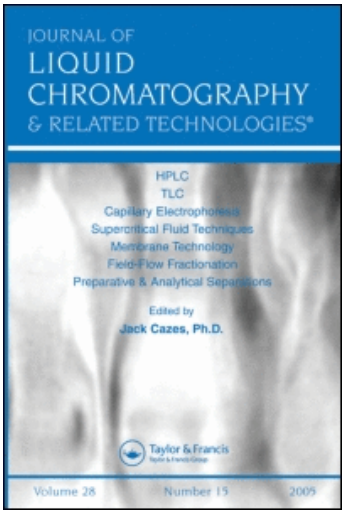

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Simultaneous Determination of Nicotine and Cotinine in Untreated Human Urine by Micellar Liquid Chromatography

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SIMULTANEOUS DETERMINATION OF NICOTINE AND COTININE IN UNTREATED HUMAN URINE BY MICELLAR LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple, precise, accurate and sensitive method for the simultaneous determination of nicotine and cotinine by direct injection of human urine was developed and validated. This method utilizes nontoxic, nonflammable and relatively inexpensive sodium dodecylsulphate (SDS) as a mobile phase and eliminates the need for pretreating the urine. Optimum conditions for the separation of cotinine and nicotine, the column efficiency, and the elution of non-alkaloid components in urine were investigated. Nicotine and cotinine in untreated human urine were separated and quantitatively eluted using 0.20 M SDS and 3% 2-propanol at a pH of 4.60 and column temperature at 40 °C. Retention times for nicotine and cotinine were: 10.85 and 9.48 minutes respectively, and all components in the urine sample were eluted within 15 minutes. This method may be suitable for use in hospitals, research centers, or in a physician's office.

INTRODUCTION

Nicotine accounts for nearly 95% of the total alkaloid content in commercial tobaccos (1). When nicotine is metabolized, 70% of it is converted to cotinine

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(2). Nicotine has a very short half-life and its excretion may be used to identify recent exposure (3). However, cotinine may indicate cigarette smoke exposure during the previous two to three days since it has a half-life of between 16 and 19 hours in various biological fluids (4,5).

A recent report on the detection of cotinine in semen, blood and urine, described the highest cotinine levels in urine and observed cotinine in 100% of the urine samples obtained from smokers (4). The authors also noted that cotinine concentrations increased with an increase in the number of cigarettes smoked per day or in the amount of passive smoke exposure. The presence of cotinine in urine has proved to be an effective marker of tobacco smoke exposure due to its sensitivity and specificity (2,5,6).

A simple and rapid analytical method for the simultaneous determination of nicotine and cotinine in urine would aid in identifying people who smoke or are exposed to environmental tobacco smoke, quantifying the correct dosage of nicotine for those who may need it for the therapeutic treatment of ailments such as Alzheimer's disease (7), or investigating the pharmacokinetics of non-tobacco-derived nicotine systems used in smoking cessation programs (8).

Various chromatographic methods have been developed and are used for the analysis of nicotine and its metabolites in bodily fluids. Nicotine and cotinine concentrations have been determined using high-performance liquid chromatography (2,3,9-15) and gas chromatography (7,8,16-18). A major drawback of these methods is the time-consuming derivatization, or solvent or solid-phase extraction process. An additional problem is the use and disposal of the toxic solvents and chemicals used for the derivatization or extraction processes.

Direct injection of urine eliminates time-consuming extraction procedures and avoids a protein precipitation step. Micellar chromatography is suited for direct injection because the proteins are solubilized by the micellar aggregates in the mobile phase and, therefore, are eluted with the void volume (19-21).

The purpose of this study was to develop a procedure for the simultaneous quantification of nicotine and cotinine by direct injection of human urine into a

micellar high-performance liquid chromatographic system. The applicability of this method to the assessment of human exposure to nicotine via tobacco use was demonstrated.

MATERIALS AND METHODS

Instrumentation

The HPLC system consisted of a Perkin Elmer Series 3 Liquid Chromatograph equipped with a variable wavelength LC-65T UV detector/oven, an LC1-100 integrator and a 20 μL Rheodyne sample injector (Model 7125, Berkeley, CA). The column (25 cm long and 4.6 mm i.d.) was packed with 5 micron Econosphere CN-bonded silica (Alltech, Deerfield, IL). A precolumn (25 cm long and 4.6 mm i.d.) packed with 50 micron silica gel (Alltech, Deerfield, IL) was located between the pump and sample injector to saturate the mobile phase with silica to minimize dissolution of the analytical column packing. The void volume of the system was calculated by using the peak of injected water. All pH measurements were performed with an ALTEX Model 3560 Digital pH meter and Corning combination glass electrode. The flow rate was fixed at 1.0 mL min^{-1}, the wavelength was set to 260 nm and the column temperature was maintained at 40.0 °C unless otherwise mentioned. The chart speed was maintained at 5 mm min^{-1} and at 40 mm min^{-1} when the column efficiency was studied. A double beam Hitachi U-2000 uv-vis spectrophotometer fitted with 1 cm cells was used for absorbance measurements.

Chemicals and Reagents

Sodium dodecylsulphate and 2-propanol were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Nicotine sulfate [(L-)-methyl-2-(3-pyridyl) pyrrolidine-sulfate] and cotinine [1-methyl-5-(3-pyridyl)-2-pyrrolidinone] were received from Sigma Chemical Company (St. Louis, MO). These chemicals were used as received. The stock solution of 0.20 M SDS was prepared in deionized water and filtered through a 0.45-μm membrane (Rainin Instrument

Co., Inc., Woburn, MA). The mobile phase was prepared in 0.05 M Na_2HPO_4 and adjusted to the desired pH with H_3PO_4. Stock solutions of 1140 ppm nicotine and 1100 ppm cotinine were prepared in methanol. Standard solutions of 36.29 ppm Nicotine and 79.00 ppm cotinine were prepared by appropriate dilution of the stock solutions with 0.10 M SDS. The stock solutions and the standard solutions were refrigerated when not used. The stability of nicotine and cotinine in the standard solutions was monitored each day by measuring their absorbance at 260 nm.

Preparation of Standards in Human Urine

Urine specimens were obtained from healthy nonsmoker and smoker volunteers. Each specimen was immediately acidified to a pH of 2.00 with phosphoric acid and filtered through a 0.20 μm Acrodisc syringe filter (Rainin Instrument Co., Inc., Woburn, MA). When the analysis was not to be performed immediately, the samples were frozen at -4.0 °C and thawed before using.

Blank urine samples containing 0.20, 0.53, 1.04, 1.98 and 2.85 ppm nicotine and 0.21, 0.35, 0.77, 1.51 and 2.87 ppm cotinine were prepared by spiking 2.00 mL of urine with the appropriate amounts of the standard solutions of nicotine and cotinine. These solutions were prepared fresh each day.

RESULTS AND DISCUSSION

Studies to determine optimal conditions for separation and elution of nicotine and cotinine were performed using the alkaloids dissolved in surfactant only. Once optimal conditions were determined, applicability of this method was validated using untreated urine from a smoker.

Separation of Nicotine and Cotinine

The effect of pH on the retention of nicotine and cotinine was examined in the range of 3.00 - 7.00. The capacity factor decreased slightly when the pH of the mobile phase increased (Figure 1). On the basis of pK_a values (pK_{a1} = 3.15, pK_{a2} = 7.85) nicotine and cotinine are predominantly cationic throughout

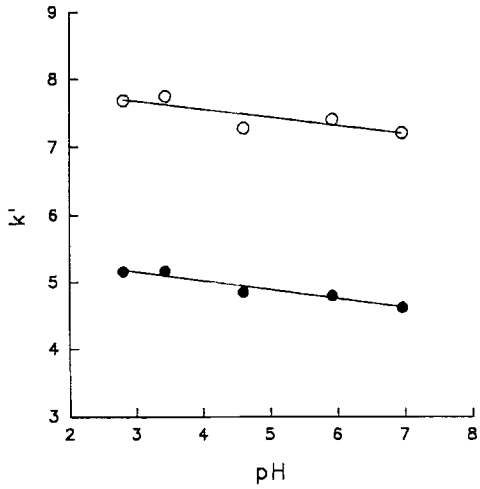

Figure 1: Dependence of capacity factor on pH of the mobile phase with 0.10 M SDS in 0.05 M phosphate buffer and 3% 2-propanol at 40.0 °C. (○) = nicotine and (●) = cotinine.

most of the pH range studied. A significant contribution from electrostatic interaction would cause an increase in retention time as the mobile phase pH increased. Therefore, the contribution from the hydrophobic interaction of the neutral species of nicotine and cotinine with the micelle and the stationary phase may be the predominate force. Based on these results, the analysis of nicotine and cotinine can be performed without careful adjustment of the pH of the mobile phase.

Figure 2 shows the dependence of the capacity factor on the surfactant concentration in the range of 0.01 - 0.20 M at pH 4.60. Increasing the SDS concentration to 0.20 M resulted in an approximately five-fold decrease in capacity factor for both nicotine and cotinine. This is most likely due to an increase in the number of micelles in the mobile phase as the concentration of surfactant increases (22). When the results were replotted as 1/k' against [SDS], a linear relationship was obtained (Figure 3). This behavior also is expected

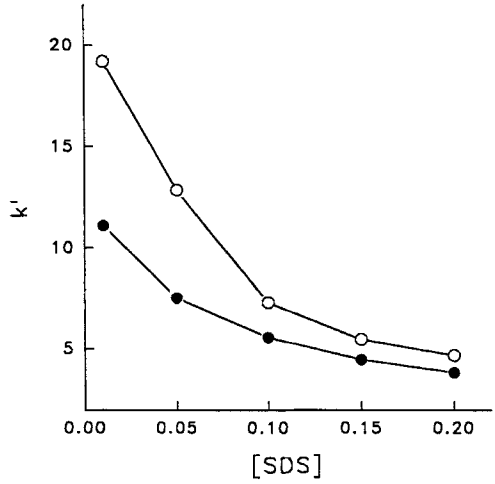

Figure 2: Dependence of capacity factor on [SDS] of the mobile phase containing 3% 2-propanol at pH 4.60 and 40.0 °C. (○) = nicotine and (●) = cotinine.

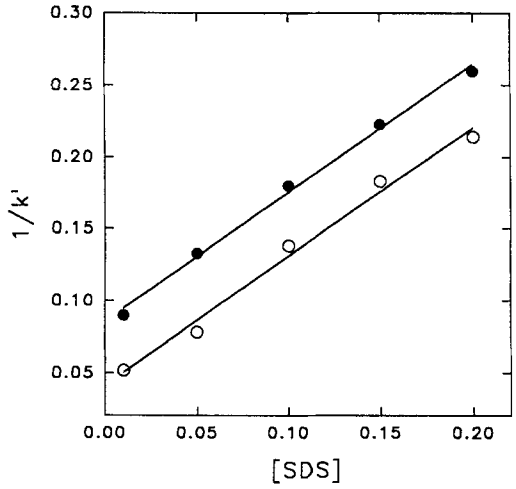

Figure 3: Variation of 1/k' with [SDS] of the mobile phase under conditions as for Figure 2. (○) = nicotine and (●) = cotinine.

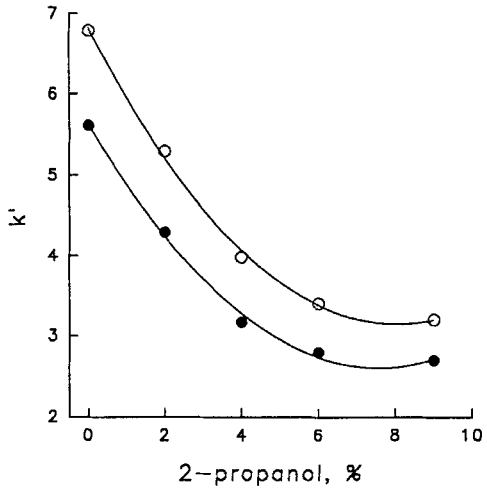

Figure 4: Dependence of capacity factor on percent of 2-propanol: 0.15 M SDS and pH 4.60. (○) = nicotine and (●) = cotinine.

when hydrophobic interaction is the predominant factor contributing to the retention time (22).

When the percentage of 2-propanol in the mobile phase was increased from 0.0 to 9%, the retention time decreased sharply between 0 and 4% and was less affected by greater concentrations (Figure 4). Increasing the percentage of 2-propanol decreases the concentration of micelles in the mobile phase and, as a consequence, the retention time should increase. The decrease in retention time may be related to a decrease in the polarity of eluent and a subsequent increase in solubility of nicotine and cotinine.

In an attempt to improve the column efficiency as reported by other workers (23), the effect of 2-propanol added to the mobile phase was studied. The number of theoretical plates (N) and the asymmetry ratios (B/A) from the observed peaks were compared. The values of N were calculated from the following equation which corrects for the asymmetry of skewed peaks (24):

$$N = \frac{41.7\ (\frac{t_R}{W_{0.1}})^2}{B/A\ +\ 1.25} \quad .....(1)$$

where: t_R is retention time,

$W_{0.1}$ is the peak width measured at 10% peak height,

B/A is the asymmetry ratio.

The results are shown in Table 1. The presence of 3% of 2-propanol doubled the plate count of nicotine and improved its peak symmetry as well as that of cotinine.

The variation of capacity factor with temperature was studied from 22.0 to 54.0 °C (under [SDS] = 0.20 M, pH = 4.60 and 3% 2-propanol). The plot of ln k′ against 1/T (van't Hoff plot) was linear with a more significant increase observed in the capacity factor of cotinine than nicotine. This difference in slopes is related to the difference in the thermodynamic distribution behavior of these species. In general, the overall changes in the capacity factor of nicotine and cotinine is low over the temperature range studied. Therefore, experiments can be performed without careful temperature control.

The effect of column temperature on both the peak symmetry and column efficiency was also studied. Again an improvement was seen in both peak symmetry and efficiency as the temperature was increased from 20.0 to 54.0 °C (Table 2).

Elution Studies on Urine Matrix

Previously reported investigations (19-21) on the use of micellar chromatography have not examined the optimum conditions for eluting urine. To determine conditions which minimize elution time of non-alkaloid components in urine, the following studies were performed.

When the surfactant concentration was increased from 0.01 to 0.20 M, the retention time of the last eluted species in the blank urine sample decreased to 6.64 min (Figure 5). In the presence of 0.20 M SDS, temperature (from 22.0 to

TABLE 1

Variation of Efficiency and Asymmetry with 2-propanol Concentration

	Nicotine		Cotinine	
2-propanol, %	N	B/A	N	B/A
0	825	2.71	2024	1.88
3	1969	1.40	2538	1.52
6	2032	1.42	2601	1.49
9	1976	1.52	2539	1.55

0.15 M SDS at pH 4.60 and 1.0 mL min^{-1}; temperature 40.0 °C; wavelength 260 nm.

TABLE 2

Variation of Efficiency and Asymmetry with Temperature

	Nicotine		Cotinine	
Temperature, °C	N	B/A	N	B/A
20.0	1126	2.15	1325	2.11
43.0	1984	1.36	2323	1.46
54.0	1737	1.42	2201	1.53

0.15 M SDS and 3% 2-propanol at pH 4.60 and 1.0 mL min^{-1}; wavelength 260 nm.

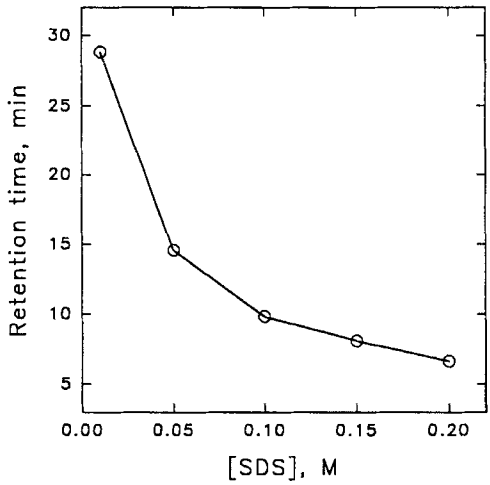

Figure 5: Variation of the retention time of the last species of the urine matrix on [SDS] of the mobile phase under conditions as for Figure 2.

54.0 °C) studied had no effect on the elution of components in urine (average retention time of 6.89 ± 0.80 min). The retention of the last eluted peak was slightly dependent on the acidity of the mobile phase. It increased from 9.68 to 10.78 min when the pH of the mobile phase (containing 0.10 M SDS, 3% 2-propanol and column temperature of 40.0 °C) was increased from 2.80 to 7.00.

Direct Sample Injection

Based on these studies a mobile phase consisting of a solution containing 0.20 M SDS and 3% 2-propanol at a pH of 4.60 (column temperature 40 °C) was chosen as optimum for quantitation of nicotine and cotinine in untreated urine. Under these conditions retention times for nicotine and cotinine were: (10.85 ± 0.33 minutes) and (9.48 ± 0.28 minutes) respectively, and all components in the urine sample eluted within 15 minutes (Figure 6). It is worth mentioning that elution of blank urine and that of a smoker were run for about three hours without noticing additional peaks. In addition, no significant changes in retention

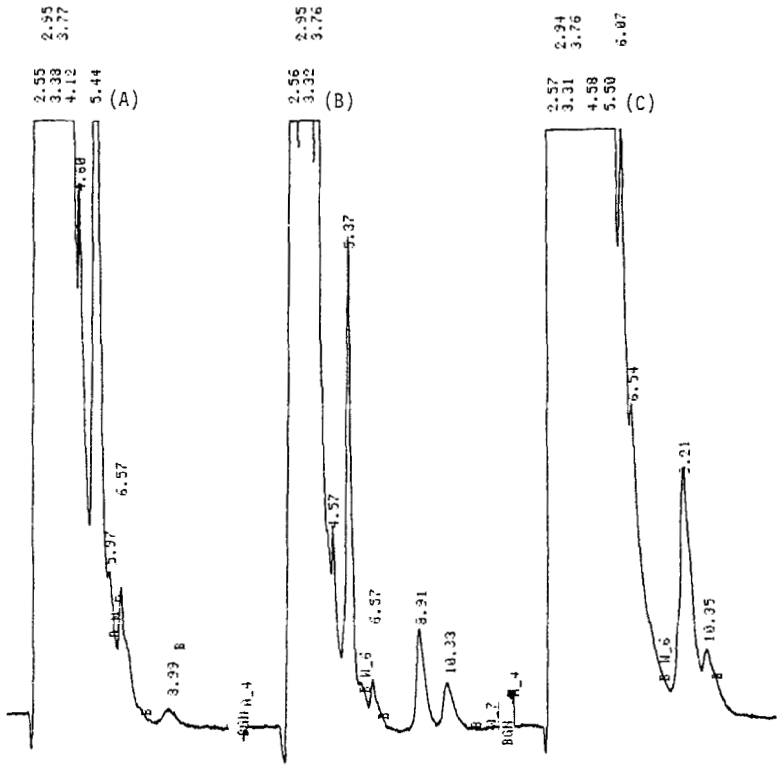

Figure 6: Typical chromatograms of (A) a non-smoker's urine (blank urine) (B) blank urine spiked with 1.23 ppm nicotine and 1.42 ppm cotinine; (C) a smoker urine. Conditions: 0.20 M [SDS], pH 4.60, 3% 2-propanol and 40.0 °C.

time, pressure, or carry over peaks from one injection to another were observed during this study.

The recovery of nicotine and cotinine from spiked urine was studied by adding different quantities of these species to blank urine samples. The results are shown in Table 3. The average percent recovery for nicotine was 100 ± 3 over a 0.53 - 1.98 ppm concentration range and the recovery for cotinine was 101 ± 2 over a 0.77 - 2.87 ppm concentration range. Based on these results both alkaloids can be quantitatively recovered.

TABLE 3

Recovery of Nicotine and Cotinine from Urine Samples

Nicotine			Cotinine		
Conc. (ppm)	Recovery %	Average Recovery	Conc. (ppm)	Recovery %	Average Recovery
0.53	109 106 100	105±4	0.77	106 100 104	103±3
1.04	100 90 97	96±4	1.51	103 97 100	100±3
1.98	97 97 101	98±2	2.87	99 100 97	99±1

0.20 M SDS and 3% 2-propanol at pH 4.60 and 1.0 mL min^{-1}; temperature 40 °C; wavelength 260 nm.

The sensitivity of the method was investigated by preparing calibration plots of 20 μL each of nicotine and cotinine in urine. Plots of the peak height against concentration injected were linear from 0.20 to 2.85 ppm nicotine and 0.21 to 2.87 ppm cotinine with correlation coefficients greater than 0.9996. The typical linear relationship for the calibration curve can be expressed by the following regression equations:

$$\textit{Peak Height} = 3.265\ [\textit{nicotine}] - 0.006 \quad .....(2)$$

$$\textit{Peak Height} = 5.268\ [\textit{cotinine}] + 0.001 \quad .....(3)$$

TABLE 4

Precision and Accuracy of Concentration Measurements of Nicotine and Cotinine in Urine Samples

Nicotine				Cotinine			
Conc. (ppm)	Average±SD	CV	E_r %	Conc. (ppm)	Average±SD	CV	E_r %
0.20	0.19±0.02	10.5	-5.0	0.21	0.20±0.02	10.0	-5.0
0.53	0.54±0.01	1.9	1.9	0.35	0.34±0.02	5.9	-2.9
1.04	1.03±0.03	2.8	-1.0	0.77	0.81±0.03	3.7	5.2
1.98	1.90±0.06	3.1	-4.0	1.51	1.48±0.03	2.0	-2.0
2.85	2.87±0.06	2.1	0.71	2.87	2.89±0.03	1.0	0.70

0.20 M SDS and 3% 2-propanol at pH 4.60 and 1.0 mL min^{-1}; temperature 40 °C; wavelength 260 nm. Four replicates at each concentration.

where the peak height is in cm and the concentration is in ppm. The minimum detectable levels of nicotine and cotinine were found to be 0.18 ppm and 0.11 ppm based on a signal-to-noise ratio of 3 with an injection volume of 20 μL. These detection limits are comparable to those values reported for nonsmokers (3).

The precision and accuracy of this technique was determined by making replicate measurements of five different standards within a concentration range of 0.20 to 2.85 ppm nicotine and of 0.21 to 2.87 ppm cotinine. Very low relative standard deviations with low relative errors were obtained (Table 4).

Analytical Applications

The amount of nicotine and cotinine in a pooled 24-h urine sample from a healthy smoker volunteer using the optimal conditions described was

determined. A 25.00 mL sample was acidified to pH 2.00 and filtered before injection. Using the regression equation from the calibration curve and recovery values, the amount of nicotine and cotinine in the urine was determined to be: 0.677 μg nicotine/mL and 5.483 μg cotinine/mL. These results are comparable to those reported for a smoker (2-4).

CONCLUSIONS

The micellar liquid chromatographic method described is a simple, precise, accurate and sensitive method for the simultaneous determination of nicotine and cotinine in human urine. It utilizes a mobile phase which is nontoxic, nonflammable and relatively inexpensive, and eliminates the need for pretreating the urine. A complete analysis can be obtained within 15 minutes. Therefore this method is suitable for use in clinical and pharmacokinetic studies, or in a physician's office.

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