An Assay Evaluation of the Methylene Blue Method for the Detection of Anionic Surfactants in Urine

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Abstract

Adding detergent to urine intended for drug testing is one of many ways to adulterate the specimen. This modified methylene blue procedure allows the detection and quantitation of anionic surfactants in urine. One-hundred urine specimens that exhibited normal foaming when shaken gave anionic surfactant values lower than 36 µg/mL with a mean of 8.73 µg/mL. Most of the suspected adulterated specimens and spiked samples with only 100 µL of detergent in 60 mL of urine had values greater than 750 µg/mL. Based on the analysis of negative samples, a urine specimen with an anionic surfactant level of 100 µg/mL or greater could be considered adulterated and most likely will have levels greater than 800 µg/mL.

Introduction

The purpose of this assay evaluation was to determine if the methylene blue method (1) was suitable for detecting anionic surfactants in forensic urine drug test specimens that were suspected of adulteration. Detergent has long been an effective method of adulterating forensic urine drug tests because detergents can interfere with the immunoassay initial test, as well as cause problems with gas chromatography–mass spectrometry (GC–MS) confirmation procedures (2–4).

Anionic, cationic, and nonionic surfactants are the three main types of detergents; the most prevalent of these are anionic surfactants (5). Linear alkylbenzene sulfonates (LAS) are the most common of the anionic surfactants (1). Sodium dodecylbenzene sulfonate (DBS) is a common example of LAS that is present in liquid hand soaps.

Methylene blue active substances (MBAS) form an ion pair with 3,9 bis-dimethylamino phenazothionium chloride (methylene blue) (6), a cationic dye that is extracted with chloroform (1). Among the chief MBAS are sulfonate salts, sulfate ester salts, and sulfated nonionic salts. Alkali salts of fatty acids (soaps) do not form an ion pair that is extractable (1). Organic sulfonates, sulfates, carboxylates, and phenols and inorganic thiocyanates, cyanates, nitrates, and chlorides do form an extractable ion pair but are eliminated with a backwash extraction (1).

Methods

General

All spectrophotometric measurements were made on a single-beam Hewlett-Packard 8451A diode-array spectrophotometer using a wavelength of 652 nm and a rectangular, open-top 10-mm pathlength, glass spectrophotometer cell (Starna Cells, Atascadero, CA). Sodium dodecylbenzene sulfonate (DBS, 96%, branched chain) was obtained from Pfaltz & Bauer (Waterbury, CT). Methylene blue reagent (0.003%) for surfactants was obtained from LabChem, Inc. (Pittsburgh, PA). Potassium phosphate (powder, anhydrous), high-performance liquid chromatography (HPLC)-grade chloroform, and 1N sulfuric acid were obtained from FisherChemical (Fair Lawn, NJ). Certified negative urine (CNU) was supplied by LabCorp-OTS Quality Control department (Research Triangle Park, NC).

Solutions

Phosphate wash solution. To a 100-mL graduated cylinder that contained 75 mL of deionized (DI) water, 25 mL of 1N H_2SO_4 was added. The solution was transferred to a 100-mL beaker that contained 5 g K_2HPO_4. The solution was stirred until the K_2HPO_4 was dissolved.

Stock sodium dodecylbenzene sulfonate (DBS) solution (1000 µg/mL). A 1-L volumetric flask was charged with 1.04 g of sodium dodecylbenzene sulfonate (96%). The flask was slowly filled to volume with DI water; the solution was stirred to avoid excessive foaming. The solution was refrigerated to minimize decomposition. A fresh solution was prepared each month. Separate preparations of stock solutions were used to prepare the calibrator and controls.

100-µg/mL DBS calibrator. In a 16-mm ×-100 mm screw-top glass tube, 500 µL of stock solution and 4500 µL of CNU were added. The solution was refrigerated to prevent decomposition and was prepared fresh monthly.

70-µg/mL DBS control. In a 16-mm × 100-mm screw-top glass tube, 350 µL of stock solution and 4650 µL of CNU were combined. The solution was refrigerated to prevent decomposition and was prepared fresh monthly.

120-µg/mL DBS control. In a 16-mm × 100-mm screw-top glass tube, 600 µL of stock solution and 4400 µL of CNU were

added. The solution was refrigerated to prevent decomposition and was prepared fresh monthly.

200-µg/mL DBS control. In a 16-mm × 100-mm screw-top glass tube, 1000 µL of stock solution and 4000 µL of CNU were added. The solution was refrigerated to prevent decomposition and was prepared fresh monthly.

Procedure

Extraction. A 200-µL aliquot of each calibrator (100 µg/mL), control (70 µg/mL, 120 µg/mL, 200 µg/mL, and CNU) and donor specimen was placed in a clean, labeled 16-mm × 100-mm screw-top glass tube. To each calibrator, control, and specimen, 3.8 mL of DI water, 2.0 mL of commercially prepared methylene blue reagent (0.003%), and 3.0 mL of HPLC-grade chloroform were added. The tubes were shaken for 20 min to extract the methylene blue-anionic surfactant ion pair into the chloroform layer. At this point, it was necessary to indicate if all the blue dye had migrated to the chloroform layer. Depletion of the blue dye from the aqueous phase occurred if the stoichiometric limit of linearity had been exceeded. The tubes were centrifuged at 3000 rpm for 5 min.

Two milliliters of the chloroform (bottom layer) was removed from each tube, with a disposable volumetric pipette and placed in a clean, labeled, 16-mm × 100-mm screw top glass tube. To each tube, 3.0 mL of chloroform and 3.0 mL of phosphate wash were added. The tubes were shaken for 30 min to back-extract any weakly bound ion pairs back into the aqueous phase. The tubes were centrifuged at 3000 rpm for 5 min.

With a disposable pipette, 3 mL of the chloroform (bottom layer) was transferred to a clean, labeled 16-mm × 100-mm screw-top glass tube.

Measurement. The absorbance of each calibrator, control, and specimen was measured using a wavelength of 652 nm against a reference of pure HPLC-grade chloroform.

Calculation. All calculations were based on Beer's Law, A = εbc, where A was the absorbance, ε was the molar absorptivity, b was the length of the light path (1 cm), and c was the concentration of absorbing molecules. Correcting for the matrix effect, A was calculated by subtracting the measured absorbance of the CNU (A_{CNU}) extract from the measured absorbance of the calibrator (A_{CAL}) extract. This corrected absorbance (A) for the calibrator was divided by the target concentration of the calibrator (C_{CAL}) to obtain ε (Eq. 1).

$$\varepsilon = \frac{(A_{CAL} - A_{CNU})}{C_{CAL}} \qquad \text{Eq. 1}$$

The concentration of the controls and specimens (C_{SAMPLE}) was calculated by subtracting the absorbance of the CNU from the specimen or control (A_{SAMPLE}). This corrected absorbance was divided by the calculated ε (Eq. 2).

$$C_{SAMPLE} = \frac{(A_{SAMPLE} - A_{CNU})}{\varepsilon} \qquad \text{Eq. 2}$$

Donor specimens were reported as positive/negative for anionic surfactants (detergent) as methylene blue active substances (MBAS), calculated as sodium dodecylbenzene sulfonate (DBS), relative to the 100-µg/mL calibrator.

Results

Linearity

The linearity of the methylene blue procedure was determined by plotting the mean of the first five results obtained for the following concentrations: 0 µg/mL, 70 µg/mL, 100 µg/mL, 120 µg/mL, 200 µg/mL, 250 µg/mL, 275 µg/mL, 300 µg/mL, and 325 µg/mL. The results of the linearity test are shown in Table I and Figure 1. (Note: The aqueous layer of the 275-µg/mL, 300-µg/mL, and 325-µg/mL specimens was completely depleted of blue color after the first extraction.)

Precision

A set of 15 replicates was analyzed to determine the precision of the methylene blue procedure for the negative, 70-, 100-, 120-, and 200-µg/mL controls. Five replicates were taken from a single batch to determine the within-run precision, and 10 were taken

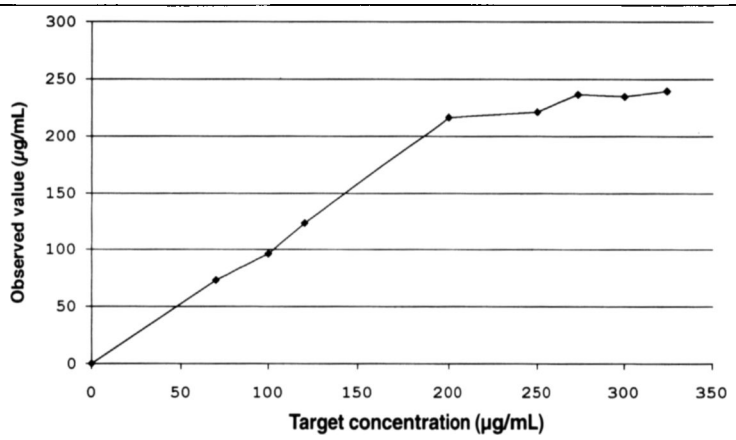

Figure 1. Plot of test results obtained from the methylene blue procedure linearity test. All controls were within 20% of target concentrations, except the 300- and 325-µg/mL controls.

Table I. Linearity of the Methylene Blue Procedure

Target value (µg/mL)	A (µg/mL)	B (µg/mL)	C (µg/mL)	D (µg/mL)	E (µg/mL)	Mean (µg/mL)	% Target
0	0.47	0.78	0.00	0.31	0.47	0.41	N/A
70	71.96	74.43	71.08	70.90	76.37	72.95	104.2
100	95.94	92.04	94.23	100.00	98.87	96.22	96.2
120	123.28	122.75	123.10	119.40	128.75	123.46	102.9
200	221.87	221.87	204.23	211.29	220.11	215.87	107.9
250	235.42	210.92	217.48	221.84	220.69	221.27	88.5
275	256.25	224.96	233.66	228.08	238.75	236.34	85.9
300	237.15	232.76	238.51	220.28	243.68	234.48	78.2
325	252.78	228.08	246.60	232.76	235.47	239.14	73.6

from separate batches to determine the between-run precision. The means of the within-run results were 0.41, 72.95, 96.22, 123.46, and 215.87 µg/mL, respectively. The standard deviations of the within-run results were 0.28, 2.38, 3.27, 3.36, and 7.85 µg/mL, respectively. The percent coefficients of variation (%CV) for the within-run results were 69.90%, 3.26%, 3.40%, 2.72%, and 3.64%, respectively. The means of the between-run results were –0.02, 69.81, 97.66, 122.98, and 200.66 µg/mL, respectively. The standard deviations of the between-run results were 0.37, 3.52, 4.59, 7.52, and 11.95 µg/mL, respectively. The %CVs for the between-run results were –15.88%, 5.03%, 4.70%, 6.12%, and 5.95%, respectively.

Table II. Accuracy of the Controls

Control (µg/mL)	Mean (µg/mL)	SD (µg/mL)	2 SD Range (µg/mL)
CNU	0.12	0.39	–0.66–0.90
70	70.86	3.45	63.96–77.76
100	97.18	4.13	88.92–105.44
120	123.14	6.30	110.54–135.74
200	205.73	12.82	180.09–231.37

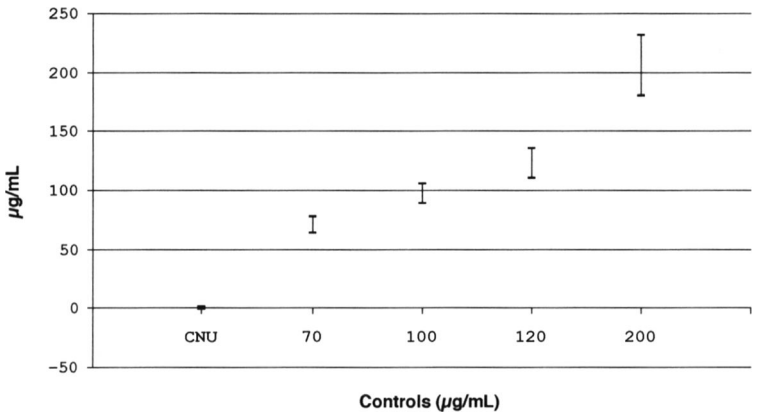

Figure 2. Plot of the calculated mean for each control ± 2 standard deviations to determine the potential for overlap. Overlap was not observed.

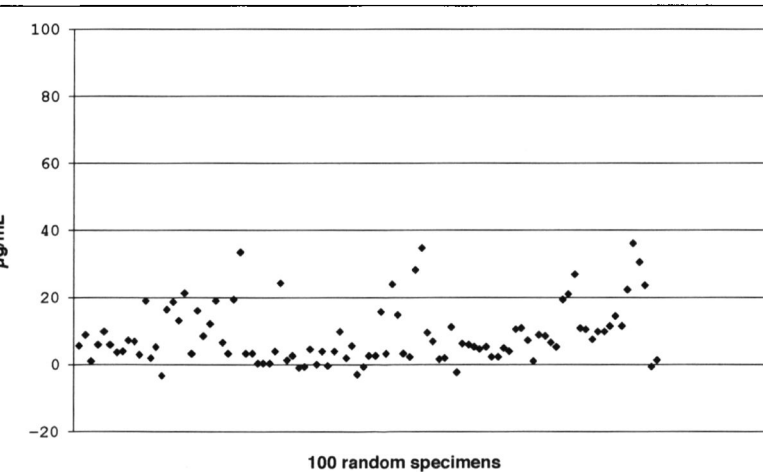

Figure 3. Distribution of test results for 100 donor urine specimens using the methylene blue procedure.

Accuracy

The accuracy of the methylene blue procedure was determined by checking the potential for overlap between the negative, 70-, 100-, 120-, and 200-µg/mL controls using a range of two standard deviations (SD) from each of their means. The mean was obtained from the first 15 replicates of each control. The results are listed in Table II. The two SD ranges around the means of the controls are depicted in Figure 2.

Negative/positive analysis

Negative analysis. A group of 100 donor urine specimens that exhibited no unusual foaming when shaken were selected and analyzed using the method described previously. The calculated mean was 8.73 µg/mL with a standard deviation also of 8.73 µg/mL. The lowest value calculated was –3.12 µg/mL and the highest specimen observed was 36.00 µg/mL. The results are shown in Figure 3.

Positive analysis. Thirteen donor urine specimens that were considered unsuitable for testing because they failed to generate valid test results in immunoassay and/or GC–MS and they exhibited unusual foaming characteristic of detergent were selected and analyzed. Some specimens required a dilution to obtain the final result. The results are shown in Table III.

Spikes. Six 60-mL CNU specimens were spiked with various household detergents or Mary Jane SuperClean 13 and were analyzed by the methylene blue procedure. Some specimens required a dilution to obtain the final result. The results are listed in Table IV.

Discussion

The methylene blue procedure outlined in *Standard Methods for the Examination of Water and Wastewater* (1) was intended for measuring trace amounts of anionic surfactants in natural waters. Several modifications were necessary to adapt this procedure to a urine matrix. The main modification was volume and the ratio of specimen/methylene blue reagent/chloroform. The original procedure called for using 100 mL of specimen, 25 mL methylene blue reagent, and 10 mL of chloroform (10:2.5:1). The modified procedure used 200 µL of specimen diluted with 3.8 mL of distilled water, 2 mL methylene blue reagent, and 3 mL of chloroform (1:10:15). These volumes allowed for smaller specimen volumes, higher relative anionic surfactant concentrations, and the use of common disposable glassware.

Another modification was ignoring the pH-adjustment step, which called for titrating with 1N NaOH to the phenolphthalein endpoint. Hon-Nami and Hanya (7) demonstrated that this was not necessary within the acceptable pH range of urine. Specimens with a pH outside of the acceptable range of 3 to 11 would be considered as adul-

Table III. Urines Exhibiting Unusual Foaming

Specimen	Result (µg/mL)
A	4940
B	4311
C	2960
D	61
E	0
F	881
G	1229
H	2202
I	2978
J	991
K	1320
L	366
M	407

Table IV. Urines Spiked with Detergents

Spike	Result (µg/mL)	Volume of spike/ 60 mL CNU
Ivory Liquid	831.13	100 µL
Palmolive Ultra	769.86	100 µL
Clairol Shampoo	812.91	100 µL
Mr. Clean	71.35	100 µL
Pine-Sol	73.61	100 µL
Mary Jane SuperClean 13	3081.63	1 vial (1.8 mL)

terated on the basis of pH alone (8).

The primary concern of modified volumes was the effect on the range of linearity. Samples that ranged in concentration from 70 µg/mL to 325 µg/mL were analyzed with the modified procedure. The samples were replicated five times and the mean was calculated at each level. The determined value must be within 20% of the target value to be acceptable for the linearity check. The means from 70 µg/mL to 275 µg/mL were within 20% of the target value (Figure 1). Samples with 300 µg/mL and 325 µg/mL were outside of 20%. The upper limit of linearity for this procedure was determined to be 275 µg/mL.

The precision of the modified procedure was determined. The five controls used in this assay [0, 70, 100 (calibrator), 120, and 200 µg/mL] were analyzed in replicate 15 times. Five replicates were obtained from a single batch to determine the within-run precision. The remaining 10 were analyzed in separate batches to determine the between-run precision. Means, standard deviations, and %CVs were calculated for each control in each group. Ignoring the negative control, all means were within 10% of target value and all %CVs were less than 10%. The precision results were considered as acceptable.

The accuracy of the methylene blue procedure was determined by comparing the two SD range around the means listed previously. Overlap was not observed in a plot of the ± 2 SD ranges (Figure 2).

A selection of 100 donor urine specimens that did not exhibit an unusual amount of foaming was analyzed. The results ranged from –3.12 µg/mL to 36.00 µg/mL (Figure 3). The average was 8.73 µg/mL with a standard deviation of 8.73 µg/mL.

Thirteen donor urine specimens that had been considered as unsuitable for testing because of difficulties performing immunoassay or GC–MS extraction and exhibited unusual foaming characteristic of detergent when shaken were analyzed. Results ranged from 61.90 µg/mL to 4940.40 µg/mL with one specimen (specimen E) at 0 µg/mL. Specimen E was re-examined. A definite detergentlike foam was observed. The specimen was re-analyzed, and the same result was observed. The final conclusion was that a cationic or non-ionic detergent could have been used in this specimen, which would explain the results of the physical examination and the methylene blue analysis.

Five specimens of certified negative urine (CNU) were spiked with common household detergents and one CNU was spiked with a product called Mary Jane SuperClean 13. To mimic the least amount of foam observed in suspect specimens at our lab, the volume of 100 µL of detergent was chosen. The results of the household detergent spiked samples ranged from 71.35 µg/mL to 831.13 µg/mL. The results for the specimens spiked with the detergents Mr. Clean and Pine-Sol were 71.35 µg/mL and 73.61 µg/mL, respectively. The result of the Mary Jane SuperClean spiked specimen was 3081.63 µg/mL. One vial of Mary Jane SuperClean was used as indicated by the instructions.

Analysis of a negative urine population (n = 100), revealed a mean level of 8.73 ± 8.73 µg/mL. Using 10 standard deviations above the mean (96.03 µg/mL), it is believed that 100 µg/mL can be set as the level above which adulteration with anionic surfactant is indicated.

Indicators for detergent testing may include immunoassay interference and/or confirmatory gas chromatography–mass spectrometry analysis difficulties. The extent of interference depends upon the type of immunoassay reagent used, the pH of the specimen, the concentration of other constituents in the specimen, the concentration of the surfactant, and the type of surfactant added.

This analysis for detecting anionic surfactants in urine can be used along with other indicators such as the physical presence of unusual foaming and/or odor to support the forensic determination that a specimen is adulterated by a detergent.

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