Buprenorphine, Norbuprenorphine, and Naloxone Levels in Adulterated Urine Samples: Can They be Detected When Buprenorphine/Naloxone Film is Dipped into Urine or Water?

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ABSTRACT: Reportedly, various urine manipulations can be performed by opioid use disorder (OUD) patients who are on buprenorphine/naloxone medications to disguise their non-compliance to the treatment. One type of manipulation is known as "spiking" adulteration, directly dipping a buprenorphine/naloxone film into urine. Identifying this type of urine manipulation has been the aim of many previous studies. These studies have revealed urine adulterations through inappropriately high levels of "buprenorphine" and "naloxone" and a very small amount of "norbuprenorphine." So, does the small amount of "norbuprenorphine" in the adulterated urine samples result from dipped buprenorphine/naloxone film, or is it a residual metabolite of buprenorphine in the patient's system? This pilot study utilized 12 urine samples from 12 participants, as well as water samples as a control. The samples were subdivided by the dipping area and time, as well as the temperature and concentration of urine samples, and each sublingual generic buprenorphine/naloxone film was dipped directly into the samples. Then, the levels of "buprenorphine," "norbuprenorphine," "naloxone," "buprenorphine-glucuronide" and "norbuprenorphine-glucuronide" were examined by Liquid Chromatography with tandem mass spectrometry (LC-MS/MS). The results of this study showed that high levels of "buprenorphine" and "naloxone" and a small amount of "norbuprenorphine" were detected in both urine and water samples when the buprenorphine/naloxone film was dipped directly into these samples. However, no "buprenorphine-glucuronide" or "norbuprenorphine-glucuronide" were detected in any of the samples. In addition, the area and timing of dipping altered "buprenorphine" and "naloxone" levels, but concentration and temperature did not. This study's findings could help providers interpret their patients' urine drug test results more accurately, which then allows them to monitor patient compliance and help them identify manipulation by examining patient urine test results.

KEYWORDS: Buprenorphine, norbuprenorphine, naloxone, urine, adulteration

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*Quotation marks were used to specify the elements found in urine and water specimens, e.g., "buprenorphine," as compared to all other forms, e.g., buprenorphine/naloxone medication.

Background

Buprenorphine

Buprenorphine was approved by the U.S. Food and Drug Administration (FDA) in 2002 to treat opioid use disorder (OUD).[1] This medication is one of the three FDA-approved medications for OUD. It is a partial *mu*-opioid receptor agonist and *kappa*-opioid receptor antagonist. The *mu*-opioid receptor causes analgesia, euphoria, and dependence while the *kappa*-opioid receptor is responsible for more dysphoria.[2,3] Buprenorphine binds to the *mu*-opioid receptors with a higher affinity than full agonists but has a much safer profile because of its "ceiling effect," in which opioid effects stop increasing at a certain point even as the dose increases.[4]

The safer profile of buprenorphine makes it suitable for use in Office Based Opioid Treatment (OBOT) programs, where patients are not monitored 24/7 but are regularly supervised with urine tests. Substance Abuse and Mental Health Services Administration (SAMHSA) recommends monthly drug tests in the maintenance phase, and even more frequently for unstable cases, to properly assess treatment progress.[4] Therefore, monitoring urine drug test results is an important part of buprenorphine OBOT, and understanding how to interpret the results is crucial for appropriate treatment for OUD patients.

Urine drug screening tests can be categorized into two types: qualitative and quantitative tests. Qualitative tests are immuno-assay tests, often used for point-of-care (POC)

testing. They are less expensive and more convenient with a shorter turnaround time; however, higher false positive / false negative rates can be expected.[5] In contrast, quantitative tests such as Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS) tests are more labor intensive and more accurate.[6] They are reported to be robust, and specific with an accuracy of 99.9%.[7-10] Quantitative urine drug screening tests for buprenorphine treatment can include buprenorphine metabolites, such as the major metabolite "norbuprenorphine" and other glucuronides, which indicate that the medication is absorbed and metabolized in the system.[11] Thus, "buprenorphine" and "norbuprenorphine" levels are often monitored together for quantitative urine drug screening tests for those who are on buprenorphine.

The levels of urinary "buprenorphine" and "norbuprenorphine" in individuals may fluctuate because their pharmacokinetics and pharmacodynamics vary. Consequently, it is difficult to accurately estimate levels of these analytes in relation to the buprenorphine dose.[12,13] However, the comparative levels of "buprenorphine" and "norbuprenorphine" in patients' urine samples can reveal various details of their behaviors regarding buprenorphine treatment compliance such as the dose, frequency and timing of buprenorphine intake. Patients who take buprenorphine intermittently have lower levels of "norbuprenorphine" than "buprenorphine" in their urine, while those who take buprenorphine daily have higher "norbuprenorphine" levels. This is because the detection window of "buprenorphine" in urine ranges from 1 to 7 days, while that of "norbuprenorphine" can be up to 14 days.[14] Furthermore, various other factors such as the timing of buprenorphine intake prior to urine collection can alter the levels of these metabolites; approximately seven hours after taking buprenorphine, the ratio between "buprenorphine" and "norbuprenorphine" inverts to a higher "norbuprenorphine" than "buprenorphine" ratio.[15] Therefore, patients' behaviors regarding the timing and frequency of buprenorphine intake as well as compliance with the buprenorphine treatment can be inferred by the levels of "buprenorphine" and "norbuprenorphine" in their urine samples.

Buprenorphine is metabolized through complicated metabolic pathways, involving various enzymes. The free "buprenorphine" is metabolized to free "norbuprenorphine" first by cytochrome CYP450 enzymes, mainly 3A4 and 2C8. Then both "buprenorphine" and "norbuprenorphine" are further glucuronidased to "buprenorphine-3-glucuronide (buprenorphine-glucuronide)," and "norbuprenorphine-3-glucuronide (norbuprenorphine-glucuronide)" respectively by Uridine 5′-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase or UGT).[16] More specifically, the metabolism from "norbuprenorphine" to "norbuprenorphine-glucuronide" requires mainly UGT 1A1 and 1A3 while that from "buprenorphine" to "buprenorphine-glucuronide" requires UTG 1A1, 1A3, and 2B7.[17] Thus, buprenorphine is an active component detected as free "buprenorphine" while "norbuprenorphine," "buprenorphine-glucuronide" and "norbuprenorphine-glucuronide" are considered as metabolites.[17]

Additional consideration must be given to a group of medications called CYP450 inducers, which increase CYP450 enzyme activity, hastening buprenorphine metabolism and increasing the metabolite levels in urine. One of the primary enzymes involved in buprenorphine metabolism is CYP450 3A4. If patients are taking these medications, their buprenorphine metabolite levels in urine may be increased. In contrast, other medications inhibit these enzyme functions and slow down buprenorphine metabolism. As a result, this group of medications increases the concentration of buprenorphine in the system and thus decreases the metabolite levels in the urine.[18] Because CYP450 enzymes are primarily found in the liver, the primary site of buprenorphine metabolism, liver disorders may also alter buprenorphine metabolism and, consequently, metabolite levels in the urine.[10,19]

One of the most common ways to adulterate urine is to dip or spike a buprenorphine/naloxone film straight into a urine sample, resulting in substantial changes in urine "buprenorphine" and "norbuprenorphine" levels. This form of manipulation has been discussed by many previous studies regarding the ratio between "buprenorphine" and "norbuprenorphine" and/or high "buprenorphine" levels. Based on the study by Warrington, et al., a summary table was re-created below;[20] a list of the previous studies on adulterated urine samples from patients who are taking buprenorphine products (Table 1).

Most of these studies set the threshold ratio for suspected urine adulteration at "buprenorphine" to "norbuprenorphine" >50, although some focus on the high level of "buprenorphine" only. For example, Warrington, et al., Hull, et al., and Suzuki, et al. found that a "buprenorphine" to "norbuprenorphine" ratio of >50 can indicate urine adulteration.[20,22,24] However, Accurso, et al. added a warning to this ratio, claiming that the >50 ratio may overlook some adulterated samples and instead recommended a ratio >50 with "buprenorphine" >1000 ng/mL.[21] Donroe, et al. and McMillin, et al. also argued that when "buprenorphine" is >1000 ng/mL, adulteration may be suspected.[10,23] Although Suzuki, et al. used "buprenorphine" to "norbuprenorphine" ratio >50, they observed that all of these urine samples have >2000 ng/mL of "buprenorphine."[22] Therefore, there is no consensus as to how to identify adulterated urine, but many studies argued that a high level of "buprenorphine" and/or a high ratio of "buprenorphine" to "norbuprenorphine" suggest urine adulteration.

Some previous studies on urine adulteration detected small amounts of "norbuprenorphine" in the suspected adulterated urine samples. For example, Suzuki, et al. found an average of 11.9 ng/mL "norbuprenorphine" among the urine samples with "buprenorphine" to "norbuprenorphine" ratio >50,[22] while McMillan, et al. observed an average of 17.83 ng/mL "norbuprenorphine" among the 12 urine samples with "buprenorphine" >1000 ng/mL.[10] So, did these small amounts of "norbuprenorphine" come from the dipped buprenorphine

Table 1. Previous studies on the ratio of "buprenorphine (bup)" and "norbuprenorphine (norbup)" versus "buprenorphine" levels (ng/mL) in adulterated urine samples.

REFERENCE	SAMPLE NUMBER	THRESHOLD	BASIS	% FLAGGED BY THRESHOLD
1. Warrington, et al[20]	136,605 urine samples at commercial lab	Bup:Norbup >50	"Identified abnormal ratio"	0.58%
2. Accurso, et al.[21]	33 patients	Bup>1000 & Bup:Norbup >50	Scatter plot estimation	27%
3. Suzuki, et al.[22]	168 patients, 2275 samples	Bup:Norbup >50 (all bup >2000)	Based on Bup:Norbup ratio	0.35%
4. Donroe, et al.[23]	51 patients, 662 samples	Bup >=1000	Endorsed & suspected cases	Endorsed 42.9% & suspected 40.6%
5. McMillin, et al.[10]	1946 samples: 786 had "bup" and "norbup"	Bup >1000	Based on bup levels	0.89%
6. Hull, et al.[24]	70 patients, 216 samples	Bup:Norbup >50	Citing prior studies	4.2%

film? Or was it residual "norbuprenorphine" in the patients' systems, which would mean that the patients have taken buprenorphine within 14 days, the expected detection time of "norbuprenorphine" in urine?[14]

Naloxone

While buprenorphine is a *mu*-opioid receptor partial agonist, naloxone is a *mu*-opioid receptor antagonist and blocks the receptor. Despite these competing pharmacological differences, naloxone is added to some buprenorphine formulations with the ratio of 4:1. Naloxone is believed to have little clinical effect due to poor absorption sublingually.[25-28] Therefore, it is added to buprenorphine to prevent abuse; if buprenorphine/naloxone is taken outside of the intended route of administration (eg, intravenously or intranasally), naloxone will be absorbed, blocking the opioid receptor and preventing buprenorphine intoxication and abuse. Thus, by harnessing complementary but competing effects of naloxone and buprenorphine, this formulation presumably discourages unprescribed use such as intravenous injection and intranasal insufflation.[29,30]

"Naloxone" levels in urine can be altered by various other factors just as "buprenorphine" levels can. For example, naloxone passes through multiple metabolic pathways, but is eventually metabolized to glucuronide conjugate "naloxone-glucuronide," mainly by CYP450 2C18, 2C19, and 3A4.[31] Thus, if patients are on medications that induce or inhibit these enzymes, then their "naloxone" levels in urine can be elevated or decreased, respectively. Furthermore, naloxone is primarily metabolized in the liver to "naloxone-glucuronide," so a malfunctioning liver can also modify the naloxone metabolism and consequently "naloxone" levels in urine.

Many previous studies have detected "naloxone" in both adulterated and unadulterated urine samples. Warrington, et al. found very high levels of "naloxone" in urine from the patients who were taking buprenorphine/naloxone medications; even 8.0% of their urine samples had naloxone >2000 ng/mL, and the average naloxone level was 633.65 ng/mL.[32] Strickland and Burson found that almost all urine samples in their study (92.7%) had >30 ng/mL of "naloxone" from OUD patients who were taking buprenorphine/naloxone medication.[33] The relatively lower levels of "naloxone" found in this study were possibly because the urine was collected >24 hours after naloxone intake. Thus, high "naloxone" levels can indicate the timing of buprenorphine/naloxone medication intake. The half-life of sublingual naloxone is approximately 30-40 minutes.[34] "Naloxone" can be detected in urine for up to 3 days,[35] while "buprenorphine" can be detected for up to 7 days, and "norbuprenorphine" up to 14 days.[14] Thus, high levels of "naloxone" might signal that patients have taken buprenorphine/naloxone shortly before their urine collection, presumably within 24 hours.

The "naloxone" found in the urine was used to argue that sublingual naloxone can be absorbed in the system and excreted in the urine.[36] In addition, "naloxone" levels in urine can be monitored to check patients' compliance to monotherapy. When OUD patients insist that they being on buprenorphine monotherapy for various reasons such as insurance coverage or adverse side effects from naloxone, "naloxone" detected in urine samples can help the provider recognize if the patients are taking the medication as prescribed. Furthermore, monitoring "naloxone" levels in urine is important because an extremely high "naloxone" level in urine samples can confirm adulteration when high levels of "buprenorphine" and/or high ratios of "buprenorphine" to "norbuprenorphine" are detected. Warrington, et al. claimed that high "naloxone" levels can highlight potentially adulterated urine samples.[32] Heikman, et al. also proved that unstable patients had an extremely high level of "naloxone" compared to stable patients.[36]

Burns, et al.[37] and Furo, et al.[38] utilized urine samples that came from healthy individuals who were not on buprenorphine/naloxone, and they found "naloxone" in urine when a buprenorphine/naloxone film was dipped into the samples. However, to our knowledge, there have been no studies that

examined "naloxone" levels in relation to buprenorphine metabolite levels in urine and water samples.

Methods

This study replicates and expands on the study by Furo, et al., which discussed "naloxone" levels in adulterated urine samples.[38] This study reviews not only "naloxone" levels but also the levels of "buprenorphine," "norbuprenorphine," "buprenorphine-glucuronide," and "norbuprenorphine-glucuronide." The following section summarizes the data analysis methods used in both studies.

Data

After the Institutional Review Board (IRB) approval was granted at the University of Texas Health at San Antonio (IRB Protocol ID 20220593HU), 15 participants were recruited through flyers and word-of-mouth. When selecting participants, the following two inclusion criteria were applied: (1) no significant medical history, and (2) not taking any medications or using substances. It was important that the participants had no significant medical history, especially as regards liver failure, which could alter the metabolism of buprenorphine and naloxone. It was also critical that the participants were not on any medications and substances because some medications could inhibit or induce CPY450 enzymes and thus modify the test results.[39] In addition, urine samples of more than 80 mL were required from each participant. Three participants were removed because they were not able to provide the required amount of urine. As a result, 12 urine samples from 12 healthy participants were utilized for this study. After the 12 participants provided urine samples, they were asked to complete a questionnaire on their demographic information. The participants received financial compensation for their time and transportation to the urine collection site.

After each urine sample was collected, it was divided into four specimens with 20 mL in each one. Then, a sublingual film of 8 mg/2 mg buprenorphine/naloxone in generic formulation by ALVOGEN Inc. was dipped directly into each urine specimen. The size of the film was 22.0mm vertical by 12.8mm horizontal. In the first specimen, 1mm of the vertical edge was dipped into the urine sample (1mm*12.8mm = 12.8mm^2) for 3 seconds, which is described as "1mm*3sec" hereafter. In the second specimen, half of the film (11.0mm*12.8mm = 140.8mm^2) was dipped into the urine sample for 3 seconds (half*3sec). In the third specimen, the full film (22.0mm*12.8mm = 281.6mm^2) was dipped into the urine specimen for 3 seconds (full*3sec). In the fourth specimen, the full film (22.0mm*12.8mm = 281.6mm^2) was dipped into the urine specimen for 30 seconds (full*30sec). This way, we could investigate to see if the area and duration of dipping altered the levels of buprenorphine metabolites and naloxone in the adulterated urine samples. Additionally, 4 samples were used as a control. One sample had purified water at room temperature (0%RT). The second sample had purified water warmed up to approximately a body temperature of 97°F (0%BT). The third sample had 2 mL of urine with 18 mL of room temperature purified water, a total of 10% of 20 mL diluted urine specimen (10%RT). The fourth sample had 2 mL of urine with 18 mL purified water at approximately 97°F, a total of 10% of 20 mL diluted urine specimen at body temperature (10%BT). These 4 samples were added to examine if buprenorphine and naloxone metabolites were detectable in water with little or no human urine at different temperatures. Thus, a total of 64 specimens (12*4 specimens plus 4*4 specimens) were used for this study. They were sent to the Associated Regional and University Pathologists, Inc. (ARUP) laboratory for LC-MS/MS testing.

Data analysis

All results were processed and stored in Microsoft Excel. The data sets were analyzed using IBM SPSS software. Student's *t*-test and Analysis of Variance (ANOVA) were employed with the alpha set at .05 ($\alpha = .05$). All analysis processes were conducted anonymously to protect participant confidentiality.

Results

The demographic information of the participants in Furo, et al. and this study is listed in Table A1 in the Appendix.[38] The urine samples from the 12 participants were examined regarding the "buprenorphine," "norbuprenorphine," "buprenorphine-glucuronide," "norbuprenorphine-glucuronide," "naloxone," and "creatinine." They were reviewed in each processed category (1mm*3sec, half*3sec, full*3sec, and full*30sec), and the results were summarized in Table A2 in the Appendix. The mean and standard deviation (SD) of each category were also included. The detectable ranges were as follows: "buprenorphine" and "norbuprenorphine": 2-1000 ng/mL, "buprenorphine-glucuronide" and "norbuprenorphine-glucuronide": 5-1000 ng/mL, "naloxone": 100-1000 ng/mL, and "creatinine": 5-2239 mg/dL. If the levels were not detectable, "–" is listed. If the levels were above the highest measurable level, the maximum cut-off levels with ">" are used as ">1000 ng/mL."

When we compared "buprenorphine" levels of 1mm*3sec, half*3sec, and full*3sec by ANOVA, there was a statistical significance ($P = .03$). Therefore, the larger the area of film dipped, the higher the "buprenorphine" levels detected. "Naloxone" levels of 1mm*3sec, half*3sec, and full*3sec were also analyzed by ANOVA, and the results indicated that there was a significant difference among the 3 groups ($P = .045$). This also indicates that the area of dipping can affect the "naloxone" levels.

When "buprenorphine" levels of full*3sec, and full*30sec samples were compared by t-test, a statistical significance ($P < .01$) emerged. This indicates that the longer the duration the film was dipped, the higher "buprenorphine" levels were detected. As Furo, et al. pointed out,[38] the "naloxone" levels of these two groups were also determined to be statistically significant by *t*-test ($P < .001$). Thus, the duration of dipping

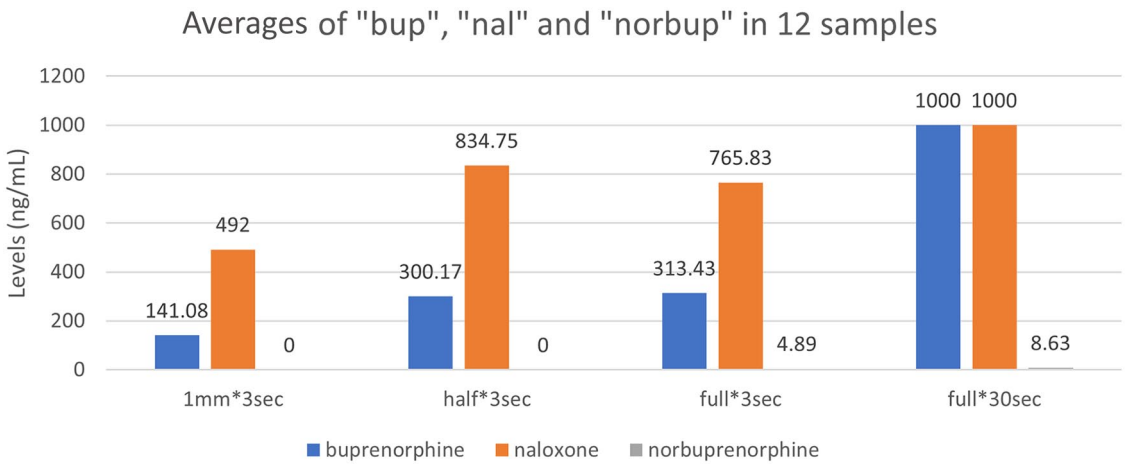

Figure 1. Average levels of "buprenorphine," "naloxone" and "norbuprenorphine" in 12 urine samples. bup = "buprenorphine", norbup = "norbuprenorphine", nal = "naloxone", 1mm*3sec = buprenorphine/naloxone film 1mm was dipped vertically for 3 seconds, half*3sec = half film was dipped for 3 seconds, full*3sec = full film was dipped for 3 seconds, full*30sec = full film was dipped for 30 seconds.

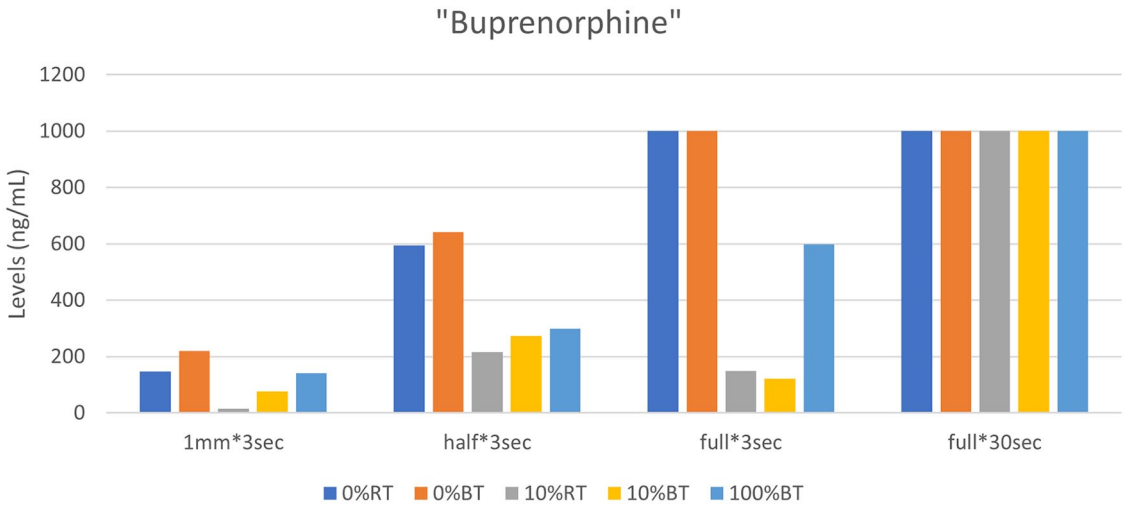

Figure 2. "Buprenorphine" levels in 5 sample types. 0%BT = 100% purified water at ~97°F, 0%RT = 100% purified water at room temperature, 10%BT = 2 mL of 100% urine with 18 mL of ~97°F water, 10%RT = 2 mL of 100% urine with 18 mL of room temperature water, 100%BT = average of 12 urine samples. The maximum cut-off level of all was 1000 ng/mL.

affects not only "buprenorphine" levels but also "naloxone" levels as well.

The following graph illustrates the results of the 12 sample mean values for "buprenorphine," "naloxone" and "norbuprenorphine." Because "buprenorphine-glucuronide" and "norbuprenorphine-glucuronide" were not detected in any samples, they were not included in this figure.

Small amounts of "norbuprenorphine" were detected in full*3sec and full*30sec specimens (Figure 1). "Buprenorphine" and "naloxone" were present in all of the groups; the mean "naloxone" levels were much higher than those of "buprenorphine" levels in all groups. The full*30sec group has maximal cut-off levels (>1000 ng/mL) for both "buprenorphine" and "naloxone."

The 5 samples, 0%BT, 0%RT, 10%BT, 10%RT, and 100%BT (the average of the 12 urine samples) were analyzed in a similar manner, and the results were listed in Table A3 in Appendix. When all "buprenorphine" levels of 0%BT and 0%RT were compared by *t*-test, the difference of "buprenorphine" levels in the two samples was not statistically significant ($P = .915$). As determined in Furo, et al.,[38] when "naloxone" levels of 0%RT and 0%BT were compared by *t*-test, there was no statistical difference ($P = .711$). Comparison of "buprenorphine" levels in 0%BT, 10%BT, and 100%BT urine samples did not show a significant difference ($P = .114$). As Furo, et al., pointed out,[38] "naloxone" levels of the same group showed no statistical difference ($P = .526$), either. Thus, the concentration of urine samples did not affect either "buprenorphine" or "naloxone" levels. The "norbuprenorphine" levels were detected but at an insufficient level to be compared statistically.

"Buprenorphine" was detected in all 5 samples, even in the 100% water samples (See Figure 2). The water specimens had

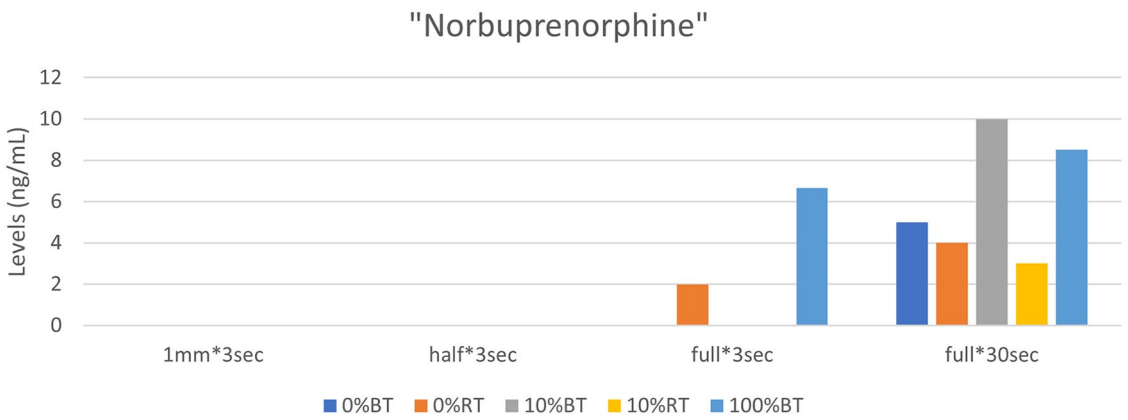

Figure 3. "Norbuprenorphine" levels in 5 sample types. 0%BT = 100% purified water at ~97°F, 0%RT = 100% purified water at room temperature, 10%BT = 2 mL of 100% urine with 18 mL of ~97°F water, 10%RT = 2 mL of 100% urine with 18 mL of room temperature water, 100%BT = average of 12 urine samples. The maximum cut-off level was 1000 ng/mL.

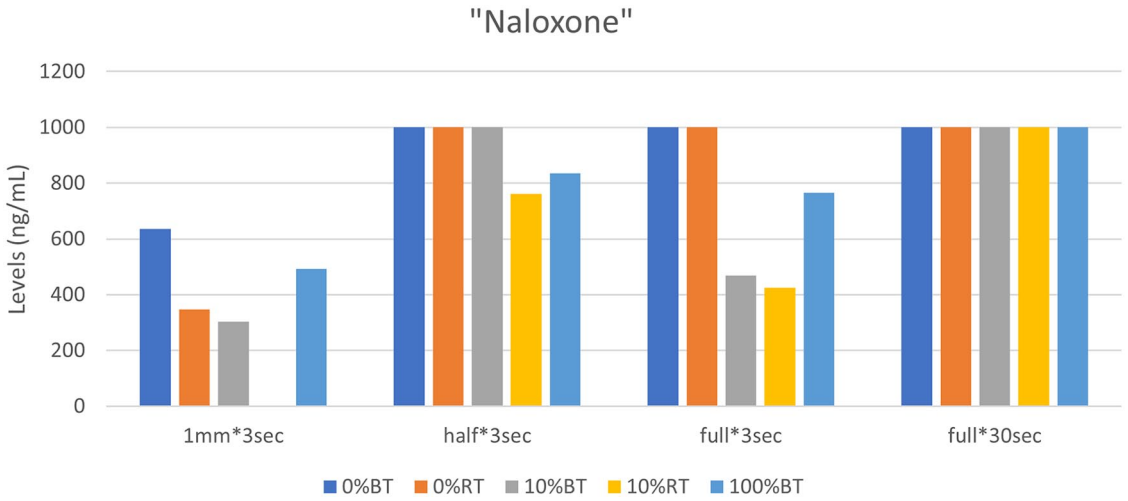

Figure 4. "Naloxone" levels in 5 sample types. 0%BT = 100% purified water at ~97°F, 0%RT = 100% purified water at room temperature, 10%BT = 2 mL of 100% urine with 18 mL of ~97°F water, 10%RT = 2 mL of 100% urine with 18 mL of room temperature water, 100%BT = average of 12 urine samples. The maximum cut-off level was 1000 ng/mL.

higher levels of "buprenorphine" than the other samples with 1mm*3sec, half*3sec, and full*3sec dipping although statistical significance in the concentration analysis was not detected. This might be due to the small number of data set. Therefore, we await tests that have higher cut-off values to conduct more accurate analyses.

"Norbuprenorphine" was detected in all of the full*30sec specimens, but none in 1mm*3sec and half*3sec (see Figure 3). 0%RT and 100% BT samples had "norbuprenorphine" found with full*3sec dipping. The detected "norbuprenorphine" amounts in these specimens were much smaller than those of "buprenorphine" and "naloxone," ranging from 2 to 10 ng/mL.

This study found that all of the specimens had "naloxone" levels higher than either "buprenorphine" or "norbuprenorphine" as indicated in Figure 1. However, there was one specimen where "naloxone" was not detected, that is, 10% RT with 1mm*3sec (See Figure 4). Moreover, Table A1 showed that "naloxone" was not detected in one of the 100% urine samples with 1mm*3sec, either. The full*30sec samples all had "naloxone" with >1000 ng/mL. Figure 4 also showed that 100% water samples (0%BT and 0%RT) have higher levels of "naloxone" than the samples with urine in all groups. More specifically, these water samples had higher levels of "naloxone" in 1mm*3sec, half*3sec, and full*3sec, compared to the samples with urine, except the 100% urine sample with 1mm*3sec, which had a higher "naloxone" level than that of 0%RT.

In summary, this study found the following results with 12 urine samples:

1) "Buprenorphine" was detected in all specimens in all samples.
2) "Naloxone" was detected in 47 out of 48 samples, and the average levels in the detected specimens were higher than those of "buprenorphine."

3) A small amount of "norbuprenorphine" was found in some full*3sec and all full*30sec samples.
4) No "buprenorphine-glucuronide" nor "norbuprenorphine-glucuronide" were found in any specimens.
5) The area and duration of dipping did alter the "buprenorphine" and "naloxone" levels.

When the 5 controlled samples (0%BT, 0%RT, 10%BT and10%RT, and 100%BT) were studied, the following results were found.

1) "Buprenorphine" and "naloxone" were detected in all of the samples except one.
2) No "buprenorphine-glucuronide" and "norbuprenorphine-glucuronide" was detected in any of these samples.
3) The area and duration affected "buprenorphine" or "naloxone" levels, but the temperature and concentration did not.

Discussion

Buprenorphine

"Buprenorphine" was detected in all specimens in this study. This result agreed with those of the previous studies, which found that urine samples with adulteration (suspected or confirmed) showed high levels of "buprenorphine" in comparison with "norbuprenorphine." In this study, the "buprenorphine" levels were much higher compared to "norbuprenorphine" levels in all of the specimens with a ratio >50. However, the "buprenorphine" levels were not always >1000 ng/mL as discussed in some previous studies.[10,23] In particular, the shorter time and smaller area of dipping led to lower "buprenorphine" levels. The corresponding "norbuprenorphine" seemed to be also lower with these specimens. As a result, urine adulteration can be identified more accurately using the ratio between "buprenorphine" and "norbuprenorphine" with >50 as opposed to high levels of "buprenorphine" alone.

In addition, "buprenorphine" was detected even in the 100% water samples without any urine. Although the statistical testing did not show a significant difference among the different concentration specimens, the water specimens had higher levels of "buprenorphine" than the other samples with 1mm*3sec, half*3sec, and full*3sec dipping. This might suggest that buprenorphine is more easily dissolved in less concentrated fluid, perhaps because the "buprenorphine" detected in these samples merely resulted from dissolving buprenorphine. To prove this hypothesis, larger-scale research studies with more data must be conducted in the future.

Naloxone

This study also found "naloxone" in most specimens except for two: one of the 1mm*3sec dipped urine specimens and the 1mm*3sec dipped 10%BT specimen. Because the 1mm*3sec in the 10%BT specimen was a diluted sample, it might have been below the threshold level (<100 ng/mL). "Naloxone" was not found in one of the 100% urine samples (1mm*3sec dipped), either. Some other specimens also had low "naloxone" levels, close to the threshold; a 100% urine specimen with half*3sec had 110 ng/mL, a full*3sec had 117 ng/mL, and a 1mm*3sec had 187 ng/mL. Thus, it is hard to designate these specimens where "naloxone" was not detected as anomalies or outliers.

Although these two specimens did not detect "naloxone," the averages of "naloxone" found in this study were 492.00 ng/mL for 1mm*3sec, 834.75 ng/mL for half*3sec, 765.83 ng/mL for full*3sec, and all 12 of the full*30sec had >1000 ng/mL. These average levels were much higher than those of "buprenorphine"; for example, 141.08 ng/mL for "buprenorphine" vs. 492 ng/mL for "naloxone" in the 1mm*3sec urine samples.

"Naloxone" levels fluctuated at a much wider range than "buprenorphine." For example, the standard deviation (SD) of "buprenorphine" was 88.95 ng/mL while that of "naloxone" was 351.82 ng/mL in 1mm*3sec urine samples, where the difference was the greatest. This might be because the lower limits of detection were different, that is, "buprenorphine" 5 ng/mL vs. "naloxone" 100 ng/mL, although the maximum detection levels for "buprenorphine" and "naloxone" were the same (>1000 ng/mL). This means that "naloxone" levels less than 100 ng/mL were listed as "undetected" and thus calculated as 0 ng/mL for the study. These different lower thresholds for "buprenorphine" and "naloxone" might affect the calculations, resulting in the wider range of "naloxone" levels.

In addition, "naloxone" levels had a wider range than "buprenorphine" levels possibly because naloxone is metabolized much more rapidly than buprenorphine. This study showed that the shorter the time of dipping, the higher the "naloxone" levels compared to "buprenorphine." For example, 1mm*3sec had an average "buprenorphine" 141.08 ng/mL vs. "naloxone" 492 ng/mL, half*3sec "buprenorphine" 300.17 ng/mL vs. "naloxone" 834.75 ng/mL, and full*3sec "buprenorphine" 313.43 ng/mL vs. "naloxone" 765.83 ng/mL. However, both "buprenorphine" and "naloxone" levels in the full*30sec samples had >1000 ng/mL. Because of this wider fluctuation of "naloxone" levels, even when a high "naloxone" level is detected, we cannot use this as the basis to identify "adulteration" of the urine samples. Also, if "naloxone" levels are low, we cannot exclude the possibility of adulteration. Even so, high "naloxone" levels were often detected in suspected adulterated urine samples.[37,41] Thus, high "naloxone" levels can be used to confirm, not identify, adulterated urine samples.

In addition, because of the quick action and short half-life of naloxone,[34,40,41] a high "naloxone" level indicates that buprenorphine/naloxone has been taken shortly before the urine collection.[33,37] As a result, high "naloxone" levels may indicate two things: (1) confirmation of adulteration if "buprenorphine" and "norbuprenorphine" ratio is >50, and (2) buprenorphine/naloxone intake shortly before the urine collection.

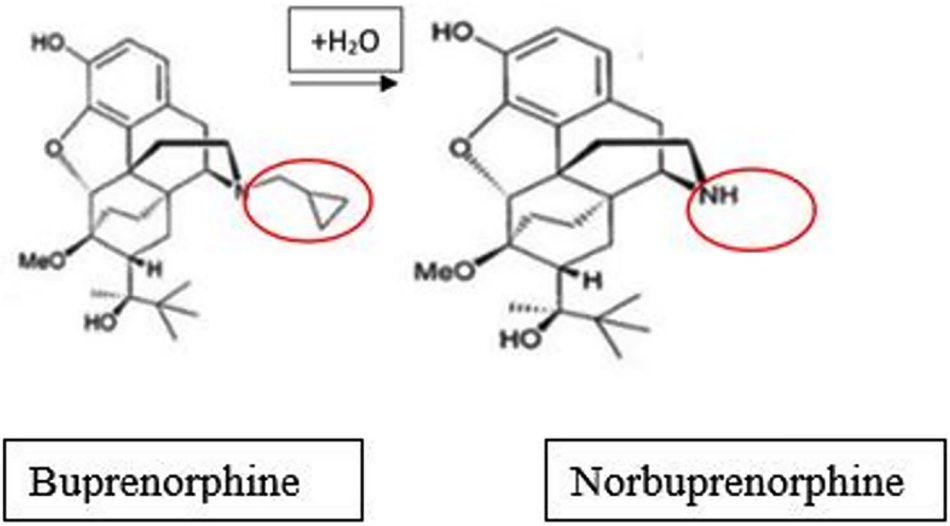

Figure 5. Buprenorphine to Norbuprenorphine metabolism.[17]

Buprenorphine-glucuronide and norbuprenorphine-glucuronide

"Buprenorphine" was found in all specimens, and "naloxone" was found in all but two specimens. Although there were no statistically significant differences in urine concentration, Figures 2 and 4 showed higher levels of "buprenorphine" and "naloxone" in the water samples than urine samples. This might be because "buprenorphine" and "naloxone" were mere solvents dissolved in the samples, and thus they might have dissolved more easily in water than in urine samples. "Buprenorphine-glucuronide" and "norbuprenorphine-glucuronide," on the other hand, were not found in any specimens, agreeing with previous studies, indicating that they are genuine metabolites, and thus require specific enzymes to be metabolized from the parent compounds.

Norbuprenorphine

Many previous studies found small amounts of "norbuprenorphine" in adulterated urine samples; for example, an average of 11.9 ng/mL "norbuprenorphine" in Suzuki, et al.[22] and an average of 17.83 ng/mL in McMillin, et al.[10] However, the average of "norbuprenorphine" in our study was lower (8.46 ng/mL). This might be because the patients in these previous studies might have had some residual "norbuprenorphine" in their systems in addition to "norbuprenorphine" that came from adulteration.

Some previous studies also argued that "norbuprenorphine" is a metabolite of "buprenorphine," a product of the metabolism with CYP450.[11,17] However, in this study, "norbuprenorphine" was found in all of the full*30sec specimens, even in the water samples, although the amounts were very small. So why was "norbuprenorphine" found in the adulterated samples, and why were the levels so low?

Some might argue that positive "norbuprenorphine" in adulterated urine can be due to the cross-reactivity of urine tests. Immuno-assay test accuracy for buprenorphine screening varies depending on the products, but one test reportedly had a 75% specificity.[42] Therefore, this cross-reactivity argument between "buprenorphine" and "norbuprenorphine" can be theoretically possible in qualitative tests with false positives of "norbuprenorphine."[43] However, this study used LC-MS/MS quantitative tests, which are highly accurate. Thus, it is unlikely that the small amounts of "norbuprenorphine" found in this study resulted from false positives due to cross-reactivity.

Then, was the "norbuprenorphine" found in this study a mere solvent? This hypothesis can explain the "norbuprenorphine" detected in this study. Namely, the small amounts of "norbuprenorphine" could be chemically dissolved into the samples. But if "norbuprenorphine" is a mere solvent, then why was the "norbuprenorphine" level so low compared with "buprenorphine" and "naloxone"? There are a few possible explanations.

First, "norbuprenorphine" could be a small byproduct of buprenorphine synthesis, and consequently "norbuprenorphine" was included in the final product of buprenorphine/naloxone film. To prevent this, the synthesis process should include a purification step. This study used generic buprenorphine/naloxone sublingual films, manufactured by ALVOGEN Inc. Therefore, an inquiry was put forward to the company regarding this issue. The response was simply a restatement of the metabolism of buprenorphine, and thus it is hard to prove or disprove this hypothesis.

Another explanation can be that buprenorphine/naloxone films suffer from some degradation during storage and transportation. Buprenorphine itself may act as an acid and attack the nitrogen group to form norbuprenorphine through *N*-dealkylation, which can then be dissolved into solute. To examine this hypothesis, a study with pH information of specimens and / or with a neutral or more basic buffer may be needed.

Finally, the small amounts of "norbuprenorphine" found in this study could result from a chemical reaction of

buprenorphine, more specifically, amine group *N*-dealkylation (See Figure 5).[17,44] This chemical reaction can take place in various forms, including amine-group *N*-dealkylation with catalysts such as metals, electrochemical *N*-dealkylation, photochemical *N*-dealkylation, and enzymatic *N*-dealkylation, and these *N*-dealkylations could transform "buprenorphine" into "norbuprenorphine."[44] In summary, the small amounts of "norbuprenorphine" found in this study could be explained as a solvent dissolved in the samples, namely, a product of degradation, or because of a chemical reaction in the samples. Future studies with more data await to examine these hypotheses.

Summary

Application of findings in this study

This study reviewed various findings from previous studies and further added some findings. We can apply these findings to day-to-day practices at OBOT programs for OUD patients who are on buprenorphine treatment. We can also apply these findings in clinical decision-making processes. The following is a list of possible applications.

1) A ratio between "buprenorphine" and "norbuprenorphine" >50 should be used to flag the urine samples as likely adulterated urine.
2) Once the "buprenorphine" and "norbuprenorphine" ratio is detected as >50, then adulteration can be confirmed with high "buprenorphine" and / or "naloxone" levels. On the other hand, a low "buprenorphine" and/or "naloxone" level should not exclude the possibility of adulteration if the "buprenorphine" and "norbuprenorphine" ratio is >50 because "buprenorphine" and "naloxone" levels can fluctuate due to various factors, such as area and duration of dipping. Thus, "buprenorphine" and "naloxone" levels can be used as confirmation, not as the primary criterion for identifying urine adulteration.
3) Even "naloxone" found in urine does not necessarily mean that naloxone is metabolized and excreted in urine because "naloxone" was even found in the purified water samples in this study, implying that "naloxone" could just represent dissolved naloxone.
4) A high "naloxone" level can indicate that the urine was collected shortly after naloxone is taken, probably within 24 hours because naloxone is metabolized quickly.
5) "Norbuprenorphine" was found in adulterated urine and even in purified water, indicating that "norbuprenorphine" is not necessarily a metabolite.

Therefore, it would be helpful to have an alert system for the laboratory results in the electronic medical record (EMR) when the ratio between "buprenorphine" and "norbuprenorphine" levels is >50. This system could help providers simplify the detection of possible urine adulteration.

Limitations

Limitations of this study are as follows. First, this study utilized 12 urine samples from 15 volunteers, yielding a small data set, resulting in low statistical analysis power. Secondly, the upper limit of measurement levels of "buprenorphine," "norbuprenorphine" and "naloxone" were 1000 ng/mL, and if the levels were above this amount, they were counted and calculated as 1000 ng/mL. On the contrary, the lower limit of detection for "buprenorphine" and "norbuprenorphine" was 2 ng/mL while that of "naloxone" was 100 ng/mL. Therefore, levels below these cut-off values were calculated as 0 ng/mL. These minimum and maximum cut-off levels could have affected the data analysis results. Thirdly, this study used urine samples that were from healthy individuals who were not on any medications. When the results of this study were applied in daily practices with actual OUD patients who are on buprenorphine/naloxone medications, the levels of buprenorphine and naloxone metabolites in urine might be affected by other factors, including patient medications and other medical conditions. As a result, some caution must be taken in applying the findings of this study to daily practice in OBOT programs. Finally, although "naloxone" levels in urine have been discussed in many previous studies, it is not a general practice to include "naloxone" in urine drug screening tests, especially in immuno-assay qualitative tests. Thus, the application of "naloxone" level results found in this study might be limited.

Conclusion

Urine drug screening tests have been crucial components of OUD treatment in OBOT programs because test results provide fundamental information about treatment progress, including the patient compliance with treatment. However, the urine drug test results should be used with caution; namely, they should never be used for punitive purposes, but instead for a better outcome of patient treatment. Keeping this in mind, this study explored the in-vitro urine experiments and compared them with the results from previous studies, providing further insights into the interpretation of urine drug screening test results in the clinical context. This information can be applied to OUD patient treatment in OBOT programs, especially in clinical decision-making processes. It can contribute to optimal OUD treatments, and consequently, improve the current problematic social issues of increasing opioid overdose incidents.

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Author Contributions

Concept and design: Furo, Elkin, Brimhall. Analyses and interpretation of the data: Furo. Drafting of the manuscript: Furo. Critical revision of the manuscript for important intellectual content: Lin, Zhou, Abdelsayed, Whitted, Brimhall, Elkin. Administrative, technical, or material support: Whitted, Brimhall, Elkin. Supervision: Furo, Brimhall, Elkin

Data

The data can be shared upon request.

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Appendix

Table A1. Demographic information of the participants.

CHARACTERISTIC (N = 12)	MEAN ± SD OR N (%)	RANGE
Age (y)	32.9 ± 11.3	20-57
Sex, male	10 (83.3.%)	
Ethnicity		
Hispanic	8 (66.7%)	
White	3 (25%)	
Other	1 (8.3%)	
Marital status		
Single	9 (75%)	
Married	3 (25%)	
Employment status		
Employed	12 (100%)	
BMI (kg/m^2)	29.1 ± 5.7	21-40.3
Smoking		
Never	11 (91.7%)	
Smoker	1 (8.3%)	
Veteran	1 (8.3%)	
Education		
Some college	8 (66.7%)	
Bachelor's	3 (25%)	
Others	1 (8.3%)	
On any meds	0 (0%)	
Any medical issues	0 (0%)	

Abbreviation: BMI, body mass index.
The data sets are presented as mean ± standard deviation (SD) or mean with n (%).

Table A2. Metabolite levels in 12 urine samples.

SPECIMENS	PARTICIPANTS	BUP (NG/ML) (2-1000)	NORBUP (NG/ML) (2-1000)	BUP-G (NG/ML) (5-1000)	NORBUP-G (NG/ML) (5-1000)	NAL (NG/ML) (100-1000)	CRE (MG/DL) (5-2239)
1mm*3sec	Subject 1	22	–	–	–	–	84
	Subject 2	113	–	–	–	331	165
	Subject 3	283	–	–	–	>1000	52
	Subject 4	118	–	–	–	407	27
	Subject 5	254	–	–	–	933	159
	Subject 6	72	–	–	–	226	45
	Subject 7	215	–	–	–	809	97
	Subject 8	80	–	–	–	233	67
	Subject 9	143	–	–	–	524	75
	Subject 10	47	–	–	–	187	138
	Subject 11	90	–	–	–	263	73
	Subject 12	256	–	–	–	991	95
	Average	141.08				492.00	89.75
	SD	88.95				351.82	43.98
half*3sec	Subject 1	54	–	–	–	337	84
	Subject 2	285	–	–	–	>1000	165
	Subject 3	46	–	–	–	110	52
	Subject 4	375	–	–	–	>1000	27
	Subject 5	282	–	–	–	>1000	159
	Subject 6	374	–	–	–	>1000	45
	Subject 7	597	–	–	–	>1000	97
	Subject 8	287	–	–	–	>1000	67
	Subject 9	382	–	–	–	>1000	75
	Subject 10	233	–	–	–	969	138
	Subject 11	155	–	–	–	601	73
	Subject 12	532	–	–	–	>1000	95
	Average	300.17				834.75	89.75
	SD	168.18				311.03	43.98
full*3sec	Subject 1	735	–	–	–	>1000	84
	Subject 2	559	–	–	–	>1000	165
	Subject 3	45	–	–	–	117	52
	Subject 4	>1000	14	–	–	>1000	27
	Subject 5	>1000	3	–	–	>1000	159
	Subject 6	437	–	–	–	>1000	45
	Subject 7	>1000	3	–	–	>1000	97

Table A2. (Continued)

SPECIMENS	PARTICIPANTS	BUP (NG/ML) (2-1000)	NORBUP (NG/ML) (2-1000)	BUP-G (NG/ML) (5-1000)	NORBUP-G (NG/ML) (5-1000)	NAL (NG/ML) (100-1000)	CRE (MG/DL) (5-2239)
	Subject 8	>1000	–	–	–	>1000	67
	Subject 9	>1000	–	–	–	>1000	75
	Subject 10	245	–	–	–	504	138
	Subject 11	100	–	–	–	354	73
	Subject 12	73	–	–	–	215	95
	Average	313.43	6.67			765.83	89.75
	SD	268.76	6.35			356.91	43.98
full*30sec	Subject 1	>1000	4	–	–	>1000	84
	Subject 2	>1000	4	–	–	>1000	165
	Subject 3	>1000	3	–	–	>1000	52
	Subject 4	>1000	16	–	–	>1000	27
	Subject 5	>1000	9	–	–	>1000	159
	Subject 6	>1000	–	–	–	>1000	45
	Subject 7	>1000	9	–	–	>1000	97
	Subject 8	>1000	4	–	–	>1000	67
	Subject 9	>1000	20	–	–	>1000	75
	Subject 10	>1000	–	–	–	>1000	138
	Subject 11	>1000	–	–	–	>1000	73
	Subject 12	>1000	–	–	–	>1000	95
	Average	1000	8.63			1000	89.75
	SD	0	6.32			0	43.98

Abbreviations: bup = "buprenorphine", norbup = "norbuprenorphine", bup-G = "buprenorphine glucuronide," norbup-G = "norbuprenorphine glucuronide," nal = "naloxone", cre = "creatinine". 1mm*3sec = buprenorphine/naloxone film 1mm was dipped vertically for 3 seconds, half*3sec = half film was dipped for 3 seconds, full*3sec = full film was dipped for 3 seconds, full*30sec = full film was dipped for 30 seconds. SD, standard deviation.

Table A3. 100% urine, 10% urine, and 100% purified water comparison.

	BUP (NG/ML) (2-1000)	NORBUP(NG/ML) (2-1000)	BUP-G (NG/ML) (5-1000)	NORBUP-G (NG/ML) (5-1000)	NAL (NG/ML) (100-1000)	CRE (MG/DL) (5-2239)
0% BT						
1mm*3sec	221	–	–	–	637	–
Half*3sec	642	–	–	–	>1000	–
Full*3sec	>1000	–	–	–	>1000	–
Full*30sec	>1000	5	–	–	>1000	–
0% RT						
1mm*3sec	147	–	–	–	347	–
Half*3sec	594	–	–	–	>1000	–
Full*3sec	>1000	2	–	–	>1000	–
Full*30sec	>1000	4	–	–	>1000	–
10% BT						
1mm*3sec	77	–	–	–	304	7
Half*3sec	273	–	–	–	>1000	–
Full*3sec	122	–	–	–	468	–
Full*30sec	>1000	10	–	–	>1000	–
10% RT						
1mm*3sec	16	–	–	–	–	6
Half*3sec	217	–	–	–	761	–
Full*3sec	149	–	–	–	426	–
Full*30sec	>1000	3	–	–	>1000	–
100% BT						
1mmg*3sec	141.08	–	–	–	492	89.75
Half*3sec	300.17	–	–	–	834.75	–
Full*3sec	598.75	6.67	–	–	765.83	–
Full*30sec	>1000	8.5	–	–	>1000	–

Abbreviations: 0%BT = 100% purified water at ~97°F, 0%RT = 100% purified water at room temperature, 10%BT = 2 mL of 100% urine with 18 mL of ~97°F water, 10%RT = 2 mL of 100% urine with 18 mL of room temperature water, 100%BT = average of 12 urine samples.