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DETERMINATION OF NICOTINE IN ALLERGENIC EXTRACTS OF TOBACCO LEAF BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

BERNARD J. L. SUDAN* and CHRISTIAN BROUILLARD
P.O. Box 1951, 4001 Basle 1 (Switzerland)
CHRISTIANE STREHLER and HENRI STRUB
Ecole Nationale Supérieure de Chimie (Laboratoire de Synthèse et de Photochimie Organiques, CNRS LA 135), Université de Haute-Alsace, 68093 Mulhouse Cedex (France)
JOSEPH STERBOUL
Clinique Saint-Louis, 16, rue de Pékin, 90000 Belfort (France)
and
JEAN SAINTE-LAUDY
Laboratoire d'Allergologie et d'Immunologie, 5, Boulevard du Montparnasse 75006 Paris (France)
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SUMMARY

A high-performance liquid chromatographic (HPLC) system has been developed for the determination of nicotine and cotinine in allergenic extracts of tobacco leaf. This analysis showed eight allergenic extracts of tobacco (leaf and Mix) to have markedly different nicotine patterns. Cotinine, a photodegradation product of nicotine, was not detected.

INTRODUCTION

We have previously studied a familial dermatitis which resembles seborrhoeic dermatitis[1–5]. For all patients, the "*in vitro*" human basophil degranulation test (HBDT) of Benveniste[6] gave positive results with either the allergen, tobacco (leaf, Hollister-Stier) or the hapten, nicotine bound to human serum albumin (HSA). The detection of specific IgE antibodies by the radioallergosorbent test (RAST) was positive for the first author (B.J.L.S.) using allergenic extracts of tobacco leaf (S. B. Lehrer, New Orleans, U.S.A.), and the passive cutaneous anaphylaxis (PCA) results were positive with tobacco extracts (Bencard) and nicotine sulphate in the guinea-pig (A. L. de Weck, Bern, Switzerland), the rabbit and the mouse. The intra-cutaneous tests read after 15 min revealed positive reactions with allergenic extracts of tobacco leaf (Institut Pasteur).

In 1928, Karrenberg[7] described an anaphylactic reaction after application of a drop of nicotine (1/1,000,000) to a woman working in the tobacco industry and afflicted with a facial dermatitis. In 1975, we initiated a specific desensitization with allergenic extracts of tobacco leaf (Institut Pasteur) at a dilution of 1/1,000,000. The

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results were very satisfactory and total protection was achieved at a final concentration of 1/100 (tobacco leaf, Hollister-Stier) with a monthly subcutaneous injection of allergen (0.2 ml): HBDT results were negative and the dermatitis disappeared. However, after 13 months the dermatitis reappeared when the same allergenic extracts were used. Apparently the allergenic potency of the tobacco leaf extracts was reduced and we decided to determine the nicotine content of different allergenic extracts (Hollister-Stier and Institut Pasteur) by high-performance liquid chromatography (HPLC). Because allergenic extracts are exposed to light during the desensitization period, we postulated that nicotine is degraded by ultra-violet (UV) light.

Seventy years ago, Ciamician and Silber[8] reported the autooxidation of nicotine under the influence of light. At the same time, Custis[9] found, as the result of studying the effect of light on nicotine solutions, that there is a change in nicotine content when solutions of nicotine are exposed to sunlight: this change is more marked in the presence of air, which suggests an oxidation process; the ability to cause the change is limited to light of short wavelengths, UV light produces a change and the alkalinity of the solutions has no effect. Wada *et al.*[10] reported an oxidation of nearly 20% after a 4-week aeration of nicotine solutions at 30°C. The oxidation products were nicotinic acid, oxynicotine, nicotyrine, cotinine and myosmine. More recently, Hubert-Brierre *et al.*[11] demonstrated that irradiation of a methanolic solution of nicotine in the presence of methylene blue and oxygen gave nicotyrine (23%), cotinine (30%) and nicotine N-oxide (7%) (Fig. 1). Therefore, we have investigated the content of the major oxidation product of nicotine, cotinine, in eight different allergenic extracts of tobacco leaf and Mix (Table I).

In general, relevant HPLC studies have been concerned with the separation of either nicotine or cotinine, its major metabolite in urine[12,13] or plasma[13]. A normal-[12] or a reversed-phase[13] system has been used for these HPLC separations. HPLC has proved to be an ideal method for the determination of alkaloids of *Nicotiana tabacum*[14]. Recently, a reversed-phase HPLC procedure was used for the determination of nicotine in liquid formulations[15]. The present study reports the

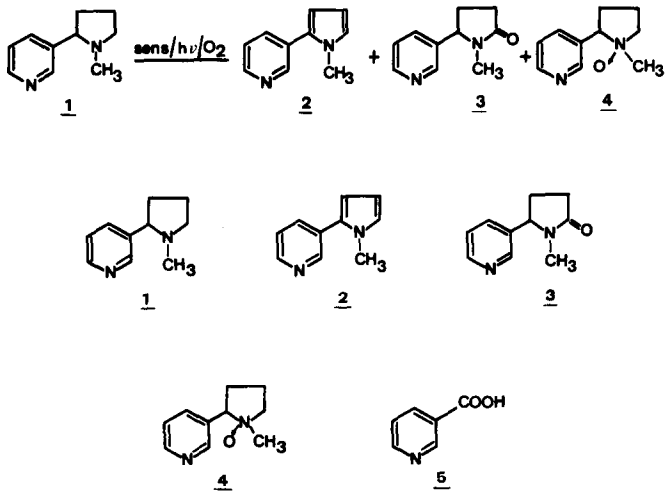

Fig. 1. Structures of nicotine (1), nicotyrine (2) cotinine (3), nicotine N-oxide (4) and nicotinic acid (5).

TABLE I

ALLERGENIC EXTRACTS OF TOBACCO LEAF AND MIX USED FOR DETERMINATION OF NICOTINE

Extract 1:	Tobacco leaf, 1/10 (w/v), Hollister-Stier Expiry date: 15.9.1980
Extract 2:	Tobacco leaf, 1/10 (w/v), Hollister-Stier Expiry date: 5.2.1983
Extract 3:	Tobacco leaf, 1/10 (w/v), Hollister-Stier Expiry date: 15.3.1986
Extract 4:	Tobacco leaf, 1/10 (w/v), Hollister-Stier Expiry date: 1.6.1986
Extract 5:	Tobacco Mix (pipe, cigarette, leaf, cigar), 1/200 (w/v), Hollister-Stier Expiry date: 2.12.1978
Extract 6:	Tobacco leaf, 22 mg/ml, Institut Pasteur Date of preparation*: May 1983
Extract 7:	Tobacco leaf, 10 mg/ml, Institut Pasteur Date of preparation*: May 1983
Extract 8:	Tobacco leaf, 8 mg/ml, Institut Pasteur Date of preparation*: May 1983

* Allergenic extracts were analysed about 10 weeks after the date of preparation.

analysis of nicotine and cotinine in freshly and expired allergenic extracts of tobacco (leaf and Mix).

EXPERIMENTAL

Determination of cotinine and nicotine

To determine the presence of cotinine and nicotine, gradient elution with the following solvent systems was employed: A, 20% methanol, 80% buffer pH 9.2 (0.02 *M* NH_4Cl–NH_4OH); B, 100% methanol. The gradient was from 5% B (24% methanol) during 2 min to 50% B (60% methanol) in 6 min.

Nicotine

To calculate the nicotine concentration, 4-chloroaniline was used as internal standard. The same amount of internal standard was added to both the calibration and the analysis samples, and the ratio between the internal standard and the sample remained the same. Gradient elution was carried out from 35% B (48% methanol) to 50% B (60% methanol) in 5 min, with the samples (Table I) diluted 10 times as described hereafter.

Apparatus

A Spectra-Physics 3500 B high-performance liquid chromatograph was equipped with a spectrophotometric detector Schoeffel Model 770. The detector was connected to a integrator Spectra-Physics SP 4100. A stainless-steel column (15 cm × 4.6 mm I.D.) packed with Ultrasphere C_8 (particle size 5 μm) was obtained from Beckman Altex. An injection loop of 10 μl was used. Detection of cotinine, nicotine and 4-chloroaniline was effected at 254 nm; O.D.: 0.02 for all solutions. The solvent was a mixture of ammoniacal buffer (0.02 *M* NH_4Cl–NH_4OH, pH 9.2) and methanol; flow-rate 1.2 ml/min.

Chemicals

Reference alkaloids. (−)-Nicotine and 4-chloroaniline were obtained from Fluka (Buchs, Switzerland) and cotinine from Roth-Sochiel (Lauterbourg, France).

Tobacco leaf extracts (Hollister-Stier). The tobaccos used for the tobacco leaf extracts from Hollister-Stier (Berkeley, CA, U.S.A.) are Pennsylvania, Burley, Bright and Mexican. The tobacco company would not reveal the details of the blending of various types and grades of tobacco[16]; therefore, the amounts of the various types are unknown. The exact method of extraction of the leaf material is also unknown. For regular process extracts (not acetone precipitated), 1/10 (w/v) means that each gram of raw source material, *i.e.*, tobacco leaf, was extracted with 10 ml of solution. The preservative was 50% glycerine. Dilutions were made with a solution containing 0.9% sodium chloride, 0.5% phenol and distilled water for specific desensitization.

Tobacco Mix extracts (Hollister-Stier). The tobacco Mix extracts comprised mixtures of leaf, cigar, cigarette and pipe tobacco. The concentration was 1/200 (w/v) and the exact method of extraction is again unknown. The preservative was 50% glycerine.

Tobacco leaf extracts (Institut Pasteur). The tobacco used for the allergenic extracts of tobacco leaf from Institut Pasteur was Kentucky, kindly supplied by Mr. Ch. Dietrich, Fabriques de Tabac Rinsoz & Ormont, Vevey, Switzerland. Extraction of this material was carried out by maceration of 20 g of dried tobacco leaves in 200 ml of an alkaline solution of Coca (0.5% sodium chloride, 0.275% sodium bicarbonate, 0.4% phenol and distilled water) during 48 h at 4°C. After shaking, the solution was filtered on a filter-paper (initial extract). To avoid the hazards of heat or chemical additives, membrane filters were used for sterilizing allergenic extracts.

Extract 6 (Institut Pasteur). Fifty millilitres of the initial extract were centrifuged (20,000 *g*) and sterilized with a 0.45-μm membrane filter. The content of the dried extract was estimated to be 22 mg/ml, without dialysis. The remaining volume of extract 6 was shaken and dialyzed simultaneously with distilled water at 4°C using a membrane filter 6000/8000 for preparation of extract 7.

Extract 7 (Institut Pasteur). At day 8 after preparation of the initial extract, the above concentrate from the membrane filter 6000/8000 was lyophilized and centrifuged. The allergenic extract was sterilized using a 0.45-μm membrane filter. The content of the dried extract was estimated to be 10 mg/ml after dialysis.

Extract 8 (Institut Pasteur). About 18 h after preparation of the initial extract, extract 8 was concentrated by lyophilization and then stored in 60 ml of distilled water. The extract was dialyzed at 4°C using a membrane filter 2000. During 3 days, distilled water was removed twice a day and a freshly solution of distilled water was added. Then the content of the membrane was separated and the allergenic extract was sterilized using a 0.45-μm membrane filter. The content of the dried extract was estimated to be 8 mg/ml after dialysis.

For analytical purposes the samples, as prepared above, were diluted ten times in 0.02 *M* NH_4Cl–NH_4OH, with the exception of extract 5 (Tobacco Mix, Hollister-Stier) which was used in the concentration indicated in Table I.

RESULTS AND DISCUSSION

HPLC has proved to be one of the most useful techniques for the separation of tobacco alkaloids, consequently this technique was adopted to analyse the leaf

extracts. The nicotine and cotinine contents of allergenic extracts of tobacco have not previously been analysed; good separations were achieved (Figs. 2 and 3). The eight allergenic extracts of tobacco (leaf and Mix) have markedly different nicotine patterns (Table II). All samples were found to contain nicotine, except extract 7 (Institut Pasteur). For this sample, a membrane filtration 6000/8000 was used.

The difference in the nicotine patterns of the allergenic extracts correlates with previous observations: RAST results were strongly positive for B.J.L.S. with extracts of tobacco leaf and negative with extracts of tobacco smoke, the later extract has a lower nicotine content. HBDT results were strongly positive when using tobacco leaf extracts but only weakly positive with tobacco Mix extracts[17]. The specific desensitization with allergenic extracts of tobacco leaves is very useful when using a freshly prepared allergen containing a threshold dose of nicotine alkaloid; the volume injected is a parameter influencing the efficiency of specific desensitization. In contrast, the use of tobacco Mix extracts (Hollister-Stier) has proved to be ineffective[18]. The provocation test by inhalation of tobacco smoke showed a more significant reaction

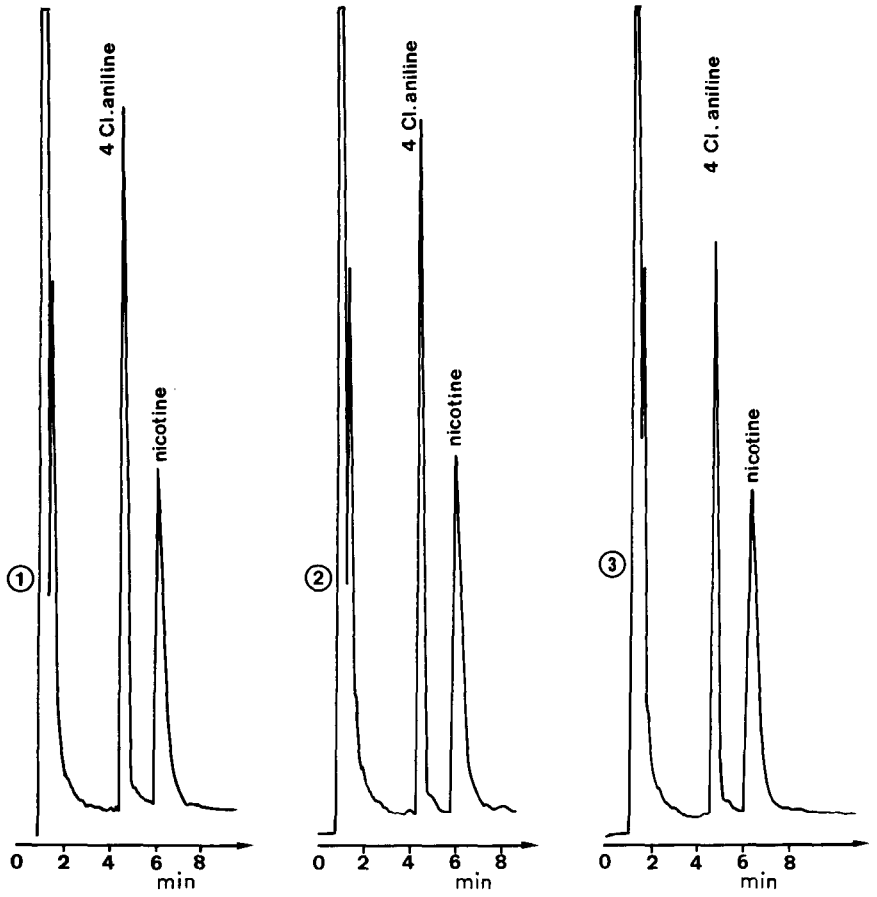

Fig. 2. HPLC determination of nicotine alkaloid in allergenic extracts of tobacco leaves (Hollister-Stier): extracts 1, 2 and 3.

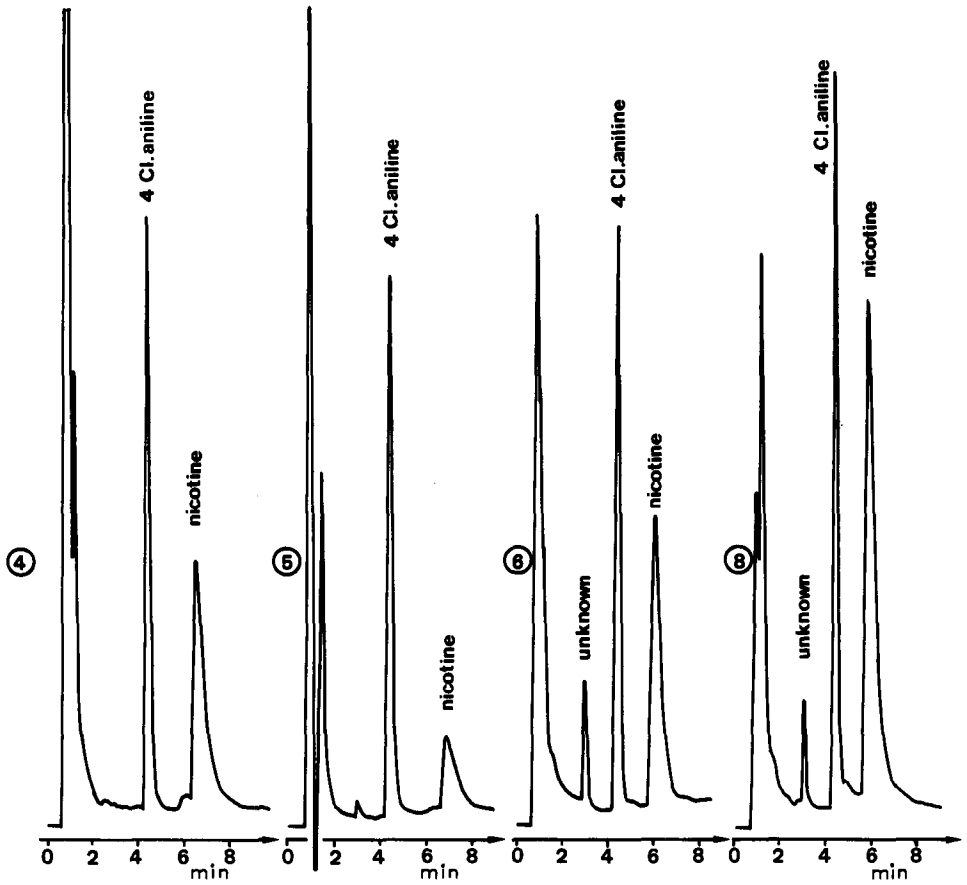

Fig. 3. HPLC determination of nicotine alkaloid in allergenic extracts of tobacco leaves and Mix: extracts 4, 5 (Hollister-Stier) and 6, 8 (Institut Pasteur).

TABLE II

NICOTINE CONTENTS IN EIGHT ALLERGENIC EXTRACTS OF TOBACCO LEAF AND MIX

Extract	*Nicotine (mg/ml)*
1	2.021
2	2.241
3	2.253
4	2.085
5	0.062
6	1.99
7	0
8	3.18

with cigar smoke than with cigarette smoke, in time and intensity, and a good correlation with haptenic content in nicotine[1].

Factors affecting the nicotine content are various, *e.g.*, genetic selection of species of *Nicotiana tabacum* leaves, culture methods and leaf selection. The lower leaves of the tobacco plant usually contain the least nicotine, and the higher the most. Exposure to sunlight raises the nicotine content. The tobacco leaves used were from different countries, which is of importance for the haptenic content of nicotine in allergenic extracts.

The nicotine content of allergenic extracts of tobacco is influenced by several factors among them oxidation of nicotine occurred upon exposure to UV light. This process is probably very slow because we were unable to demonstrate the presence of cotinine, a major photo-degradation product of nicotine. The different origins of the tobacco leaves would suggest variations in the exposure to sunlight. Tobacco from the U.S.A. has the highest nicotine content. In Canadian and Zimbabwean tobacco the nicotine level is similar but a little lower, in South African it is low and in Philippine and Thailand tobacco the content is very low, averaging around 0.7% in the latter[19].

The level of nicotine is also dependent on the extraction method, maceration time, sterilization method and membrane filtration. The capacity of human individuals to develop "blocking antibodies IgG" and T "suppressor" lymphocytes upon various doses, dilutions and injection volumes may explain the variability in the response to specific desensitization with allergenic extracts of tobacco (leaves and Mix). Environmental nicotine from cigar, pipe and cigarette tobacco inhaled passively in public places seems to be another important factor influencing the humoral response of allergic individuals. In future, the nicotine content of allergenic extracts of tobacco should be determined precisely by HPLC and the same allergenic extract should not be used for specific desensitization for more than 6 months.

This rapid and convenient procedure for the determination of nicotine in allergenic extracts of tobacco leaves could be useful in an international program to control the standardization of such extracts[20].

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