The Certification of Cocaine and Benzoylecgonine in a Human Urine Standard Reference Material*

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Abstract

The concentrations of cocaine and benzoylecgonine (BE) in Standard Reference Material (SRM) 1508, cocaine and metabolites in freeze-dried human urine, were determined at the National Institute of Standards and Technology (NIST, formerly NBS) by two independent methods. For cocaine, one method was based on gas chromatography/mass spectrometry (GC/MS); the other was based on high-performance liquid chromatography (HPLC). For BE, one method was based on GC/MS; the other was based on flow injection analysis/thermospray mass spectrometry (FIA/MS). The results for each pair of methods were statistically evaluated. Concentrations were determined in the SRM for three levels of cocaine and three levels of benzoylecgonine. Methylecgonine, although present in the material, was not determined. For cocaine, the concentrations were 90, 263, and 429 ng/mL of human urine. For BE, the concentrations were 103, 259, and 510 ng/mL of human urine.

Introduction

Drug testing has experienced tremendous growth over the past few years. Concerns over the inaccuracy of drug testing have prompted the National Institute of Standards and Technology (NIST, formerly the National Bureau of Standards) to undertake the development of reference materials to validate accurate measurements in drug testing laboratories. NIST has previously developed a standard reference material (SRM 1507) that contains the major metabolite of marijuana in a lyophilized urine, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid. This report describes the determination of cocaine and benzoylecgonine concentrations in a lyophilized urine, SRM 1508.

When cocaine is administered to humans by oral, intravenous, or intranasal routes, it is quickly metabolized and excreted into the urine (1). The principal metabolites are benzoylecgonine (BE) and ecgonine methyl ester (methyl ecgonine), and typically they make up about 40% each of the total cocaine excreted. However, with the advent of "crack," freebase cocaine, there is evidence that free cocaine may be found in the urine at a relatively high level (1). Chronic users have been reported to have persistent levels of cocaine in urine and in saliva (2). For this reason, cocaine and both major metabolites are present in SRM 1508. However, only the BE and cocaine concentrations were determined.

For the detection of cocaine use, the metabolite BE is usually determined. For Federal workplace drug-testing programs, the BE cutoff level, defined as that concentration below which a test for the presence of BE will be reported as negative, was set at 150 ng/mL urine by the National Institute on Drug Abuse (3). No cutoff level has been established for cocaine because cocaine is not usually among the analytes determined in the urine. SRM 1508 consists of four vials of material: one vial of material at a concentration less than 150 ng/mL of each analyte, two at more than 150 ng/mL of each analyte, and one with no detectable concentration of the analytes for use as a blank.

Two independent methods were used to determine the concentration of each analyte, one of which was an isotope dilution GC/MS method, which is similar to methods generally used by drug-testing laboratories for confirmatory measurements. For cocaine, the first method used was isotope dilution GC/MS after a solid-phase extraction using a specially bonded, silica-based, mixed-phase solid-phase cartridge. The second method used was HPLC. A cyclodextrin solid-phase cartridge was used for sample clean-up. Quantitation was then achieved by passing the sample through a C_{18} HPLC column, using cocaethylene as the internal standard, and detecting the cocaine at 233 nm, its absorbance maximum. For BE, the first method used was an isotope dilution GC/MS method which was the same as that for cocaine, except that the BE was converted to the trimethylsilyl ester. The second method developed for BE used the same sample clean-up procedure as the HPLC method for cocaine, with quantitation of the extracts from the cleanup by isotope dilution flow injection analysis mass spectrometry (FIA/MS).

Experimental

Materials

All chemicals were commercially available and, after charac-

*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 National Institute of Standards and Technology, nor does it imply that the the equipment, instruments, or materials are necessarily the best available for the purpose.

terization, were used as received. Analytical valves reported below have been corrected for the measured purities of the cocaine and BE used in the preparation of standards. BE, described by the commercial supplier as being anhydrous, was found to be in the tetrahydrate form. BE-(*N*-methyl-d_3) was also a tetrahydrate. Cocaine was the hydrochloride, and cocaine-(*N*-methyl-d_3) was the free base.

The BE was verified as the tetrahydrate by Karl Fischer titration. Samples were taken from previously opened and unopened bottles. The measured water content was 20.2%; the calculated value for four water molecules of hydration was 19.94%. The melting point of the BE was also determined. A sample taken from a previously unopened bottle and placed in a dried and sealed melting point tube underwent a change in appearance at 90°C with the appearance of water and then underwent final melting at 193–195°C. The reported melting point of the anhydrous form is 195°C, and for the tetrahydrate is 86–87°C (4).

The ethyl ester of benzoylecgonine (cocaethylene), chosen as internal standard in the HPLC method, was synthesized by refluxing 127 mg BE for 4 h in about 3 mL ethanol, to which 10 drops of trimethylchlorosilane had been added as a catalyst (HCl source). Then 2 mL of water was added, followed by about 200 mg sodium bicarbonate. The solution was extracted 4 times with 10-mL portions of pentane. The pentane fractions were combined and evaporated to dryness under argon. A white, wax-like solid residue (cocaethylene) was collected. The residue was characterized by HPLC using the method described below, and its molecular weight was confirmed by mass spectrometry. No evidence of BE or other impurities was observed.

Bond-Elut Certify™ solid-phase extraction cartridges, which contain a specially bonded, silica-based mixed-phase, were obtained from Analytichem International. Cyclodextrin cartridges were obtained from ASTEC. Bakerbond™ C_{18} wide pore HPLC columns (25 cm × 4.6 mm, 5-µm particle diameter) were obtained from J.T. Baker.

The material for the SRM was prepared by a commercial supplier from a single all-male urine pool, which was sterile-filtered and divided into four portions. Three of the portions were spiked with appropriate amounts of BE, cocaine, and methyl ecgonine; the fourth was retained as a blank.

GC/MS methods

Standard solutions of BE were prepared as follows. Stock solutions of BE and deuterated BE (5–6 mg/L, accurately weighed) were made up in water. Two independent preparations of BE stock solutions were used. The first was used for Set 1, and the second for Sets 2 and 3. Four BE standards were prepared for each sample set by combining appropriate volumes (0.1–0.9 mL, measured to 0.001 mL) from the BE and deuterated BE stock solutions to make solutions with weight ratios of BE to deuterated BE ranging from about 0.8 to 1.2. The mixtures were dried under nitrogen at 60°C and the residue was treated with 100 µL of bis(trimethylsilyl)acetamide (BSA) for at least one hour.

Each set consisted of three bottles of lyophilized urine at each of the three levels, plus two bottles of blank. Each bottle of urine was reconstituted with 10 mL of water delivered from a calibrated automatic pipettor. The bottles were allowed to stand for an hour, with occasional gentle swirling. One 5-mL aliquot was taken from each bottle and placed in a 50-mL glass centrifuge tube, and to each tube was added 2 mL of 0.10M phosphate buffer (pH 6) and an appropriate quantity of the deuterated BE spike. The amount of spike was calculated to give an approximately 1:1 ratio of BE to deuterated BE. The samples were mixed, and each sample checked with pH paper. If the pH was not in the range of 4–6, it was adjusted to be within that range. If the pH was outside that range, recovery was poor.

Bond-Elut Certify solid-phase extraction cartridges were used to isolate the BE from the matrix. One cartridge was used per sample. Cartridges were washed with 2 mL methanol, followed by 2 mL 0.1M phosphate buffer (pH 6). Each sample was then slowly aspirated (2–3 mL/min) through a cartridge. The cartridge was then rinsed successively with 3 mL distilled water, 3 mL 0.1M HCl, and finally 9 mL methanol. The BE was eluted with 2 mL of a solution of methylene chloride–isopropyl alcohol (80:20) containing 2% concentrated aqueous ammonium hydroxide, which was made fresh daily and shaken just before use. The eluate was dried under nitrogen, and 100 µL of BSA was added to the vial. Samples were clear and colorless with no debris.

Cocaine samples were prepared in the same manner as the BE samples, with the following exceptions. Cocaine standards were made up in methanol solution. Two independently weighed stock solutions of cocaine were prepared. The first was used for Sets 4 and 5; the second was used for Set 6. A single solution of deuterated cocaine in methanol was used as the spike for standards and samples. At the final step of sample preparation, samples were dissolved in BSA.

The mass spectrometric measurements were made with a triple quadrupole mass spectrometer attached to a GC. The GC injector and transfer line were set to 270°C. Helium head pressure was set to about 103 kP (15 psi). The column was a 30-m × 0.25-mm i.d. nonpolar [95% dimethyl–5%-phenylmethyl-polysiloxane] fused-silica capillary column of 0.25-µm film thickness. Split injections were made with a vent to column flow ratio of 10:1. The GC temperature program used was 1 min at 235°C, then 5 min at a gradient of 11°C/min up to 290°C.

The mass spectrometer was operated in the electron ionization mode, scanning the third quadrupole. The electron energy was 70 eV and the ion source was set to 200°C. The selected ion monitoring window was set at 0.6 amu. The mass spectrometer filament was turned on an hour before a run was started. The filament was kept on at all times, except when the solvent front came through, so that instrumental conditions were more stable. For both BE and cocaine, the intensities of the molecular ions were too low to be used for measurement. BE was measured at *m/z* 240 and corresponding *m/z* 243 because it was the most intense of the higher mass ions and no interferences were evident. Cocaine was measured at *m/z* 82 and 85 because other fragment ions either had an interference present *m/z* (182 and 185) or were too low in intensity. The instrument was programmed to cycle every 100 ms between *m/z* 82 and 85 until the cocaine peak eluted, and then to cycle between *m/z* 240 and 243 until the BE peak eluted. The detection limits for this method are 12 ng/mL for BE, and 5 ng/mL for cocaine.

For each set of samples, the following measurement protocol was used. A standard curve was run consisting of single injections of each of the four standards in order of increasing weight ratio. Then duplicate injections of each sample at a particular level were made. Finally, a standard curve was run in reverse weight ratio order. Blanks were run at the end of the set.

For each level, a composite standard curve was prepared by combining the data on the two standard curves run before and after the samples and calculating the regression line. This equation was then used to convert the measured intensity ratios of samples to weight ratios. The weight ratio calculated for each injection was multiplied by the quantity of deuterated compound added to give the concentration for each sample. These concen-

trations were combined to give the final answer.

In a separate BE recovery study, reconstituted blank urine was spiked at NIST with BE at 100, 300, and 500 ng/mL. The samples were processed to isolate the BE as described. After the BE was eluted, the eluant for each sample was then spiked with deuterated BE, dried under nitrogen, and derivatized with 100 µL of BSA. The samples were analyzed as described. Recovery of BE was 80, 76, and 96% for the 100-, 300-, and 500-µg/mL levels, respectively. Because an isotopically labeled standard was used to spike the samples before the solid-phase extraction, the recovery of the internal standard should be the same as the recovery for the unlabeled compound. Therefore no correction need be made for recoveries.

HPLC method

Cocaine concentrations in the SRM were determined using the following HPLC method. Three calibration standard solutions were prepared by dilution from common stock solutions. Concentrations in water ranged from about 1.1 to 7.8 µg cocaine per milliliter of solution. Standard curves were prepared for each run from blank urine samples spiked with known amounts of cocaine from these solutions; the concentrations of the spiked urines used to generate the standard curves were comparable to those of the reconstituted SRM. Cocaine levels in unknown samples were determined using these calibration curves. The concentrations of the unknowns were determined from a linear regression fit of the spiked urine standards. Quantitation was carried out by the internal standard method. Because of the specificity of the sample cleanup method, an internal standard was selected with similar structure to cocaine, i.e., the ethyl ester of benzoylecgonine (cocaethylene). The retention time of cocaethylene was about 10.3 min; the retention time of cocaine was about 8.2 min. 10 mL of water and 1.00 mL of aqueous internal standard solution (13.83 µg/g) were added to each bottle of SRM urine. The same internal standard solution was added to both standards and samples. Cocaine was isolated by solid-phase extraction using cyclodextrin cartridges. The contents of an entire SRM bottle were loaded onto a cyclodextrin cartridge using a 25-mL syringe, assisted by aspiration. The flow rate through the cartridge was approximately 10 mL/min. The cartridge, as recommended by the manufacturer, was not pre-equilibrated with organic solvent. The cartridge was washed with 10 mL of water and aspirated for approximately 2 minutes. Cocaine was eluted from the cartridge using approximately 3 mL methanol. The solvent was evaporated under a stream of argon, and the residue was redissolved in approximately 60 µL water and then transferred to a conical sample vial for analysis by HPLC.

Cocaine levels were determined by a gradient elution HPLC method. Gradient elution was used to improve the separation of the analyte from interferences in the urine matrix. A Bakerbond C_{18} wide-pore column (25 cm × 4.6 mm, 5-µm particle diameter) had suitable chromatographic properties (i.e., symmetrical peak shape and adequate retention), and was used in the development of the chromatographic method. The pH of the aqueous mobile phase component was adjusted with a phosphate buffer. A 7.0-g sample of sodium phosphate (Na_2HPO_4) was added to 1 L HPLC grade water, and the solution was adjusted to pH 2.1 with H_3PO_4. Separations were carried out at 60°C using this buffer with acetonitrile, under the following program. Step 1: initial conditions, 95% buffer/5% acetonitrile at a flow rate of 2 mL/min; Step 2: linear gradient to 80% buffer–20% acetonitrile over 5 min followed by a 10-min hold; Step 3: linear gradient to 50% buffer–50% acetonitrile over 1 min; Step 4: a linear gradient to the initial conditions over 1 min, followed by reequilibration for 10 min.

The separation occurred within the first 15 min of the program. The remainder of the program was included to clean and equilibrate the column after each analysis. Detection was at 233 nm. Two or three samples at each of the three levels were analyzed on two different days.

FIA/MS method

BE in the SRM was also determined by FIA/MS. One bottle of each level plus a blank was analyzed. Each bottle was reconstituted and spiked with an ethanol solution of deuterated BE to a concentration approximating the expected level of BE in the sample; i.e., so that weight ratios of deuterated and unlabeled materials were about one. The blank was spiked at the same concentration as the lowest level sample. Standard solutions were made by spiking water at concentrations approximating those expected in the samples and with the same volume of deuterated BE solution used in the respective samples.

Both the standards and samples were subjected to the cyclodextrin solid-phase extraction used in the HPLC method for cocaine. Aliquots of 2–10 µL were injected for analysis. The extract from each bottle was measured 12–13 times.

Table I. GC/MS Determination of BE in SRM 1508 (ng/mL)

Level	Set 1	Set 2	Set 3
100	108	108	99
	105	111	93
	113	104	97
	112	110	101
	102	109	97
	93	109	98
Day Avg	105.5	108.5	97.5
Overall Average		103.8	
CV*		3.2%	
300	249	261	264
	257	239	261
	237	244	269
	245	248	254
	240	253	241
	229	245	240
Day Avg	242.8	248.3	254.8
Overall Average		248.6	
CV*		1.4%	
500	535	470	459
	502	498	559
	529	468	500
	508	493	519
	476	478	512
	468	471	469
Day Avg	503.0	479.7	503.0
Overall Average		495.2	
CV*		1.6%	

* At each level, the three day-averages were averaged and the coefficient of variation of the overall average was calculated from the day-averages.

The extracts were injected into a thermospray LC/MS interface, for which the operating conditions were as follows. The mobile phase was water containing 0.1M ammonium acetate, the flow rate was 1.2 mL/min, and the block temperature was 275°C. No chromatographic separation was used. Positive ions at *m/z* 290 and 293, the $(M+H)^+$ ions of the BE and deuterated BE, were used for quantitation.

This FIA/MS method gave rapid results and high sensitivity, but it was found that an interference present in the extract from the blank urine gave an ion at *m/z* 290 and thus interfered with the measurement. Because the same urine pool was used to make all four levels of the standard reference material, the measured value for the blank was subtracted from the measured value for the other levels.

The validity of this assumption was verified by using minimal separation on short columns. Under conditions where the blank approached zero, the other levels approached that of the FIA/MS method after subtracting the blank. However, the sensitivity, and hence the precision, were not as good because of the wider peaks and the presence of methanol.

Both standard solutions and urine samples were spiked with the same amounts of internal standards and were subjected to the same extraction procedures. Concentrations were calculated by comparing ratios of areas of the peaks at *m/z* 290 and and 293 for the samples and for the standards; i.e., sample concentration = standard concentration × $(R_{sample}/R_{standard})$, where R = $area_{290}/area_{293}$.

Table II. GC/MS Determination of Cocaine in SRM 1508 (ng/mL)

Level	Set 1	Set 2	Set 3
100	84.5	82.0	81.2
	80.7	83.1	82.1
	84.8	85.1	79.2
	89.3	87.4	76.0
	89.2	83.0	85.9
	84.2	88.1	83.9
Day Avg	85.4	84.8	81.4
Overall Average		83.9	
CV*		1.5%	
300	254	260	249
	250	267	253
	242	276	249
	258	256	247
	241	263	241
	251	237	245
Day Avg	249.3	259.8	247.3
Overall Average		252.1	
CV*		1.5%	
500	433	406	426
	432	425	424
	419	409	402
	436	434	426
	420	401	416
	416	428	413
Day Avg	426.0	417.2	417.8
Overall Average		420.3	
CV*		0.7%	

*At each level, the three day-averages were averaged and the coefficient of variation of the overall average was calculated from the day-averages.

Results and Discussion

The primary approach used at NIST to certify an analyte, that is, to publish a particular value as an accurate one, is to use the results from two independent and reliable methods. This approach is based on the rationale that the likelihood of two independent methods being biased by the same amount, in the same direction, is small. It is with this approach that the concentrations of BE and cocaine in this SRM were certified. At present, we do not have the two independent and reliable methods necessary to certify a value for ecgonine methyl ester.

The GC/MS measurements for BE and cocaine are listed in Tables I and II, respectively. The coefficients of variation (CV)

Table III. HPLC Determination of Cocaine in SRM 1508 (ng/mL)

Level	Day 1	Day 2
100	90	109
	93	112
	92	95
Day Avg	91.7	105.3
Overall Average		98.5
CV*		6.9%
300	274	300
	268	264
	266	
Day Avg	269.3	282.0
Overall Average		275.6
CV*		2.3%
500	443	433
	426	467
	451	420
Day Avg	440.0	440.0
Overall Average		440.0
CV*		0.0%

*At each level, the two day-averages were averaged and the coefficient of variation of the overall average was calculated from the day-averages.

Table IV. FIA/MS Determination of BE in SRM 1508 (ng/mL)

Sample level	BE signal	BE signal minus blank*	Number of measurements
Blank	37	0	13
100	140	103	13
300	306	269	12
500	561	524	13

* The coefficient of variation of each average value is estimated to be 2%.

Table V. Comparison of Cocaine and BE Results by Method

Substance	Level	GM/MS	Std error	HPLC	Std error	Difference betw. methods (%)
Cocaine	100	83.9	1.2	98.5	6.8	−16%
	300	252.1	3.9	275.6	6.4	− 9%
	500	420.3	2.8	440.0	0.0	− 5%
Benzoylecgonine	100	103.8	3.3	103	2	+ 1%
	300	248.6	3.5	269	5	− 8%
	500	495.2	7.8	524	10	− 6%

Table VI. Certified Values for Cocaine and BE in SRM 1508

Substance	Level	Certified value	Estimated uncertainty*
Cocaine	100	90	14
	300	263	22
	500	429	20
Benzoylecgonine	100	103	10†
	300	259	20
	500	510	29

* Two standard deviations of the certified value.
† An estimated uncertainty of 4 ng/mL was calculated from the data in Tables I and IV. However, the authors, based on scientific judgement, believe that an uncertainty of 10 ng/mL is a more realistic estimate.

of the overall average GC/MS values vary from 0.7 to 3.2%.

Solutions for the preparation of cocaine standards were prepared in methanol to avoid the conversion of cocaine to BE. Two independently prepared sets of standards for each analyte were used to prepare the standard curves for quantitation to ensure that no single weighing error could bias the results. On each of three separate days, new sets of standards and samples were prepared and measured.

The literature was surveyed to find a second method suitable for cocaine. No non-GC/MS method was found that was sensitive enough or specific enough for our purposes. Therefore, a second method, using HPLC, was developed at NIST. The HPLC cocaine measurements are listed in Table III. The coefficients of variation (CV) of the overall average HPLC values vary from 0.0 to 6.9%.

The cyclodextrin solid-phase extraction cartridges used to isolate cocaine in this method provided a degree of specificity for cocaine that was not achieved with C_{18} cartridges. HPLC column selection was an important factor in the analysis. A variety of C_{18} columns were evaluated because column properties (peak shape and selectivity) were found to be critical in the development of the method. A Bakerbond C_{18} wide pore (25 cm × 4.6 mm, 5-μm particle diameter) column was selected as suitable. On each of two days, a new bottle at each concentration was measured either in duplicate or in triplicate. This method was also investigated for its suitability in determining BE, but the precision was not satisifactory.

The FIA/MS method for BE is rapid and sensitive. The SRM 1508 results from the FIA/MS method are shown in Table IV. Because the method does not use a chromatographic separation, it is subject to non-analyte contributions to the analyte signal. A blank urine is run to correct this problem.

The overall averages for each of the analytes for the methods are summarized and compared in Table V. The data of this table show that the GC/MS results tend to be slightly lower than the results obtained from the HPLC or FIA/MS methods. We have no evidence for bias in any of the independent methods used. All methods used provided accurate results on blank urine samples spiked with known amounts of analytes.

For the certification of SRM 1508, an initial statistical analysis was made for each analytical method and for each analyte and concentration level. The final certification analysis of variance was then made, recognizing both the within- and between-method components of variance. A weighted average and its uncertainty was obtained for each analyte and concentration level (5). The weights of each average were based on both the within- and between-method imprecisions. The SRM 1508 certified values ± 2 SD of the certified values are given in Table VI.

Approximately one year later the concentrations of BE and cocaine were again measured by GC/MS in order to investigate the stability of the material. The new results gave no evidence for any decomposition having occurred.

The SRM is intended for use as a control material and an accuracy benchmark for the drug testing community. The precision of the measurements is suitable for the intended use.

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