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Short Communication

Simple selected ion monitoring capillary gas chromatographic–mass spectrometric method for the determination of cotinine in serum, urine and oral samples

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ABSTRACT

A capillary gas chromatographic–mass spectrometric method using selected ion monitoring was developed for the analysis of cotinine in urine, serum and oral samples. The procedure requires 500 μl of an oral sample, 250 μl of a serum sample and 50 μl of urine and can detect 5 ng/ml cotinine in oral samples, 10 ng/ml in serum and 50 ng/ml in urine with good precision and accuracy. The method was used to determine the cotinine concentration in samples of all three fluids collected from a group of smokers and non-smokers.

INTRODUCTION

Cotinine is a major metabolite of nicotine whose presence has been used as an indicator of smoking status or passive exposure to smoke in serum, urine and saliva [1–5]. The use of a newly developed oral sampling system as an alternative to serum for the detection of clinically important analytes has recently been described [6]. To test the feasibility of this system for the determination of cotinine, a simple gas chromatographic–mass spectrometric (GC–MS) procedure for cotinine in serum, urine and oral samples was required. A number of selected ion monitoring (SIM) GC–MS methods have been described for the analysis of cotinine in urine and/or serum samples [7–10]. We developed a procedure which can be applied to both these matrices and also to oral samples. This paper describes the method, its validation and its use in the determination of cotinine in samples of all three biological fluids from a group of smokers and non-smokers.

EXPERIMENTAL

Instrumentation and chromatography

GC–MS analyses were carried out using a Model 5890A gas chromatograph with a 7673 automatic liquid sampler, a split–splitless capillary inlet system and a capillary direct interface to a Model 5970B quadrupole mass spectrometer

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(Hewlett Packard, Palo Alto, CA, USA). Data were stored and processed using a MS Chem Station, Version BA.02.01 (Hewlett Packard).

Injections (2 μl) were made in the splitless mode using a 12 m × 0.25 mm I.D. column coated with a 0.25-μm film of cross-linked 5% phenylmethylsilicone (J & W, Folsom, CA, USA). A freshly silanized injection port liner containing a small plug of silanized glass wool was used on each day of analysis. The injection port temperature was 250°C, the carrier gas (helium) flow-rate was 2 ml/min, and the septum purge on-time was 0.8 min. The temperature program began at a temperature of 70°C. This temperature was held for 1 min, after which the temperature was ramped to 250°C at a rate of 25°C/min. The instrument was first tuned using the Autotune program and then the ion current at m/z 69 of perfluorotributylamine (PFTBA) was maximized by manual adjustment of the repeller, the ion focus, the entrace lens, the ramp entrance lens offset and the ramp X-ray. The electron multiplier was programmed to carry out the analyses at 200 V above the Autotune value. Analyses were carried out in the SIM mode. The molecular ions at 98.2, 118.2 and 176.2 were monitored for cotinine and the ions at 101.2, 121.2 and 179.2 for the internal standard, deuterocotinine. The ions were monitored with a dwell time of 50 ms.

Quantitation was achieved by extraction of the most abundant ions at m/z 98.2 and 101.2 between 5.6 and 5.9 min followed by integration. Standard curves were generated by plotting the ratio of the integral of the ion at 98.2 to that at 101.2 against the amount of cotinine.

Chemicals and reagents

Cotinine used as an analytical standard was obtained from Alltech (Deerfield, IL, USA). Deuterocotinine (methyl-D_3), the internal standard, was obtained from Cambridge Isotope Labs. (Woburn, MA, USA). Analytical-grade toluene and butan-1-ol and reagent-grade sulfuric acid and hydrochloric acid were provided by Mallinkrodt (Chesterfield, MI, USA). Anhydrous reagent-grade potassium carbonate (Malinkrodt) was used in the preparation of solutions for extraction. Solutions of ammonia were prepared by dilution of a 4.96 *M* volumetric standard from Aldrich (Milwaukee, WI, USA) with water. Water used in the preparation of reagent solutions was glass-distilled.

Surfasil reagent from Pierce (Rockford, IL, USA) was used to deactivate glassware after cleaning with RBS-35 detergent, also from Pierce. B & J brand methylene chloride form Burdick and Jackson (Muskegon, MI, USA) and Mallinkrodt UltimAR-grade methanol were used in the silanization procedure.

Preparation of glassware

All test tubes, GC–MS vial inserts (Hewlett Packard), volumetric flasks and the GC inlet port liner were cleaned by soaking overnight in 2% RBS 35, followed by careful rinsing with distilled water. The glassware was then silanized using Surfasil. Reagent bottles were pre-treated by soaking in 2% RBS 35 overnight. They were then rinsed with water, soaked overnight in 0.1 *M* hydrochloric acid, rinsed with water once again and dried.

Sample collection and storage

Oral samples were collected with a paper pad (approximately 2.5 cm × 1.5 cm × 2.0 mm; liquid holding capacity approximately 1 ml) attached to a plastic handle [6]. The pad was saturated with a sodium chloride solution (3.5% sodium chloride, 0.3% citric acid, 0.1% potassium sorbate, 0.1% sodium benzoate, 0.1% gelatin, pH 7.2, adjusted with sodium hydroxide) and then dried. The pad was inserted in the subject's mouth between the lower cheek and gum, rubbed gently back and forth a few times to moisten and then held in place for 2 min. The pad was placed in a tube containing 0.8 ml of a bacteriostatic solution, and the diluted oral sample was recovered from the pad by centrifugation (10 min, 1300 *g*). Samples collected with this oral collection system (patent pending) will be referred to as OraSure samples. Blood samples were collected by standard venipuncture technique, allowed to stand for 30 min at room temperature and centrifuged (10 min, 1300 *g*). Urine samples were col-

lected in a urine collection container. All samples were aliquoted and frozen at −20°C.

Standards

Cotinine was dried *in vacuo* for 48 h. An accurately weighed amount (*ca.* 100 mg) was dissolved in 1 l of 12 m*M* hydrochloric acid. Further dilutions of this solution were prepared to obtain solutions of *ca.* 10, 1 and 0.1 ng/μl. Deuterocotinine (*ca.* 10 mg) was dissolved in 100 ml of 12 m*M* hydrochloric acid and dilutions of this solution were prepared as required. All standard solutions were used within two weeks [7].

Standard curves were generated by addition of aliquots containing cotinine in hydrochloric acid to phosphate-buffered saline (PBS). An aliquot containing 10 ng of deuterocotinine was then added as an internal standard, and the sample was subjected to the extraction procedure described below.

Extraction procedure

The following method was adapted from a procedure for cotinine determination in plasma [8]. A 0.5-ml aliquot of an Orasure sample was dispensed into a 2-ml siliconized Eppendorf tube. An aliquot containing 10 ng of deuterocotinine in 12 m*M* hydrochloric acid was added to each sample as an internal standard. A 0.5-ml aliquot of 50% (w/v) potassium carbonate in 0.2 *M* ammonia was then added, followed by 0.8 ml of 9:1 toluene–butanol. The tube was vortex-mixed for 5 min, centrifuged for 5 min (1400 *g*) and then placed in liquid nitrogen to freeze the aqueous layer. The organic layer was poured into a 2-ml Eppendorf tube, and the aqueous phase was discarded. A 0.5-ml aliquot of 0.5 *M* sulfuric acid was then added, the tube was vortexed and subsequently centrifuged (1400 *g*, 5 min). The organic layer was discarded after the aqueous layer had been frozen in liquid nitrogen. In the final extraction step, 0.5 ml of 50% (w/v) potassium carbonate in 0.2 *M* ammonia was added followed by 0.8 ml of toluene–butanol (9:1). The tube was vortex-mixed for 5 min and then centrifuged (1400 *g*, 5 min) to break the emulsion. Once again, the tube was frozen in liquid nitrogen, and the organic layer transferred to a silanized test tube. It was concentrated to dryness in a heating block at 80°C under a stream of nitrogen. The residue was dissolved in 50 μl of methylene chloride, vortex-mixed and transferred to a silanized 250-μl GC–MS autosampler vial insert. The test-tube was rinsed two more times with methylene chloride and the solutions transferred to the autosampler vial insert. The methylene chloride was removed by heating in an oven at 65°C *in vacuo*. Finally, 25 μl of 9:1 toluene–butanol were added, the vials were crimped and sealed and then placed in the autosampler tray for GC–MS analysis.

Analysis of cotinine in urine was performed after dilution of 50 μl of urine in 0.45 ml of PBS. Serum was diluted by addition of 0.25 ml of the sample to 0.25 ml of PBS.

Accuracy and precision

A three-day validation study was carried out to test the precision and accuracy of the method. A four-point calibration curve in triplicate together with aliquots from a pool of OraSure, urine and serum samples collected from a group of smokers were analyzed each day. In addition, analytical recovery was determined by spiking OraSure, urine and serum samples with known amounts of cotinine.

RESULTS AND DISCUSSION

Ion monitoring

The mass spectrum of cotinine exhibits a base peak at *m/z* 98 and a molecular ion at *m/z* 176. The corresponding peaks for deuterocotinine are 3 a.m.u. greater. Other investigators have used the molecular ions for quatitation of cotinine to minimize interference from other compounds present in plasma [9,10]. We therefore analyzed several samples by monitoring the molecular ions (*m/z* 176 and 179) of both cotinine and deuterocotinine, as well as the most abundant ions, *m/z* 98 and 101. It was noted that the molecular ion chromatograms were not cleaner than those of the most abundant ions. Therefore the base peaks at 98 and 101 were selected for monitoring to maximize the sensitivity of the GC–MS meth-

od. A typical SIM extracted ion chromatogram is shown in Fig. 1.

Standard curves

At first, the calibration curve for OraSure samples was prepared by spiking known amounts of cotinine and deuterocotinine into OraSure samples obtained from non-smokers since these samples contain no detectable cotinine. In contrast, it was found impossible to obtain urine and serum samples completely free of cotinine. Thus a standard curve for the analysis of cotinine in these two matrices was generated by spiking known amounts of cotinine and internal standard into PBS. It was subsequently observed that the OraSure standard curve was identical to that generated in PBS and therefore the PBS standard curve was used for analysis of all three biological fluids. A typical standard curve had the equation: $y = 0.1049 + 0.11159x$ ($r^2 = 1.000$). The standard curves were found to vary little from day to day and were linear up to 250 ng cotinine. Since serum samples were diluted 1:2 and urine samples were diluted 1:10 before analysis, this corre-

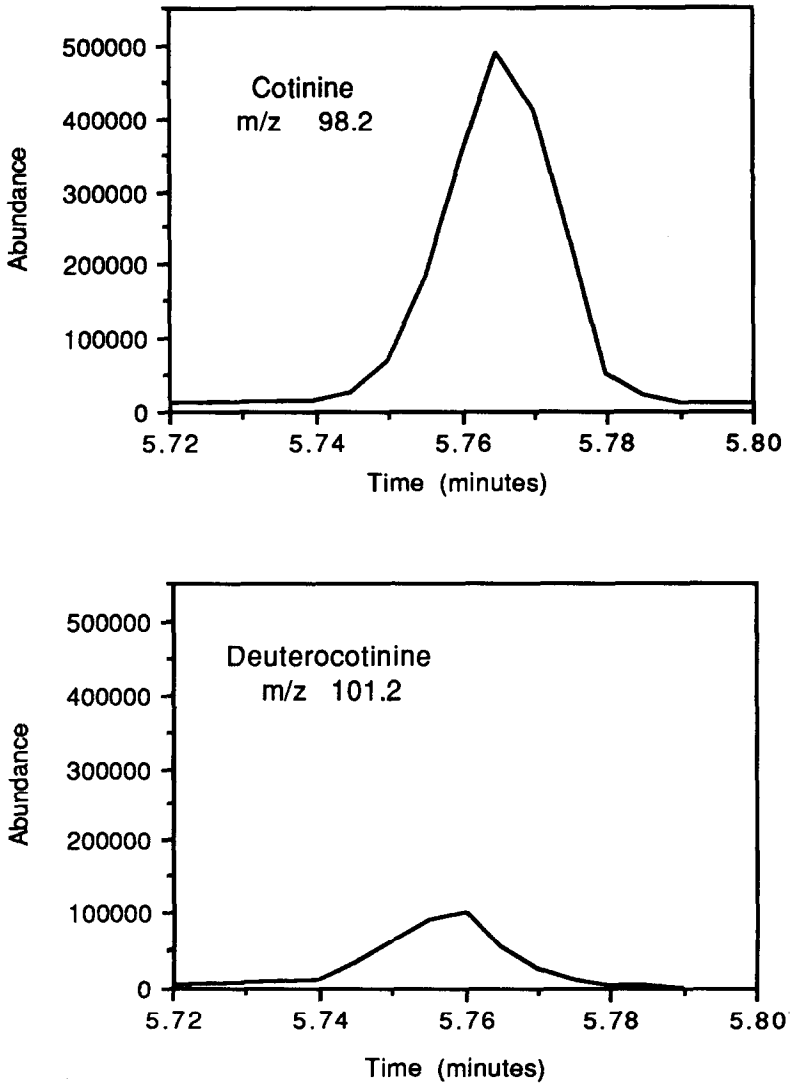

Fig. 1. Ion chromatogram of an extract of OraSure from a smoker. The cotinine concentration was 128 ng/ml.

TABLE I

DAY-TO-DAY VARIATION OF IDENTICAL ORASURE, URINE AND SERUM SAMPLES

Day	Cotinine concentration (mean ± S.D., n = 3) (ng/ml)	Coefficient of variation (%)
OraSure		
1	58.4 ± 0.362	5.47
2	59.0 ± 0.151	2.28
3	60.9 ± 0.042	0.577
Urine		
1	121 ± 0.348	2.56
2	118 ± 0.137	1.04
3	122 ± 0.791	5.48
Serum		
1	86.7 ± 0.798	8.15
2	91.7 ± 0.112	1.10
3	91.7 ± 0.843	7.73

sponds to a limit of detection of 10 ng/ml for cotinine in serum and 50 ng/ml in urine.

Accuracy and precision

Table I shows the results of a three-day validation study for replicate analyses of aliquots of pooled OraSure, urine and serum samples from a group of smokers. The coefficients of variation in all three matrices were less than 9%. The slopes of the calibration curves for the three days remained virtually identical.

Analytical recovery was determined by addition of known amounts of cotinine to OraSure, urine and serum samples. The results summarized in Table II indicate that the recoveries ranged from 84 to 100%.

TABLE II

INTRA-DAY ASSAY PRECISION AND ACCURACY FOR COTININE IN ORASURE, URINE AND SERUM

Cotinine (ng/ml)		Accuracy (%)
Actual	Measured (mean ± S.D., n = 3)	
OraSure		
24.2	22.3 ± 6.76	92.1
96.6	94.1 ± 6.80	97.4
Urine		
24.2	23.6 ± 3.74	97.5
96.6	96.4 ± 0.65	99.8
Serum		
24.2	20.3 ± 3.40	83.9
96.6	93.7 ± 7.26	97.0

Application

The cotinine levels in OraSure, urine and serum samples from a group of twenty subjects were determined. All samples were collected as described in the Experimental section and frozen at −20°C until ready for analysis. Samples were then subjected to the extraction and GC–MS methodology described above. OraSure samples were run undiluted, while urine samples were diluted 1:10 and serum samples were diluted 1:2 in PBS. A four-point calibration curve was also generated. Cotinine amounts were determined from the ratio of the peak area of cotinine to the internal standard, deuterocotinine, by interpolation from the regression line of the standard curve. The cotinine concentrations are summarized in Table III. The self-reported smoking status is also indicated. Benowitz [11] has suggested a cut-off of 10 ng/ml for saliva to assign smokers and non-smokers, and this level was also used in a recent study using OraSure samples [12]. The use of a serum cut-off of 10 ng/ml and a urine cut-off of 50 ng/ml has been reported [4]. When these cut-off values were used to assign smokers and non-smokers, the assignments in all three matrices match the self-reported status of the subjects perfectly.

CONCLUSION

A sensitive, accurate and precise analytical method for the determination of cotinine in three biological fluids has been developed. Application of this method to samples collected from a group of subjects indicate that OraSure samples provide a viable alternative to urine and serum for assay

TABLE III

COTININE CONCENTRATIONS IN A GROUP OF SMOKERS AND NON-SMOKERS

Subject	Self-reported status	Cotinine[a] (ng/ml)		
		OraSure	Urine	Serum
1	Non-smoker	ND	ND	ND
2	Non-smoker	ND	ND	ND
3	Non-smoker	ND	ND	ND
4	Non-smoker	ND	ND	ND
5	Smoker	58.3	547	198
6	Smoker	64.2	1600	285
7	Smoker	118	884	447
8	Non-smoker	ND	ND	ND
9	Smoker	63.9	986	224
10	Smoker	54.9	1550	357
11	Smoker	80.4	1070	284
12	Non-smoker	ND	ND	ND
13	Non-smoker	ND	ND	ND
14	Smoker	91.7	2820	315
15	Non-smoker	ND	ND	ND
16	Smoker	85.8	2030	286
17	Smoker	108	1510	352
18	Non-smoker	ND	ND	ND
19	Non-smoker	ND	ND	ND
20	Smoker	55.5	647	438

[a] The limits of detection of the method are 5 ng/ml in OraSure, 50 ng/ml in urine and 10 ng/ml in serum. ND = not detectable or below the lower limits of the assay.

of cotinine as an indicator of smoking status. Further studies on cotinine in OraSure fluid are in progress [12].

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