National Institute of Standards & Technology

Report of Analysis

EPA/NIST Reference Material 8444

Cotinine in Freeze-Dried Human Urine

This Reference Material (RM) is intended primarily for use in validating methods for the determination of cotinine in human urine. A unit of RM 8444 consists of four vials, each containing cotinine in 5 mL human urine, which has been freeze dried. Two vials are "blank" concentration levels, typical of nonsmokers without exposure to cigarette smoke; one vial is a "low" concentration level corresponding to nonsmokers with passive exposure to side-stream smoke; and one vial is a "high" level, typical of smokers.

Recommended Concentrations of Cotinine

The recommended concentrations of cotinine in the blank, low, and high level urine materials after reconstituting the freeze dried materials with 5.00 mL of distilled water are:

Blank level	0.8	± 0.3	ng/g
Low level	54	$^{+\ 2}_{-\ 5}$	ng/g
High level	488	$^{+\ 4}_{-\ 10}$	ng/g

The low and high level concentrations are based on the total mass of cotinine added to the urine and experimental measurements by gas chromatograph-mass spectrometry (See Table 1). The estimated total uncertainties are based on judgement and represent method imprecision, possible systematic errors, and allowances for possible degradation.

To convert concentrations from ng/g to ng/mL, multiply by the density of the reconstituted urine, 1.01 g/mL.

Expiration of Recommended Concentrations: The recommended concentrations are valid within the reported uncertainties for one year from the date of shipment. In the event that the concentrations change before then, users will be notified by NIST. Please return the attached registration card to facilitate notification.

Storage: RM 8444 should be stored in a freezer at or below 0 °C and should not be exposed to sunlight or ultraviolet radiation.

Use: To reconstitute a sample, pipet 5.00 mL distilled water into the vial, taking care to wet all solid material. Shake and/or sonicate the reconstituted sample until all solid material has dissolved (approximately one minute). Use sample immediately after dissolving (do not store reconstituted sample). The "blank" level samples can be used as a diluent for the low and high level concentrations.

Toxicity: This RM contains freeze-dried pooled human urine, and the pathogenicity and/or toxicity has not been determined. This material should be treated as a potential health hazard and appropriate care should be exercised in handling and disposal.

Preparation of this reference material and analytical determinations for this material were made in the Organic Analytical Research Division, Center for Analytical Chemistry, by L. C. Sander and G. D. Byrd.

The coordination of the technical measurements were performed by L. C. Sander and W. E. May.

The technical and support aspects involved in the preparation, measurement, and issuance of this Reference Material were coordinated through the Office of Standard Reference Materials by R. Alvarez.

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Preparation and Analysis

Pooled human urine was used in the preparation of RM 8444. Separate urine collections were made for the three materials. Donors (nonsmokers) were advised to avoid any exposure to tobacco smoke or caffeine for at least 48 h prior to the collection. The low and high level cotinine solutions were prepared gravimetrically using cotinine perchlorate. Appropriate amounts of cotinine perchlorate were weighed and dissolved in distilled water and the solutions were transferred quantitatively to the pooled urine. Cotinine was not added to the blank level material.

Five-mL aliquots of the urine reference material were pipetted into vials and then freeze dried. Before freeze-drying, the samples were frozen at -80 °C. Freeze drying was considered complete when the shelf and probe temperatures were at ambient temperature, and the pressure was less than 13.3 Pa (100 μm Hg pressure). The freeze-dried samples were sealed in the vials under a slight negative pressure.

Primary Cotinine Standards:

Because the free base form of cotinine is hygroscopic, cotinine perchlorate was used as the primary standard for this reference material. Cotinine perchlorate was synthesized from cotinine (Aldrich Chemical Co.) and 70% perchloric acid using the method of Jacob and Benowitz (1).

Purity was determined by differential scanning calorimetry over the temperature interval 210-220 °C. The purity was determined as 99.91 mole percent (melting point 218 °C). No evidence of organic impurities was observed by LC or GC analyses. A sample of the product was submitted to Galbraith Laboratories (Knoxville, TN) for elemental analyses. Duplicate determinations were made for carbon, hydrogen, nitrogen, chlorine, and oxygen. On the basis of these characterizations, product purity was accepted to be greater than 99 mole percent, and no corrections for sample impurity were made.

Deuterated cotinine (cotinine-d_3) was obtained from Cambridge Isotope Laboratories (Woburn, MA). The isotopic purity determined by GC-MS was 98.7%. Corrections were made for the undeuterated cotinine impurity in all calculations. Because deuterated cotinine was obtained in the free base form and is hygroscopic, possible weight gain from adsorbed water precluded the actual concentration of the internal standard solution from being known accurately. The approximate concentration of this solution (12.4 μg/g) was used only in minor corrections in response factors involving isotopic purity of the internal standard. The same internal standard solution was used throughout this study for both unknowns and response factor solutions.

GC-MS Analysis

A solution of cotinine-d_3 (approximately 12.4 μg/g) was prepared in methanol and was ampouled for later use. This solution was used as an internal standard for both calibration standards (response factor solutions) and for the unknowns (urine reference materials). Four separate response factor solutions were prepared. Independent response factors were determined for each level. Separate response factors were calculated for 98/101 and 176/179 mass fragments, and cotinine concentrations were determined based on both mass sets.

Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the U.S. Environmental Protection Agency or the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for this purpose.

Six vials from the low and high level materials were selectedby a stratified random sampling scheme. Because the blank material has a very low cotinine concentration special procedures were required for an estimate of the cotinine in these samples (described below). The spiked urine materials were reconstituted with 5 mL distilled water by shaking and "sonicating" for 60 s. Each sample was spiked with 50 µL cotinine-d_3 internal standard solution, followed by an addition of 10 drops of 10 M KOH. The samples were then extracted with 5 mL methylene chloride. The methylene chloride layer was removed with a pipet and treated with 1-2 g sodium sulfate. The methylene chloride was decanted into a centrifuge tube and evaporated to dryness under argon. The residue was dissolved in 50 µL methanol and analyzed by GC-MS.

A DB-210, 250 µm i.d. x 25 m capillary column, was used for the GC-MS analysis of the urine extracts. The following temperature program was used in the GC separation: 185 °C initial temperature, linear ramp of 5 °C/min, and final temperature of 225 °C. Between GC runs, the column was briefly ramped to 230 °C. A typical chromatogram from the analysis is illustrated in Figure 1.

The concentration of cotinine in the unspiked urine "blank", was estimated by combining and analyzing ten vials from the beginning, middle, and end of the blank preparation. The uncertainty of this determination is large because of the low signal-to-noise ratios. Similar measurements were performed for unspiked samples of the pooled urine used for low and high level reference materials.

Cotinine levels were again determined after 160 days. During this period, the samples were stored at ambient temperature for four months, and at -20 °C for one month. The results of these determinations are listed in table 1. Cotinine levels were found to be slightly lower than previously determined; however, the values were still within the uncertainty of the measurements. The recommended concentrations for cotinine and associated uncertainties are derived from the gravimetric preparation of the material and two sets of measurements by GC/MS. The cotinine levels in SRM 8444 will be periodically monitored. Users will be notified of any changes in cotinine levels.

Table 1 Calculated, GC-MS, and recommended concentrations, in ng/g, for cotinine in urine reference materials.

Level	Calculated[a]	Initial Concentration by GC-MS	Concentration by GC-MS after 160 days	Recommended Concentrations
Blank	---	0.8 ± 0.3	---	0.8 ± 0.3
Low	55.47	56 ± 2	52 ± 3	54 $^{+2}_{-5}$
High	489.88	491 ± 6	485 ± 12	488 $^{+4}_{-10}$

[a] The calculated concentration is based on the weighed mass of cotinine added to the urine pool.

Reference

1. P. Jacob and N.L. Benowitz. Determination of cotinine in human biologic fluids. Recommendations for a quality control/quality assurance program. Cotinine Analytical Workshop (EPA), Research Triangle Park, NC, November 10-11, 1986.

Typical Ion Chromatogram for Analysis of RM 8444