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Use of solvent optimization software for rapid selection of conditions for reversed-phase high-performance liquid chromatography of nicotine and its metabolites

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Abstract

Solvent optimization software was used to develop a reversed-phase HPLC method for nicotine, five of its metabolites and caffeine under isocratic conditions, using UV detection and a LC_8DB reversed-phase column. Starting from a methanol–buffer gradient, the software helped to find a quaternary solvent composition consisting of 2.3% methanol, 4.3% acetonitrile, 0.3% tetrahydrofuran and 93.1% buffer for the separation. The method was applied to human spiked serum and to serum of 12 cigarette smokers using a simple purification treatment before chromatography.

1. Introduction

In recent years, the metabolism of nicotine has been found to be complex, leading to more than twenty metabolites, which differ in structure and polarity [1–3]. At present, high-performance liquid chromatographic (HPLC) methods able to simultaneously quantify more than two metabolites, always require lengthy gradient mobile phases [4–7]. Moreover, different extractions from biological matrices are needed depending on the polarity of the metabolites investigated [4,8].

During the last decade, several schedules have been developed to aid the chromatographer to obtain appropriate separations in the shortest possible period of time [9]. In some cases this has led to the production of commercially available systems, either as complete instruments or as separate software packages [10,11].

We have developed a reversed-phase HPLC method for nicotine and five of its metabolites (Fig. 1) under isocratic conditions, using UV detection. Diamond solvent optimization software (Unicam, Cambridge, UK) [11,12] was used together with the HPLC system to optimize the separation of nicotine, its metabolites and caffeine using N′-ethylnorcotinine as an internal standard. The method was applied to the serum of 12 smokers using a simple purification treatment before chromatography.

2. Experimental

2.1. Materials

Cotinine (COT), nicotine (NIC) and caffeine (CAF) were purchased from Sigma (St. Louis,

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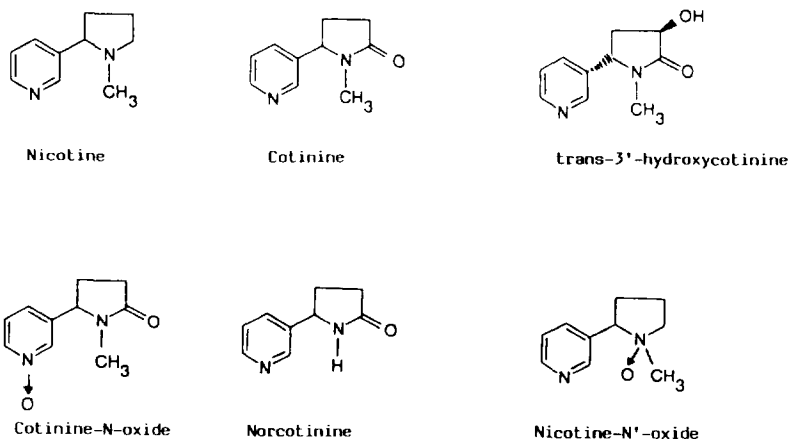

Fig. 1. Structural formulae of nicotine and five of its metabolites.

MO, USA). *trans*-3′-Hydroxycotinine (THOC), cotinine-N-oxide (CNO), nicotine-N′-oxide (NN′O), norcotinine (NCOT) and N′-ethylnorcotinine (NENC) were obtained from Dr. Georg B. Neurath (Hamburg, Germany). All solvents were analytical grade.

2.2. Chromatography

The HPLC system consisted of a Crystal 200 Series HPLC pump (Unicam, Cambridge, UK), a Crystal 240 diode array detector (Unicam), a PU4247 LC Autosampler (Philips Analytical, Cambridge, UK) and a Crystal Integrated Control Software. Both the HPLC control software and the Diamond solvent optimization software were run on a Dell 433/M computer, and were operated under a Windows 3.0 environment. Chromatographic data were collected and stored using the UICS operating software (Unicam). Then, data could be accessed from the Diamond environment, from where they could be displayed and interpreted, following any appropriate manipulation. For biological samples analyses, the HPLC pump was coupled with a Beckman 160 fixed-wavelength detector, set at 254 nm, and a Varian 4290 chromato-integrator.

A Supelcosil LC_8DB column(5 μm particle size, 25 cm × 4.6 mm I.D.; Supelchem, Rome, Italy) was used at room temperature at a flow-rate of 1.4 ml/min. The four solvents were methanol (MeOH), acetonitrile (MeCN), tetrahydrofuran (THF), and a buffer containing triethylamine (2 ml/l), 0.012 *M* each of sodium heptanesulphonate, K_2HPO_4 and citric acid, adjusted to pH 4.7 with citric acid. The injection volume was 20 μl.

2.3. Biomedical applications

Solutions of stock reference standards (1 mg/ml, 10 μg/ml, 1 μg/ml) were prepared in methanol and stored below 0°C. Dilutions were made fresh daily to create serum standards.

Drug-free human sera (1 ml) spiked with different amounts of nicotine, its five metabolites, caffeine and N′-ethylnorcotinine were transferred in a tube and rapidly deproteinized by 100 μl of perchloric acid (70%). The deproteinized samples were centrifuged at 2000 *g* for 10 min, the supernatants were removed, evaporated under nitrogen at 40°C and redissolved in 100 μl of water before chromatography. These spiked sera were used throughout the entire procedure to create calibration curves, and to determine analytical recoveries, intra-day and inter-day variabilities.

Blood samples (5 ml) from 12 smokers were obtained by venipuncture with silicone-coated vacutainers. Samples were collected at 8:00 a.m. after consumption of two cigarettes, the last one 10 min prior to collection. Immediately after collection the samples were centrifuged at 1000 *g*

for 5 min, extracted as described above and injected into the HPLC column.

3. Results and discussion

The HPLC method was developed using a LC_8DB column, used earlier to separate nicotine and four metabolites under gradient mobile-phase conditions [8]. Initially, the analytes mixture was chromatographed using a methanolic gradient consisting of 5% methanol and 95% buffer at the beginning and changed to 30% methanol and 70% buffer in 30 min (Fig. 2). This gradient was extrapolated from previous investigations [8]. Using the retention times of the first and last eluting peaks, the t_0 of the system, the methanolic profile information, and the number of components in the mixture, the software supplied an isocratic aqueous methanolic solvent composition of 14% which should result in elution of the final peak with a k' of approximately 10, as the number of components expected was 8. The mixture was run with this solvent composition and the retention time of the last peak was input to the software program, to check if the prediction was correct. An equivalent acetonitrile binary composition was obtained, the entire process was repeated and a THF binary composition was suggested. Finally, when all three binary solvent compositions had been checked, the software defined an isoeluotropic plane of 10 solvent compositions of approximately equal solvent strength (Fig. 3).

When all 10 chromatographic runs were collected, the one containing most peaks, which was the one with the THF binary solvent composition, was chosen to create a library of reference UV spectra for each of the substances present in the mixture. Then, for each of the 10 chromatograms, the spectrochromatographic data obtained with the diode array detector were interrogated. The chromatograms were divided into segments, the positions of the peaks were de-

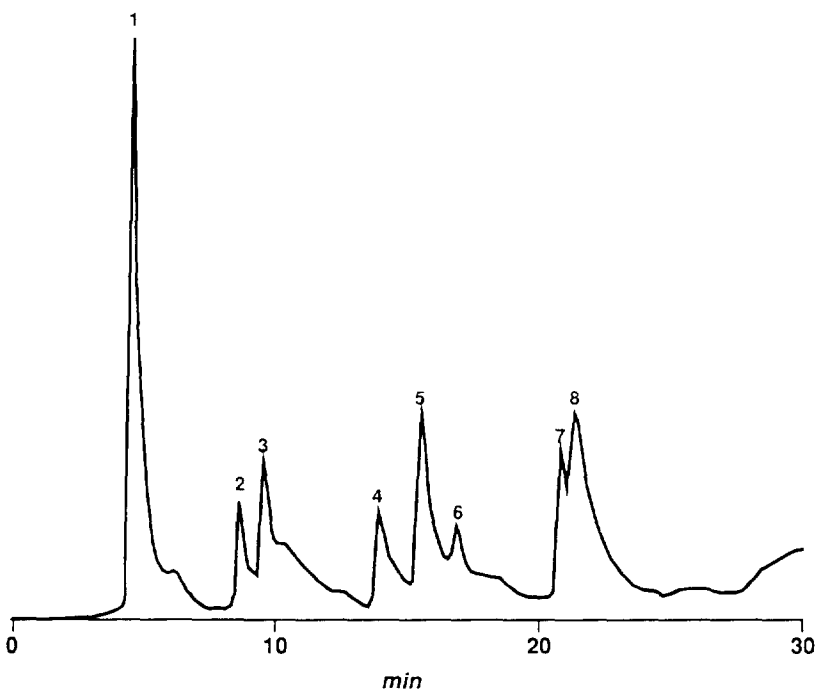

Fig. 2. Chromatogram of a standard mixture using a methanol–buffer gradient. Peaks: 1 = cotinine-N-oxide; 2 = *trans*-3′-hydroxycotinine; 3 = norcotinine; 4 = cotinine; 5 = caffeine; 6 = nicotine-N′-oxide; 7 = nicotine; 8 = N′-ethylnorcotinine.

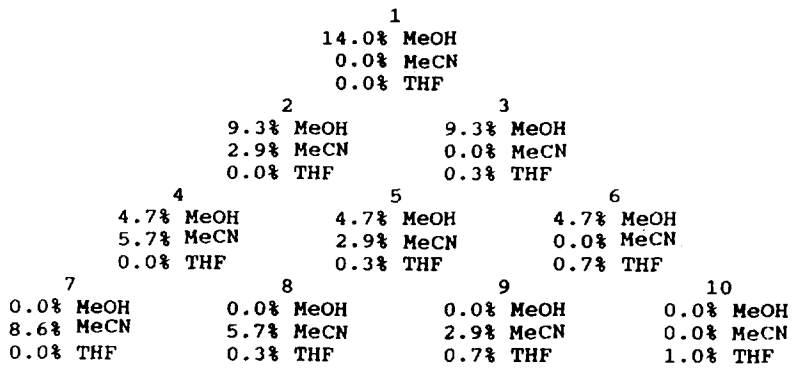

Fig. 3. The ten-solvent compositions used to define the isoeluotropic solvent plane, as determined using the software. The figures represent the percentage of each component in the mobile phase at each point, with buffer making the composition up to 100%.

termined from the second derivative of the chromatogram, and the spectral data of each peak were extracted using deconvolution where two or more peaks overlapped. When the extracted spectra were accepted, they were matched against those in the reference library and the retention times of the components were stored in the database.

Once the retention information for all 10 chromatographic data sets were achieved, the retention maps for each of the components were synthesized as shown in Fig. 4 for nicotine. The retention maps for nicotine, its metabolites, caffeine and NENC were combined to produce the final resolution map, from which the optimal separation conditions were derived (Fig. 5) using the response function ('TNE') for the minimum separation and retention time of the last peak. A quaternary solvent composition consisting of 2.3% methanol, 4.3% acetonitrile, 0.3% tetrahydrofuran and 93.1% buffer was proposed for the separation of nicotine, its metabolites, caffeine and the internal standard.

The chromatographic separation performed using the solvent composition suggested by the software showed to be very similar to the predicted one (Fig. 6).

An example of the method's application was the HPLC determination, using UV fixed-wave-

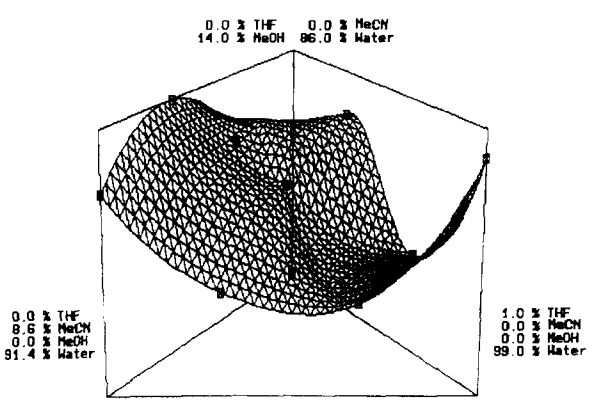

Fig. 4. Retention map for nicotine viewed towards the methanol corner.

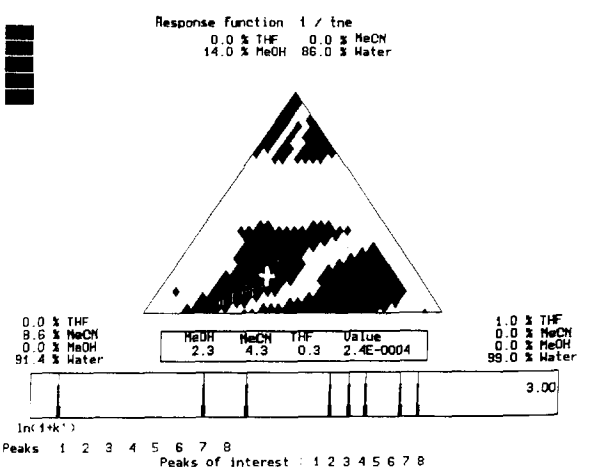

Fig. 5. Final resolution map for the separation of nicotine, five of its metabolites, caffeine and N'-ethylnorcotinine, calculated using the 'TNE' function (see text), presented as a contour plot. The white cross indicates the optimal predicted solvent composition.

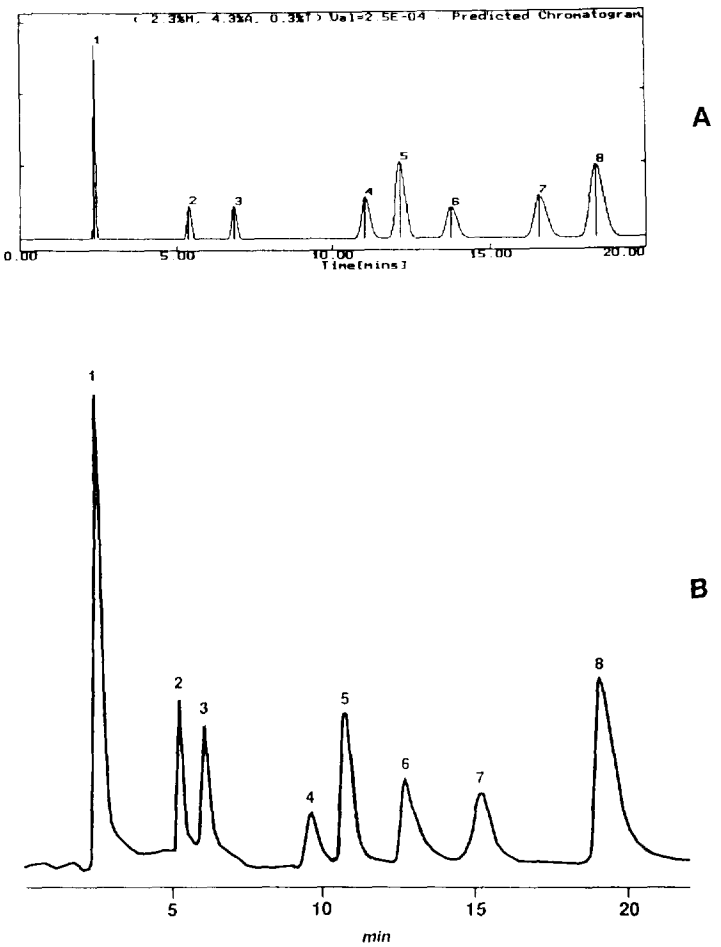

Fig. 6. A comparison of the simulated chromatogram for the optimal separation derived from the software prediction (A), and the actual separation obtained using the predicted optimal mobile phase composition calculated by the software (B). Peaks: 1 = cotinine-N-oxide; 2 = *trans*-3′-hydroxycotinine; 3 = norcotinine; 4 = cotinine; 5 = caffeine; 6 = nicotine-N′-oxide; 7 = nicotine; 8 = N′-ethylnorcotinine.

length detection, of nicotine, its metabolites and caffeine, which is present in 80% of smokers' serum samples [13], in human sera of 12 smokers, who had smoked just prior to blood collection. The results obtained are shown in Table 1.

The limits of detection (signal-to-noise ratio of 3) observed with this method were 10 ng/ml for nicotine and nicotine-N′-oxide, 5 ng/ml for cotinine, *trans*-3′-hydroxycotinine, caffeine and norcotinine, 3 ng/ml for cotinine-N-oxide. The calibration curves of the peak area vs. the amount of analytes (μg/ml) were linear over the range of 10–500 ng/ml for NIC ($y = 0.14x +$

Table 1
Concentrations of nicotine and its metabolites in serum samples of 12 smokers

Compound	Concentration, mean ± S.D. (ng/ml)
NIC	42.2 ± 35.1
COT	307.1 ± 81.6
THOC	96.5 ± 31.0
CNO	3.5 ± 1.3
NCOT	9.1 ± 2.7
NN′O	12.5 ± 1.6[a]

[a] Detected only in three samples.

0.06; $r = 0.99$) and NN'O ($y = 0.16x + 0.10$; $r = 0.98$); over the range of 5–500 ng/ml for COT ($y = 0.01x + 0.14$; $r = 0.90$), THOC ($y = 0.016x + 0.10$; $r = 0.93$), NCOT ($y = 0.03x - 0.13$; $r = 0.99$) and CAF ($y = 0.02x + 0.05$; $r = 0.98$), and over the range of 3–500 ng/ml for CNO ($y = 0.05x + 0.4$, $r = 0.99$). Over the concentration ranges tested in the analysis, recoveries were above 90% for all analytes. The within-day and between-day coefficients of variation were always less than 4 and 6% for 100 and 500 ng/ml analytes, respectively.

In conclusion, solvent optimization software proved to be a versatile and useful tool, simple to use, and interactive with the analyst, thus reducing the time spent in achieving an acceptable separation using a mathematical approach.

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