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Increasing urinary cotinine concentrations at elevated temperatures: the role of conjugated metabolites[1]

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Abstract

The presence of cotinine, a nicotine metabolite, in urine above a specified cutoff concentration is commonly used to distinguish smokers from nonsmokers, as in smoking cessation studies. A stability study of cotinine in urine was carried out after questions arose concerning analyte stability at elevated storage and shipment temperatures. Aliquots from a smokers urine pool were stored at 5, 25, 40, 50 and 60°C for 30 days. Another set of aliquots, obtained by diluting the smokers pool 1:1 with nonsmokers urine, were stored under the same conditions. Free cotinine levels, determined by a stability-indicating gas chromatographic/mass spectrometric (GC/MS) assay, increased over the 30-day period at higher storage temperatures. Cotinine concentrations in the aliquots stored at 60°C, for example, nearly doubled over 30 days (1301 to 2476 ng/ml), with similar proportional increases observed in the aliquots diluted with nonsmokers urine. Since cotinine can be excreted to a large degree as cotinine-*N*-glucuronide, this conjugated metabolite was determined by an indirect method. As the storage temperature increased, the free/conjugated cotinine ratio dramatically increased, pointing to cotinine-*N*-glucuronide as the source of the additional free cotinine at the higher storage temperatures. The results of this study are of much practical importance, since urine samples with free cotinine concentrations just below a cutoff value may become positive for smoking status if suitably low temperatures cannot be maintained during sample handling and shipment. © 1997 Elsevier Science B.V.

Keywords: Nicotine; Cotinine; Stability; Urine; Gas chromatography/mass spectrometry

1. Introduction

The alkaloid nicotine (**I**, Fig. 1) has received increased attention in recent years, both in the news media and the scientific literature, mainly due to the public health concerns surrounding its addictive properties and presence in tobacco products. Nicotine is ubiquitous in the environ-

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ment due to the widespread use of tobacco products, being detected in the blood of both smokers and non-smokers alike [1]. The determination of nicotine in various body fluids to distinguish between smokers and non-smokers is of limited value due to the short time course of nicotine in the body, having a plasma half-life of approximately 2 h. However, cotinine (**II**), the 5′-oxo metabolite of nicotine, has proven to be a more reliable indicator of smoking status due to its longer plasma half-life ($\sim$ 20 h), resulting in relatively constant blood levels throughout the smoking day [2]. Although blood (plasma) and saliva are considered the specimens of choice when making quantitative assessments of tobacco smoke exposure and/or nicotine intake [3], the convenient and non-invasive nature of urine make it an attractive specimen, particularly when assessing the smoking status of a large number of individuals, as in smoking cessation programs [4].

Following the completion of a large-scale smoking cessation research project at one of our institutions, questions arose concerning the stability of cotinine in other urine specimens. A protocol required that follow-up urine specimens be obtained from remote locales, often with little or no provisions for frozen shipment. Another proposed study, designed to assess the incidence of smoking in a large military population, called for the use of forensic urine specimens deemed negative for commonly encountered drugs of abuse. Due to chain of custody procedures, the negative specimens could not be released from room temperature storage for up to several weeks. To ensure the validity of these and future studies where specimen storage and shipment conditions may be questionable, we undertook an accelerated stability study of cotinine in human urine.

An initial experiment involved storing samples of a smokers urine pool at room temperature and at three elevated temperatures over 30 days. Cotinine was analyzed using a commercial fluorescence polarization immunoassay kit (FPIA, Abbott TDx) no longer marketed. Cotinine levels increased over the 30-day storage period, almost doubling at higher temperatures. These unexpected and possibly questionable results prompted us to repeat the study, now employing a stability-indicating gas chromatographic/mass spectrometric (GC/MS) method for analysis. Herein we describe the results obtained in the experiment employing GC/MS, provide an explanation for the seemingly anomalous data and comment on the practical implications of these findings.

2. Experimental

2.1. Reagents

Methanol (Fisher Scientific, Fairlawn, NJ) and ethyl acetate (Aldrich, Milwaukee, WI) were HPLC grade. Diethyl ether (Fisher) was reagent grade. Cotinine and deuterated cotinine (cotinine-d_3, internal standard) were obtained from Sigma (St. Louis, MO). Stock solutions of cotinine (1.0 mg/ml^{-1}) and cotinine-d_3 (200 μg ml^{-1}) were prepared using methanol and stored at -20°C in sealed volumetric flasks. A working internal standard solution was prepared by diluting cotinine-d_3 stock solution 1:10 with methanol. Standards were prepared by first diluting the cotinine stock solution 1:100 with water (10 000 ng ml^{-1}), then further diluting this solution to 3000, 2500, 1000, 500, 250 and 125 ng ml^{-1}.

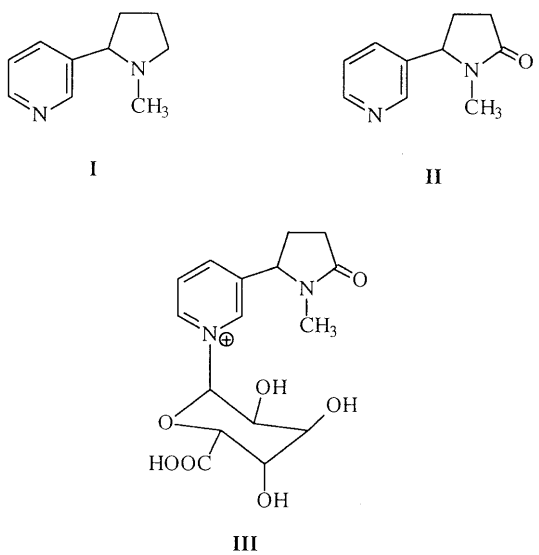

Fig. 1. Structures of nicotine (**I**), cotinine (**II**) and cotinine-*N*-glucuronide (**III**).

2.2. Urine storage and sampling

Urine from five smokers was pooled and aliquoted into teflon-lined screw cap glass test tubes. To possibly account for any concentration-dependent degradation of cotinine, a second urine pool was made by diluting smokers urine 1:1 with urine from a non-smokers pool. The urine aliquots were separated into five groups and stored under refrigeration (5°C), at room temperature (25°C) and in incubators maintained at 40, 50 and 60°C. Allowable variation was ± 2°C for all storage temperatures. A staggered sampling protocol was used, so that each group of urine aliquots was sampled at least five times throughout a 30-day period. On each sampling day, a tube of urine was removed from storage at the appropriate temperature, aliquoted into Eppendorf-type snap-closure centrifuge tubes and stored at -80°C until analysis. Urine pH was measured at each sampling time with a pH meter (model 6007, Jenco Instruments, San Diego, CA). Visual observations were also noted.

2.3. Sample preparation

Prior to extraction, samples were allowed to thaw and equilibrate to room temperature. Urine (1 ml) was added to a 13×100 mm teflon-lined screw cap glass tube followed by 100 μl cotinine-d_3 working solution and brief vortex mixing. Sodium hydroxide (12 N, 100 μl) was added to the mixture, followed by 4 ml diethyl ether. The tube was tightly capped and placed on a rotary mixer for 30 min. The diethyl ether layer was then transferred via a Pasteur pipet to a second 13×100 glass tube and evaporated to dryness under a stream of nitrogen using a Reacti-vap/Reacti-therm module (Pierce, Rockford, IL) maintained at 40°C. The resulting residue was dissolved in 50 μl ethyl acetate and tranferred to an amber 2 ml autosampler vial containing a 100-μl microvial insert. The vial was capped and placed in the autosampler tray for analysis. Aqueous cotinine standards were extracted using the same procedure. Urine samples and aqueous standards were prepared in duplicate.

2.4. GC/MS

The GC/MS components were all from Hewlett Packard (Palo Alto, CA), consisting of a 5890 Series II gas chromatograph and a 5971A mass selective detector operated via HP Chemstation software (version B.00.02). A 12 m $\times$ 0.2 mm $\times$ 0.33 μm film thickness cross-linked methyl silicon gum column was used (HP-1, Hewlett-Packard) with helium as the carrier gas. A 1-μl aliquot was introduced into the injection port (200°C) with a solvent delay of 3.5 min and purge valve on time of 0.75 min. The temperature program was as follows: 100°C (1 min) then 15°C/min to 260°C, resulting in a total run time of 11.67 min. The mass spectrometer was tuned prior to each use with perfluorotributylamine. The ionization energy was 70 eV with a source temperature of 280°C. The GC/MS was operated in the selected ion monitoring (SIM) mode. SIM ions were 176 and 119 amu (cotinine) and 179 and 122 amu (cotinine-d_3).

Standard curves were prepared by plotting 176/179 ion ratios versus standard concentrations and using regression analysis to arrive at best-fit linear equations. Sample cotinine levels were determined by solving the equations for concentration and substituting the appropriate 176/179 ion ratios.

2.5. Assay validation and stability-indicating capacity

The assay was linear over the 125–3000 ng ml^{-1} range ($r = 0.9951$ to 0.9999). Within-day and between-day precision were both less than 5% relative standard deviation (R.S.D.) ($n = 5$). System precision, determined by making five replicate injections of a prepared sample, was 0.27% R.S.D. Absolute recovery was 78.6%, determined by comparing the 176 ion peak area of spiked non-smoker urine with a cotinine standard prepared in ethyl acetate (1000 ng ml^{-1}). Relative recovery (104%) was determined by comparing 176/179 ion ratios for extracted aqueous standards (1000 ng ml^{-1}) with the responses of spiked non-smokers urine ($n = 5$). Prepared samples were shown to be stable for at least 18 h while stored in the autosampler rack, based on lack of change in 176/179 ion ratios.

Urine samples were subjected to extremes of pH and temperature to test for possible interference from degradation products. Three aliquots (1.0 ml) from the smokers urine pool were treated with either 1 N HCl (200 μl), 1 N NaOH (200 μl) or left untreated. All aliquots were subjected to a standard autoclave cycle (121°C for 15 min) to force degradation of urine components. Sample pH was readjusted, cotinine was extracted and GC-MS analysis was carried out as described above. No peaks interferring with cotinine were present in the extracted ion chromatograms (176 and 179 amu).

2.6. Cotinine glucuronide analysis

Selected frozen urine aliquots were shipped via overnight courier to the San Francisco General Hospital Medical Center location for cotinine-*N*-glucuronide analysis. Cotinine-*N*-glucuronide concentrations were determined as described previously [5] by measuring free cotinine concentrations before and after hydrolysis with β-glucuronidase using an established GC/MS method [6,7].

3. Results and discussion

Operation of the GC/MS in the SIM mode is standard procedure when quantitation is desired and makes for a very specific assay. Representative chromatograms are shown in Fig. 2. The urine extraction procedure and GC/MS method worked well for the purposes of this experiment. However, with a limit of detection of approximately 20 ng ml^{-1} (3 times the signal-to-noise ratio), this method would not be suitable for measuring the extremely low cotinine levels associated with environmental exposure to nicotine and/or tobacco smoke.

Table 1 shows the change in urinary cotinine concentrations during the course of the experiment. Cotinine levels remained relatively constant in the refrigerated urine specimens, but increased over the 30-day period when stored at room temperature and higher. The rate of appearance of cotinine was generally greater as the storage temperature increased, with cotinine concentrations in the aliquots stored at 60°C nearly doubling over 30 days (1301 to 2476 ng ml^{-1}). A very similar pattern was observed for the pool diluted 1:1 with non-smokers urine.

Urinary pH was approximately 5.8 at the outset of the experiment, for both the diluted and undiluted pools and remained essentially unchanged in the refrigerated samples. An overall increase in pH was noted at higher temperatures, with the 40°C samples exhibiting the the most rapid change (5.84 to 8.66 by Day 7). This was expected since 40°C is near optimum for microbial growth and the concomitant production of ammonia. Samples stored at 50 and 60°C increased approximately one pH unit, from $\sim$5.8 to 6.8. Interestingly, the samples stored at 25°C that were diluted 1:1 with non-smokers urine maintained a relatively constant pH of $\sim$5.8, whereas the smokers pool became more basic ($\sim$pH 5.8 to 8.6). It is noteworthy that the cotinine concentration increased to a much greater extent in the undiluted smokers urine samples stored at 25°C than in the diluted smokers samples. This can be explained by the much greater pH increase in the undiluted sample, since it is known that cotinine glucuronide is hydrolyzed under basic conditions [5]. The undiluted smokers urine stored at 25 and 40°C also became flocculated, with visible crystals. Microscopically, the isolated crystals were irregularly-shaped, variable in size, occasionally enclosed, highly polarizing and unidentifiable by a clinical laboratory on premises. Crystals were not present in the samples from pool diluted with nonsmokers urine.

The results from the preliminary stability experiment, utlizing the immunoassay (FPIA) to quantitate cotinine, were initially thought to be anomalous, perhaps due to one or several confounding factors. The FPIA method for cotinine is not stability-indicating; therefore, the possibility existed that urinary degradation products formed at higher temperatures, in addition to increasing urinary pH, interferred with the FPIA, resulting in falsely elevated cotinine levels. Initial adsorption of cotinine to the plastic containers used in that experiment, followed by desorption at higher temperatures was also considered. However, when

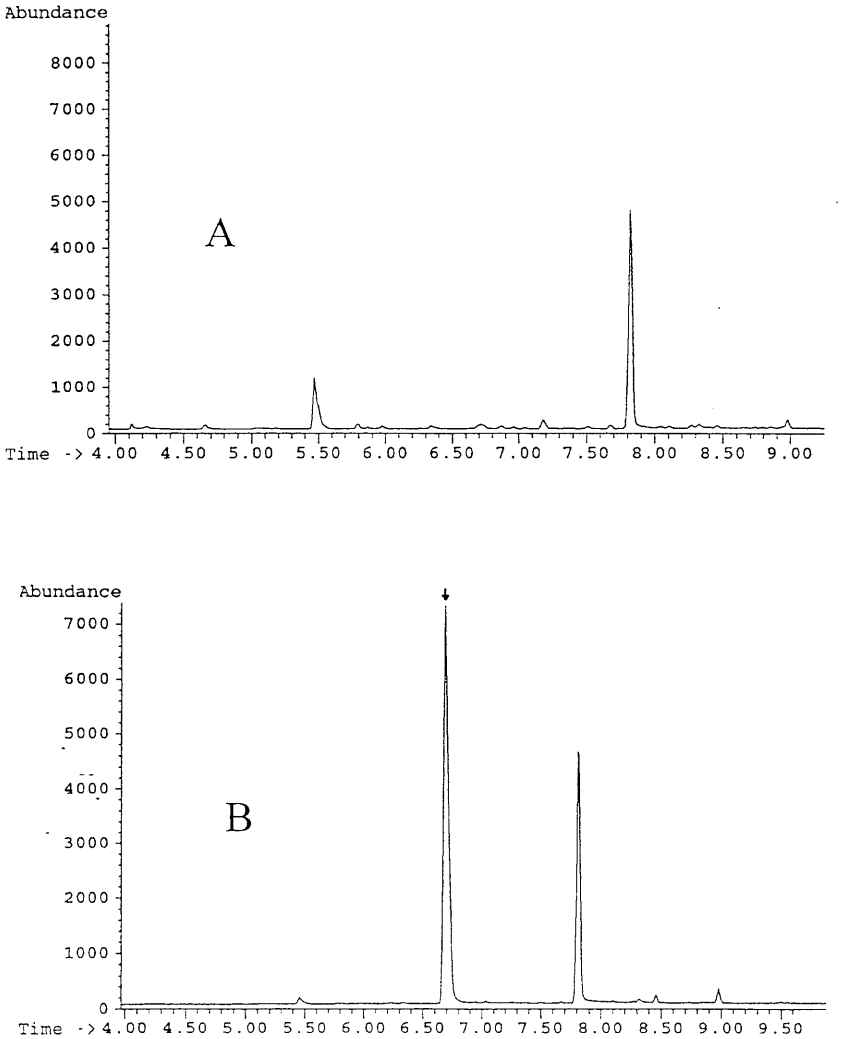

Fig. 2. Representative ion chromatograms (176 m/z) obtained by GC/MS analysis of urine extracts. Chromatogram A, a non-smoker's urine; chromatogram B, urine from a smokers' urine pool. Cotinine had a retention time of approximately 6.7 min (arrow). An unidentified peak/artifact at approximately 7.8 min was present in both samples. Note that the time scale for chromatogram A is 4.00–9.00 min, compared to 4.00–9.50 min for chromatogram B.

similar results were obtained using the stability-indicating GC/MS method described herein, another explanation was sought.

The possibility of extensive metabolic conjugation of cotinine and subsequent hydrolysis at elevated temperatures was proposed to account for the large increase in free cotinine levels over time. Recently published research revealed that approximately 50% of total excreted cotinine could be accounted for as cotinine-*N*-glucuronide (**III**) [5]. To investigate the contribution of cotinine-*N*-glucuronide to the increasing free cotinine concentrations observed at elevated temperatures, final urine samples from either Day 26 or 30, depending on the sampling protocol, were assayed for cotinine-*N*-glucuronide as described above. The final day cotinine concentrations, reported as free and conjugated cotinine are shown in Fig. 3. It is

graphically evident from Fig. 3 that free and conjugated cotinine were present in roughly equal amounts in the samples stored at 5°C. As the storage temperature increased, the free/conjugated cotinine ratio dramatically increased, pointing to cotinine-*N*-glucuronide as the source of the additional free cotinine at the higher storage temperatures.

Based on the results presented in Table 1 and Fig. 3, along with the observed pH and visual changes, the rate of appearance of free urinary cotinine seemed to be highly temperature-dependent and relatively pH-independent. However, urinary pH may be highly dependent on microbial content and may vary with source and handling procedures.

The findings of this study are of much practical importance to persons involved in the analysis of urinary cotinine, particularly if suitably low temperatures cannot be maintained during sample handling and shipment. Although samples with cotinine concentrations well above a cutoff value will not be affected if only a qualitative result is desired, samples just below a cutoff value upon collection may be positive at analysis if exposed to higher temperatures. Obviously, samples exposed to higher temperatures for cumulatively greater time periods will be at the most risk for misleadingly high cotinine levels. This problem could be circumvented by incorporating a glucuronide hydrolysis step in the sample preparation procedure and reporting results as total urinary cotinine. However, most studies involving urinary cotinine determinations have used free cotinine. It is also possible that adding acid to stored urine samples would retard hydrolysis of the glucuronide, since quaternary *N*-glucuronides are resistant to acid catalyzed hydrolysis [8]. Experiments designed to assess free urinary cotinine changes during actual shipment may be useful in assuring consistent results.

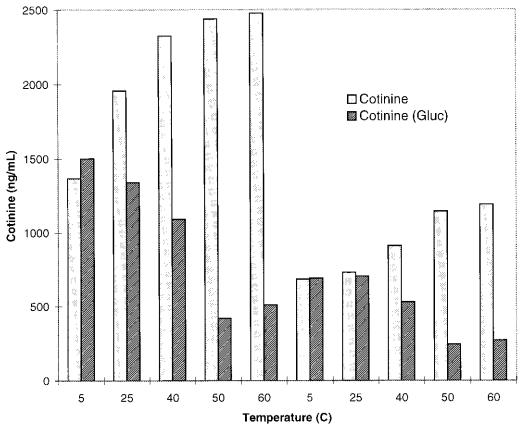

Fig. 3. Bar graph depicting final day cotinine concentrations, given as both free cotinine and cotinine conjugated with glucuronic acid, in undiluted (left) and diluted (right) smokers' urine pools.

Table 1
Free cotinine concentrations (ng ml^{-1}) in undiluted and diluted smokers' urine pools at the indicated storage temperatures

Storage day	Storage temperature (°C)				
	5	25	40	50	60
Undiluted smokers' urine					
0	1301	1301	1301	1301	1301
1		1324	1409	1379	
3	1357		1427		1517
6		1488		1875	
7	1365		1889		1843
10		1483		2121	
14	1549		1891		1955
17		1762		2333	
21	1418		2055		2186
26		1956		2437	
30	1366		2323		2477
Diluted smokers' urine[a]					
0	643	643	643	643	643
1		679		719	606
3	648		710		770
6		681		816	
7	685		760		821
10		705		1035	
14	668		796		975
17		716		1094	
21	677		849		1064
26		729		1143	
30	683		910		1189

[a] Represents a pool of smokers' urine diluted 1:1 with urine from a non-smokers' pool.

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