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Rapid method for the simultaneous measurement of nicotine and cotinine in urine and serum by gas chromatography–mass spectrometry

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Abstract

A simple, sensitive, and rapid gas chromatographic–mass spectrometric method is described for the simultaneous detection and quantitation of nicotine and its metabolite, cotinine, in urine and serum. The analytes and their respective deuterated internal standards were extracted by liquid–liquid extraction coupled to centrifugation and evaporation. The detection limit of the assay was 0.16 ng/ml for both nicotine and cotinine. The limit of quantitation for each analyte was 1.25 ng/ml. © 1998 Elsevier Science B.V.

Keywords: Nicotine; Cotinine

1. Introduction

Nicotine is a natural alkaloid obtained from the leaves of the tobacco plant, *Nicotiana tabacum* [1]. It is a tertiary amine composed of a pyridine and a pyrrolidine ring and can exist as one of two stereoisomers [2]. L-Nicotine is the major tobacco alkaloid and the most pharmacologically active form [3]. Cotinine, on the other hand, is a major oxidized metabolite of nicotine and is structurally very similar to it. However, cotinine has a much longer elimination half-life (about 20 h compared to 2 h for nicotine) and can be used as a reliable measure of nicotine intake [4]. Cotinine may also exert pharmacological activity of its own [5]. Currently, nicotine is used as an aid in smoking cessation in the forms of chewing gum [6], transdermal patch [7], and a nasal spray [8]. Until recently, medical importance of nicotine was attributed to its high toxicity and presence in tobacco. Lately, it has been suggested that nicotine may have therapeutic applications in a variety of disorders including Alzheimer's disease, Parkinson's disease, obesity, depression, anxiety, ulcerative colitis, Tourette's syndrome, and attention deficit disorder [9]. Indeed, clinical trials of nicotine in a number of these disorders with positive outcomes have been reported [10–12]. As a result, a simple, rapid, and sensitive assay for the measurement of nicotine and its major metabolite, cotinine, is fundamental in the effective development and clinical evaluation of nicotine treatments.

A wide variety of methods for the analysis of nicotine and cotinine in biological samples has been published. These methods utilize radioimmunoassay [13], high-performance liquid chromatography [14], and gas chromatography using electron-capture,

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chemical ionization [15], or alkali flame ionization [16] detectors. Additionally, gas chromatography combined with mass spectrometry [17] or with liquid chromatography [18] has also been applied. Some of these methods require large volumes of sample, utilize many extraction steps and reagents while others are unable to measure both nicotine and cotinine simultaneously. This paper describes a rapid and relatively simple and inexpensive method for the simultaneous determination of nicotine and cotinine in urine and serum utilizing gas chromatography–mass spectrometry.

2. Experimental

2.1. Chemicals and reagents

Deuterium-labeled (*N*-methyl-d_3) nicotine and cotinine, the internal standards, were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). (±)-Nicotine and (−)-cotinine were purchased from Sigma (St. Louis, MO, USA). Toxi-Lab A extraction tubes were obtained from Toxi-Lab (Irvine, CA, USA). Methanol and methylene chloride, HPLC-grade, were obtained from EM Science Company (Gibbstown, NJ, USA).

2.2. Instrumentation

A Hewlett-Packard model HP 5890A gas chromatograph (GC) equipped with a mass selective detector (MSD), model 5970B, an HP 7673A auto-injector, and an HP 310 computer (Wilmington, DE, USA) was used for the analysis. Extraction of nicotine and cotinine was done by a process of liquid–liquid extraction coupled to centrifugation. A Brinkman Sample Concentrator, model SC/27R (Westbury, NY, USA), was used to concentrate the drugs. This instrument is a variable temperature aluminum heating block designed for the efficient concentration of drug samples.

2.3. Chromatographic conditions

An HP-1 fused-silica capillary column (12 m×0.2 mm I.D.×0.33 μm film thickness, and consisting of cross-linked methyl silicone gum phase) was used. Operation of the MSD was in the electron-impact (EI) mode at 70 eV. The major ion peaks using the scan mode (50–180 a.m.u.) were m/z 84 and 162 for nicotine; m/z 87 and 165 for d_3-nicotine; m/z 98 and 176 for cotinine; and m/z 101 and 179 for d_3-cotinine. Quantitation ions using SIM were m/z 84 and 87 for nicotine and d_3-nicotine, respectively, and m/z 98 and 101 for cotinine and d_3-cotinine, respectively. A 25-ms dwell time was used for all ions. The multiplier was set at the autotune voltage. Perfluorotributylamine (PFTBA) was used to autotune the instrument daily. The carrier gas (helium) flow-rate was 0.59 ml/min at a linear velocity of 31.25 cm/s. The temperatures of the injection port and transfer line were 200 and 280°C, respectively. The initial column temperature was 60°C, which was increased by a rate of 50°C/min up to 210°C. Samples were injected with the splitless valve on for 0.75 min. The retention times for nicotine and cotinine were 2.43 and 3.45 min, respectively.

2.4. Assay procedure

To a 10-ml test tube, a volume of 2 ml of human urine or 200 μl of serum together with 1.8 ml distilled water was spiked with aqueous solutions of the internal standards, d_3-nicotine (400 μl, 250 ng/ml) and d_3-cotinine (400 μl, 500 ng/ml). The sample was extracted in a Toxi-Lab A tube for 5 min using a rotating shaker. The Toxi-A tube was centrifuged at 2665 *g* for 10 min. The organic phase was transferred to a concentration vial to which was added 1 drop of acidified methanol (0.1% HCl in methanol) to prevent excessive evaporation of the compounds. The specimen was evaporated to dryness at 50°C under vacuum and reconstituted with 100 μl of methylene chloride. A volume of 2 μl was injected automatically into the GC–MS and analyzed.

2.5. Calibration curves

Solutions of various concentrations of nicotine and cotinine standards (400 μl each) to give a concentration range of 1.25–100 ng/ml for nicotine and 1.25–1000 ng/ml for cotinine were prepared in distilled water daily for each analysis. Spiked human urine and serum standards were prepared from the known concentrations of the analytes in conjunction

with the internal standards to create calibration curves.

3. Results and discussion

This report describes a relatively simple, quick, and reliable GC–MS method for the simultaneous analysis of nicotine and its metabolite, cotinine, in spiked urine and serum. Fig. 1a and Fig. 1b show typical chromatograms of extracts from spiked urine run in the scan mode (50–180 a.m.u.) The base peak of nicotine is 84 m/z and that of cotinine is 98 m/z. In Fig. 2a,b, the major peaks of 84 and 162 m/z for nicotine and 87 and 165 m/z for d_3-nicotine, and 98 and 176 m/z for cotinine and 101 and 179 m/z for d_3-cotinine, are shown in chromatograms run in the SIM mode of spiked serum samples. Fig. 3 shows

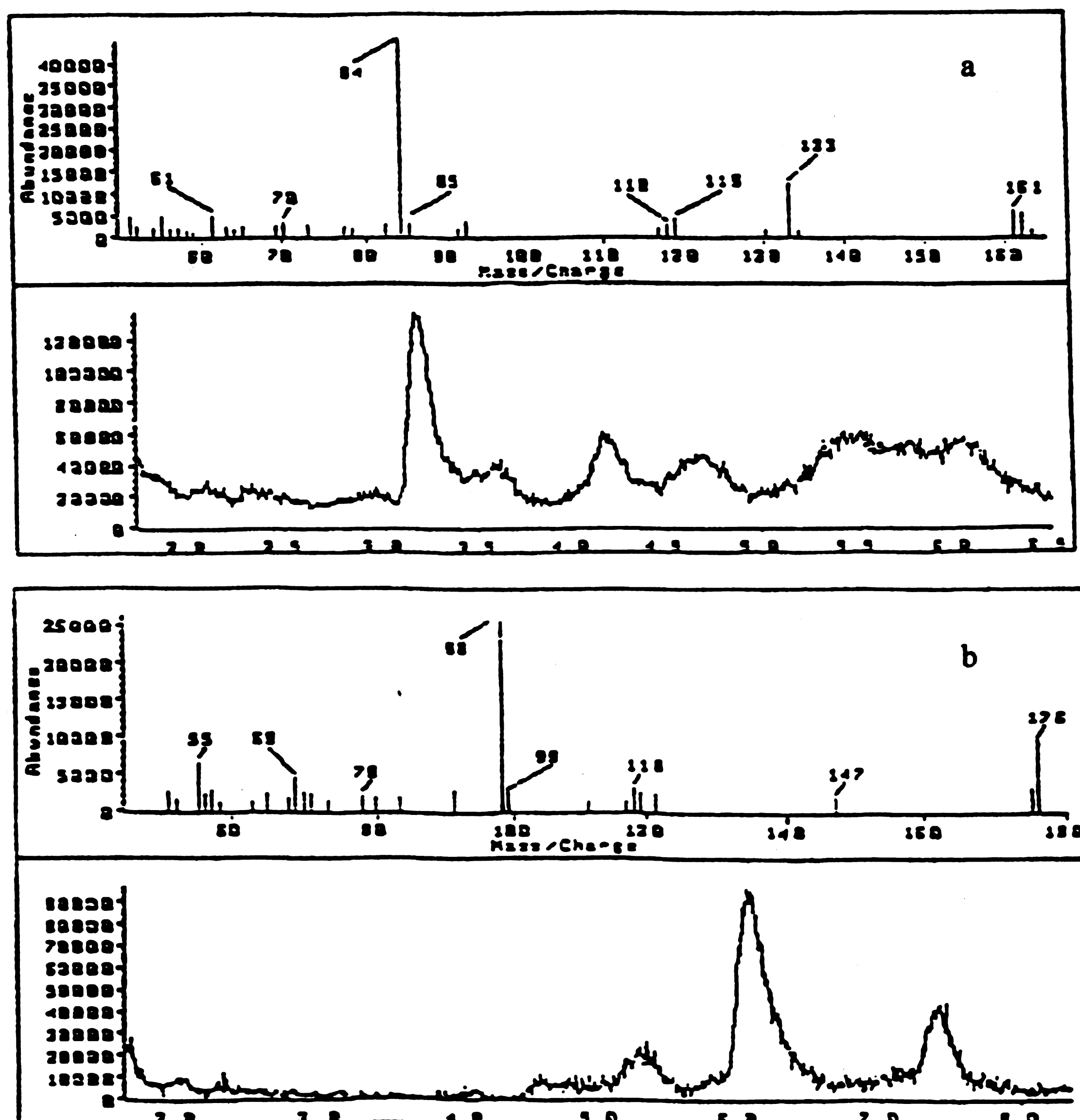

Fig. 1. Total ion chromatogram of nicotine (a) and cotinine (b) extracted from spiked urine. Run under scan with 10 ng/ml and 2-μl injection.

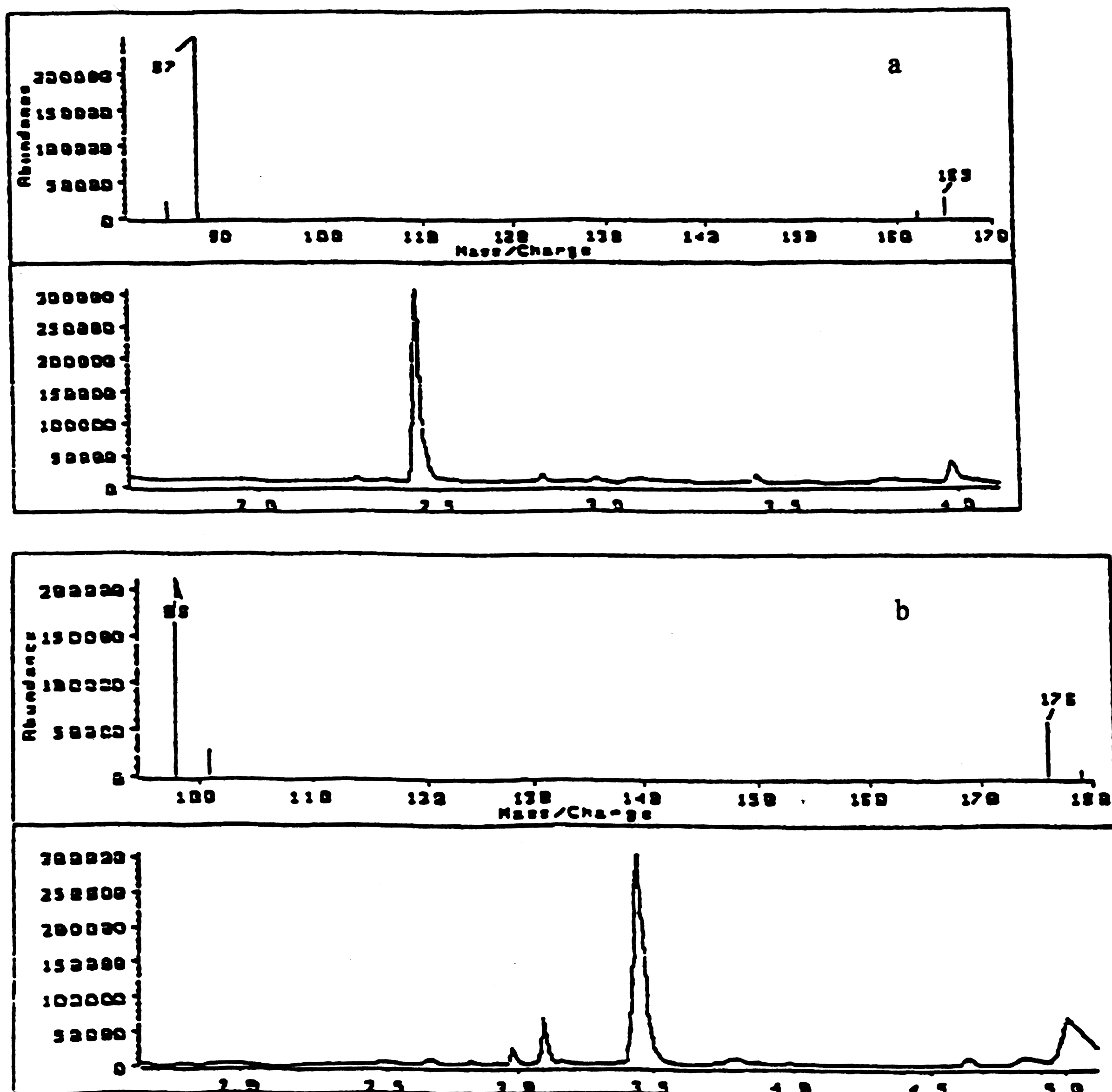

Fig. 2. Total ion chromatogram of 10 ng/ml nicotine and 250 ng/ml d_3-nicotine (a) and 1000 ng/ml cotinine and 500 ng/ml d_3-cotinine (b) extracted from spiked serum. Run under SIM with 2-μl injection.

chromatograms of the same compounds in spiked urine run simultaneously under the SIM mode. Similar results were obtained for spiked serum samples (data not shown). The reproducibility profile over the selected concentration ranges for nicotine and cotinine in spiked human urine represents six determinations and is shown in Table 1. Similar reproducibility was obtained for spiked serum samples (data not shown). The linear regression coefficient of each calibration curve was as follows: nicotine in urine, 1.0; cotinine in urine, 1.0; nicotine in serum, 0.99; and cotinine in serum, 0.97. These curves were linear and passed through the origin.

Extraction of nicotine and cotinine was accom-

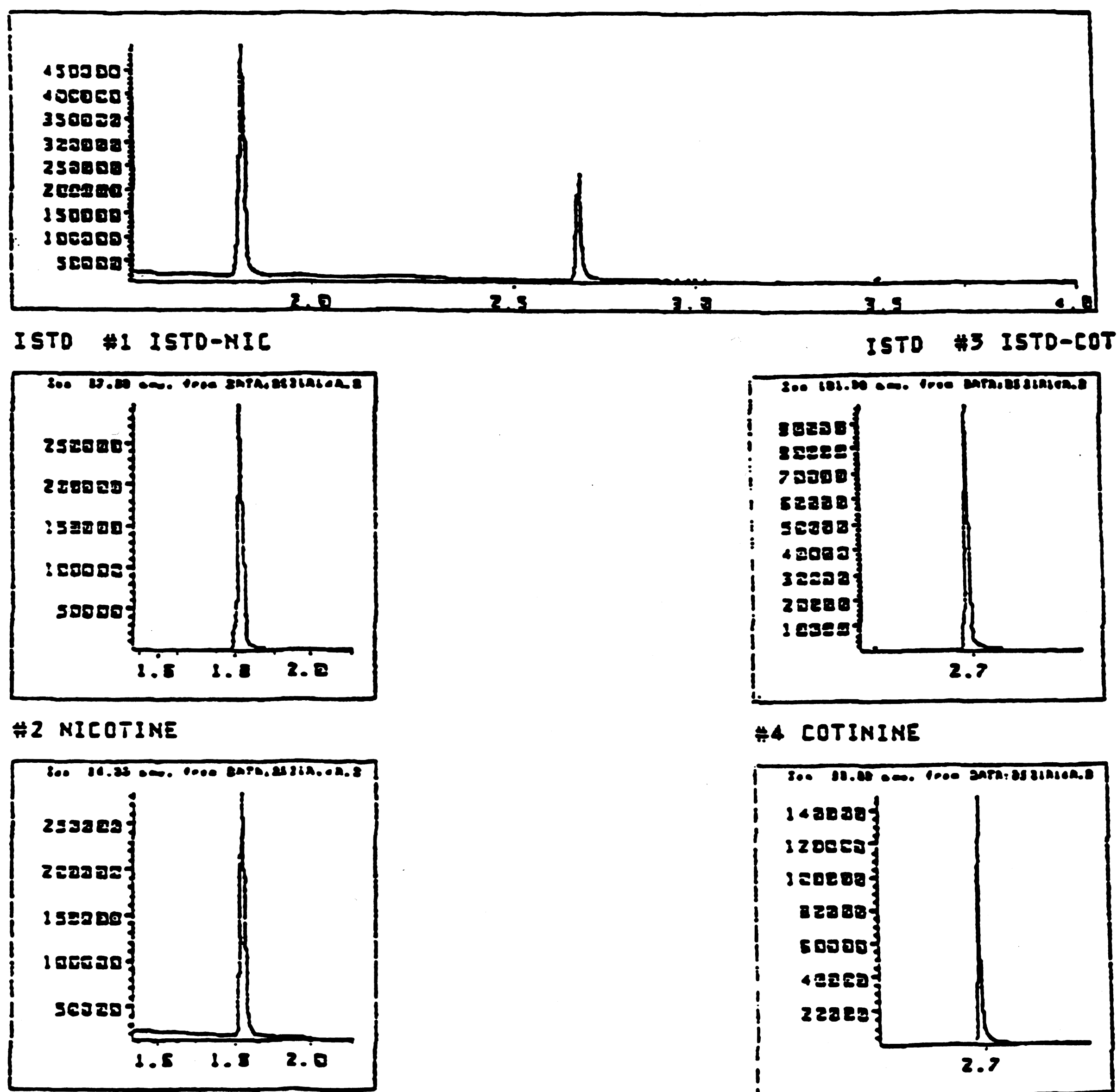

Fig. 3. Total ion chromatogram of nicotine (75 ng/ml), d_3-nicotine (250 ng/ml), continine (100 ng/ml), and d_3-cotinine (500 ng/ml) extracted from spiked urine with 2-μl injection. Run under SIM.

plished by using Toxi-Lab A extraction tubes. This is a simple procedure and results in a recovery of over 95% of the analytes. The Toxi-Lab A extraction tube is a relatively new product that facilitates easy extraction of drug samples for GC–MS analysis. It consists of an organic liquid mixture and buffered salts. In addition, the use of an initial column temperature of 100°C (versus 60°C) and a final temperature of 280°C (versus 210°C) reduced the length of the retention time to 1.81 min for nicotine and 2.68 min for cotinine, versus 2.43 and 3.45 min, respectively, at the lower temperatures. This temperature manipulation resulted in improved cotinine detection without affecting that of nicotine. The limit

Table 1
Results of various concentrations of nicotine and cotinine in urine[a]

Nicotine			Cotinine		
Concentration added (ng/ml)	Concentration determined (mean±S.D.) (ng/ml)[b]	C.V. (%)[c]	Concentration added (ng/ml)	Concentration determined (mean±S.D.) (ng/ml)[b]	C.V. (%)[c]
1.25	1.27±0.23	18.1	1.25	1.24±0.23	46.8
2.5	2.52±0.09	3.4	2.5	2.45±0.65	26.5
5.0	5.00±0.11	2.2	5.0	4.95±0.50	10.1
10.0	9.97±0.11	1.1	10.0	10.08±1.59	15.8
25.0	25.08±1.59	6.3	25.0	24.30±1.15	4.7
50.0	50.10±2.01	4.0	50.0	51.05±3.51	6.9
75.0	74.47±0.42	0.6	100.0	101±0.30	0.3
100.0	101.11±1.21	1.2	250.0	249.43±10.69	4.3
			500.0	500.27±14.62	2.9
			750.0	757.61±16.09	2.1
			1000.0	1000.27±4.25	0.4

[a]Six urine samples were assayed on six different days.
[b]Standard deviation of the mean.
[c]Percent coefficient of variation.

of detection of the assay was 0.16 ng/ml for both nicotine and cotinine. The quantitation limit for each analyte was 1.25 ng/ml. The average coefficients of variation over the nicotine and cotinine ranges were 4.6 and 10.9%, respectively, in urine and 8.6 and 18.0%, respectively, in serum. In spite of a vast number of analytical runs, performance of the column was not compromised.

The method described here has several advantages over previously published ones. It uses one calibration curve for both low and high concentrations of nicotine and cotinine. Additionally, the method of quantitation of the analytes is through isotopic dilution in which the intensities of specific nicotine and cotinine ions (84 and 98 m/z, respectively) are compared to those of analogous ions (87 and 101 m/z) of suitably labelled forms of the compounds of interest (d_3-nicotine and d_3-cotinine, respectively). The deuterated compounds, internal standards, are added to the analytical matrix in known concentrations. As a result, this technique provides greater sensitivity, reliability, and analytical specificity to the assay. Furthermore, extraction with Toxi-Lab A tubes is a one-step procedure that is quick and has a high degree of recovery of the analytes.

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