assay system oxynicotine, nornicotine and l-cotinine in which a modification is done on the pyrrolidine ring (Fig. 1), show only cross-reactivities of 0.04 %, 0.03 % and 0.006 % of nicotine respectively. With their assay system, however, cross-reactivities of these compounds were calculated to be 0.54 %, 0.90 % and less than 0.4 % of nicotine, respectively, (5) (Table 1). In contrast, 6-hydroxynicotine, an analogue with a hydroxyl residue side chain on the pyridine ring, gave higher cross-reaction in our assay than in the method of Langone et al. (5) (1.7 % vs 0.02 % as shown in Table 1), indicating that their antisera were more specific for the pyridine ring. The difference in specificity of

Table 2. Recovery of nicotine added to pooled plasma and assayed by radioimmunoassay.

Addition	Nicotine found (ng/ml)	Recovery (%)
Pooled plasma	7 ± 1.6* (11)**	
Pooled plasma + 10 ng/ml of nicotine	15 ± 0.8 (14)	80
Pooled plasma + 100 ng/ml of nicotine	97 ± 9.7 (14)	90

*Values represent the means ± SE of repeated determinations
**The numbers of samples are shown in parentheses

these antisera is not surprising. We used as an antigen 6-(p-aminobenzamido) nicotine coupled to BSA, in which the pyrrolidine ring was distal from the point of attachment to protein (Fig. 1) and therefore became antigenic. Langone et al. (5) used trans-3-succinylmethylnicotine conjugated to a protein where the pyridine ring was away from the linkage between nicotine and a protein and probably became an antigenic determinant. The use of antiserum more specific for the pyrrolidine ring in a radioimmunoassay of nicotine in biological fluids containing several nicotine metabolites appears to be desirable because of the fact that the pyrrolidine ring of nicotine is first metabolized in the body (7).

The preliminary experiment on recovery of nicotine added in pooled plasma shows fairly satisfactory results (Table 2), indicating that this specific and sensitive assay of nicotine can be applicable to the measurement of nicotine in blood.

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