

Urine pH: the Effects of Time and Temperature after Collection*

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Abstract

The Mandatory Guidelines for Federal Workplace Drug Testing Programs provide criteria for specimen validity testing, including urine pH cut-offs, to report a urine specimen as adulterated or invalid. Since the urine pH criteria for invalid classifications, ≥ 3 and < 4.5 or ≥ 9 and < 11 , became effective in November 2004, a number of specimens with results within the upper invalid limits, typically in the range of 9.1 to 9.3, have been reported with no evidence of adulteration. This study evaluated the hypothesis that these pH findings were the result of exposure to increased environmental temperatures during specimen standing and transport. Indeed, increased storage temperatures were associated with increased urine pH, with the magnitude of the change related to both storage time and temperature. The pH values of specimens stored at -20°C are relatively stable, whereas pH results > 9 are achieved at storage temperatures of room temperature or higher. It is noteworthy that no condition(s) produced a specimen with a pH > 9.5 . Degradation of nitrogenous urine analytes is most likely responsible for the noted increases in pH. These findings are intended to supplement information used by the Medical Review Officers who are responsible for interpreting such marginally invalid pH results.

Introduction

Public Law 100-71 (July 11, 1987) required the United States Department of Health and Human Services (HHS) to publish

Mandatory Guidelines that: 1. establish comprehensive standards for all aspects of laboratory drug testing and laboratory procedures to be applied in carrying out Executive Order 12564 (September 15, 1986) for the Drug-Free Federal Workplace, including standards which require the use of the best available technology for ensuring the full reliability and accuracy of drug tests and strict procedures governing the chain of custody of specimens collected for drug testing; 2. specify the drugs for which federal employees may be tested; and 3. establish standards and procedures for periodic review of laboratories and criteria for certification and revocation of certification of laboratories to perform drug testing for Federal agencies (1,2).

The National Laboratory Certification Program (NLCP) was established to certify laboratories, as mandated by Executive Order and Public Law, to conduct forensic workplace drug testing for federal agencies and for some federally regulated industries (3). Since the Mandatory Guidelines were first established in 1988, there has been a continuous review process in the development of standards for determining the validity of a urine specimen based on program experience with attempts by some specimen donors to adulterate, dilute, or substitute their specimens in order to mask the detection of illicit substances. As part of an ongoing effort to keep up with evolving practices and products used to try to "beat the drug test," detailed forensic standards for determining the validity of urine specimens, collected under the Mandatory Guidelines for Federal Workplace Drug Testing Programs, were published by the Department of Health and Human Services (HHS) in the Federal Register (69 FR 19644, April 13, 2004) (4). HHS, through the Substance Abuse and Mental Health Services Administration (SAMHSA), provides regulatory and program operational oversight using an inspection and performance testing process to ensure that laboratories, certified under the Mandatory Guide-

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lines, adopt and accurately follow the specimen validity testing and reporting procedures in the Mandatory Guidelines. The pH of a specimen collected for workplace drug testing is an important criterion to measure for two reasons: 1. FDA-cleared immunoassay kits used to perform the initial tests for marijuana metabolites, cocaine metabolites, opiate metabolites, phencyclidine, and amphetamines are designed to perform optimally in a pH-dependent fashion, and 2. products manufactured and sold with the intent to be added to the donor's specimen to facilitate passing a drug test sometimes contain components that have a very low pH (acidic) or very high pH (basic) in order to disrupt the immunoassay test or destroy the drugs in the specimen. Current specimen validity testing criteria for reporting a specimen as adulterated, substituted, invalid, or dilute are provided for certified laboratories performing workplace drug testing for federal agencies and some federally regulated industries (4). Urine pH cutoffs, < 3 or ≥ 11 , have been established to classify a urine specimen as adulterated (4). In addition, a urine specimen is reported as invalid when the urine pH is ≥ 3 and < 4.5 or ≥ 9 and < 11 (4). A colorimetric pH test or pH meter is used for the initial test and a pH meter is used for the confirmatory test (4).

Following the publication of the April 13, 2004, Mandatory Guidelines, some urine specimens subject to testing under the new standards have been reported with pH results greater than 9 and in the range of 9.1 to 9.3. No known adulterants were found as the cause of these invalid pH results. In addition, recollection under direct observation in some instances still produced urine pH results greater than 9. Investigations into these urine specimens, commonly referred as pH 9.x urine specimens, yielded the following observations: nitrite levels indicative of possible bacterial infection, greater incidence during the warm summer months, and a predominance of females as specimen donors. This study was undertaken to clarify the mechanism of urine pH 9.x production. The findings provide valuable new information to all parties in the specimen collection, transportation, testing, and reporting continuum, and helpful guidance to Medical Review Officers (MROs) in the interpretation of pH 9.x urine results.

Transportation conditions for urine specimens submitted under the Mandatory Guidelines for workplace drug testing to HHS-certified laboratories are not specifically stated in the regulatory text. Typically, packaged samples are delivered from the site of collection to the certified testing laboratory by couriers, express carriers, or the postal service. In some situations, delivery times may take up to two weeks, especially when these urine specimens were collected from distant locations, such as overseas, and shipped to the U.S. with no precautionary measures taken to avoid the extremes of temperature that specimens may experience during transit. Urine specimens tested in accordance with the Mandatory Guidelines in an HHS-certified laboratory that are reported as positive, adulterated, substituted, or invalid are stored for up to one year at -20°C after testing is completed (4).

In contrast to workplace drug testing, a random (untimed) clinical urine specimen submitted for pH testing performed as part of the urinalysis profile has specific transportation re-

quirements. Rapid transport to the laboratory is recommended, and testing is ideally performed within 2 h of collection (5). If testing is delayed, refrigeration is suggested (5). Some reference laboratories, in which the 2-h testing time constraint is impractical, use specialized collection containers to maintain urine specimen integrity. For instance, one manufacturer of urine collection containers incorporates a proprietary, insoluble, broad-spectrum antimicrobial agent into the wall of the polypropylene plastic that helps preserve formed elements and does not interfere with urinalysis test results such as pH and specific gravity (6).

Because of the time constraints imposed by clinical laboratories on urine pH testing, medical literature references on long-term stability of urine pH are scarce. This study was undertaken to evaluate the effects of time and temperature on urine pH under various physiologically simulated conditions.

Experimental

The study hypothesis is that a urine specimen will become alkaline upon long-term standing, especially at high temperatures.

In the study design, a freshly collected, random (untimed) urine specimen pool was aliquotted into working pools; each pool spiked with certain physiological additives (Table I). From the working pools, a unique aliquot was prepared for each additive condition, at each temperature, and for each time point. Aliquots were stored at the various temperatures for a maximum of two weeks. On each specified day, the pH of each aliquot was measured in duplicate and recorded. The change in pH with time was compared for each condition and incubation temperature.

Apparatus

Urine pH measurements, recorded to two decimal places, were determined using an Accumet Basic AB15 pH meter (Fisher Scientific, Atlanta, GA), calibrated each day of use with pH 4.0, 7.0, and 10.0 calibrators. Urine creatinine, urea, and uric acid concentrations were determined using the Bayer ADVIA 1650 Chemistry System (Siemens Medical Solutions Diagnostics, Tarrytown, NY). Urine osmolality was determined

Table I. Conditions Employed in the Urine pH Study

Additives	Temperature ($^{\circ}\text{C}$)	Incubation Times, Days Post Void
None	-20	1
Bacteria	4	2
Glucose	25	3
Glucose + bacteria	37	7
Protein	93	8
Protein + bacteria		9
Yeast		10
Yeast + glucose		14

using the Advanced model 3220 Micro-osmometer (Advanced Instruments, Norwood, MA). Urine specific gravity was determined using a TS Meter Refractometer (Reichert Analytical Instruments, Depew, NY) to four decimal places. The 37°C incubation was in the American IC 42 incubator (American Scientific Products, Columbus, OH), and the 93°C incubations were in the VWR Standard Heating Blocks (West Chester, PA) and the Denville Scientific Incublocks (South Plainfield, NJ).

Materials

Pseudomonas aeruginosa ATCC 27853 (gram-negative bacteria) and *Rhodotomula minuta* (yeast) were obtained from Dr. Amy Horneman, University of Maryland, Baltimore. The pH buffer solutions (4.0, 7.0, 9.0, and 10.0) and the osmolality Clinintrol 290 Reference Solution were obtained from Fisher Scientific (Atlanta, GA). Sabouraud dextrose broth, Luria broth, and Luria agar plates were obtained from Becton, Dickinson and Company (Sparks, MD). Phosphate-buffered saline (PBS) and the 2.0-mL screw-top microfuge polypropylene tubes for aliquot incubation were purchased from VWR International (Philadelphia, PA). D-(+)-Glucose, human albumin, and sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Creatinine, urea, and uric acid chemistry reagents were obtained from Siemens Medical Solutions Diagnostics (Tarrytown, NY). The Liquichek urine chemistry controls were purchased from Bio-Rad Laboratories (Hercules, CA).

Methods

Urine pH reference interval verification. The urine pH reference interval was verified by specimens obtained from three different locations in Baltimore, MD: the Clinical Laboratories of the University of Maryland Medical Center, a hospital-based clinical laboratory; Quest Diagnostics, a large clinical reference laboratory; and the Office of the Chief Medical Examiner, a forensic postmortem laboratory. For the hospital laboratory, random urine specimens were analyzed for pH within 2 h of collection using a urine dipstick method. For the reference laboratory, urine specimens, collected in the proprietary urine cups described previously, were analyzed for pH by a dipstick method within 24 h of collection. The forensic urine samples were removed by needle aspiration from bladders within 24 h of death and stored frozen until analyzed for urine pH using a pH meter at the Armed Forces Institute of Pathology, Washington, D.C.

Subjects. Under a study protocol approved by the Institutional Review Board of the University of Maryland, Baltimore, three healthy, ambulatory volunteers each donated a fresh, random, untimed morning urine sample.

In vitro alkalinization. To determine the extent of urine alkalinization achievable with sodium bicarbonate addition, three fresh random urine specimens were spiked with a freshly prepared 8.4% sodium bicarbonate solution. Additionally, sodium bicarbonate crystals were added directly to the urine samples. The urine pH was measured both before and after the addition of the sodium bicarbonate.

Bacteria and yeast preparation. Bacterial and yeast strains

were cultured in Luria and Sabouraud dextrose broths, respectively, and grown overnight at 37°C in a shaking incubator. Fresh broth was inoculated with 100 µL/10 mL of the respective microorganisms and incubated until the microorganisms had grown to mid-log phase as determined by the absorbance at 600 nm. The cells were pelleted by centrifugation and resuspended to the appropriate concentration in PBS. The bacterial concentration was confirmed by serial dilutions plated on Luria agar.

Specimen preparation. The three random urine samples provided by the subjects were promptly pooled, and the pH of the mixed pool was determined (pH 6.93). The pool was further divided into eight working pools, each with a minimum volume of 45 mL. These eight working pools were used for each of the following study conditions: neat urine, urine + bacteria, urine + glucose, urine + glucose + bacteria, urine + protein, urine + protein + bacteria, urine + yeast, and urine + yeast + glucose (Table I). For those working pools requiring glucose and protein, D-glucose and human albumin were each added to final concentrations of 2000 mg/dL. For those working pools requiring bacteria and yeast, *Pseudomonas aeruginosa* and *Rhodotomula minuta* were each spiked into the appropriate working pools to final concentrations of 49,000 colonies/mL. The pH was determined on each completed working pool. Each working pool was subsequently further divided into 40 individual 1.0 mL portions, one for each incubation temperature (−20, 4, 25, 37, and 93°C) and for each of the eight analysis days. The portions were aliquotted into appropriately labeled sterile cryovials and securely capped. The respective aliquots were incubated at their designated temperatures for the indicated periods of time. Vials were stored in the freezer at −20°C, in the refrigerator at 4°C, on the bench top at 25°C, in an oven at 37°C, and in heating blocks at 93°C (Table I). The temperatures of the freezer, refrigerator, incubator, and heating blocks were verified and monitored with NIST-certified thermometers.

pH changes by time, temperature, and composition. On incubation days 1, 2, 3, 7, 8, 9, 10, and 14 post-void, the appropriate aliquots were removed from incubation and brought to room temperature (Table I). The vial contents were transferred to a test tube, mixed, and the pH of each aliquot was immediately determined in duplicate. All duplicate pH results for each aliquot had to agree within ± 0.2 pH units or additional measurements were taken until the desired reproducibility was achieved. Each batch of five specimens was bracketed by pH 7.0, 9.0, and 10.0 quality control materials that had to read within ± 0.2 pH units.

Changes in urine creatinine, urea, uric acid, osmolality, and specific gravity by temperature. Additional aliquots of the neat urine were stored at −20, 4, 25, 37, and 93°C for two weeks and subsequently stored frozen at −20°C until analysis. The aliquots were thawed, mixed, and immediately analyzed for urine creatinine, urea, uric acid, osmolality, and specific gravity. Clinical quality control material tested along with the urine specimens was within acceptable limits.

Data analysis. The mean was calculated for each aliquot pair. The means were plotted chronologically for each of the various conditions and incubation temperatures.

Results

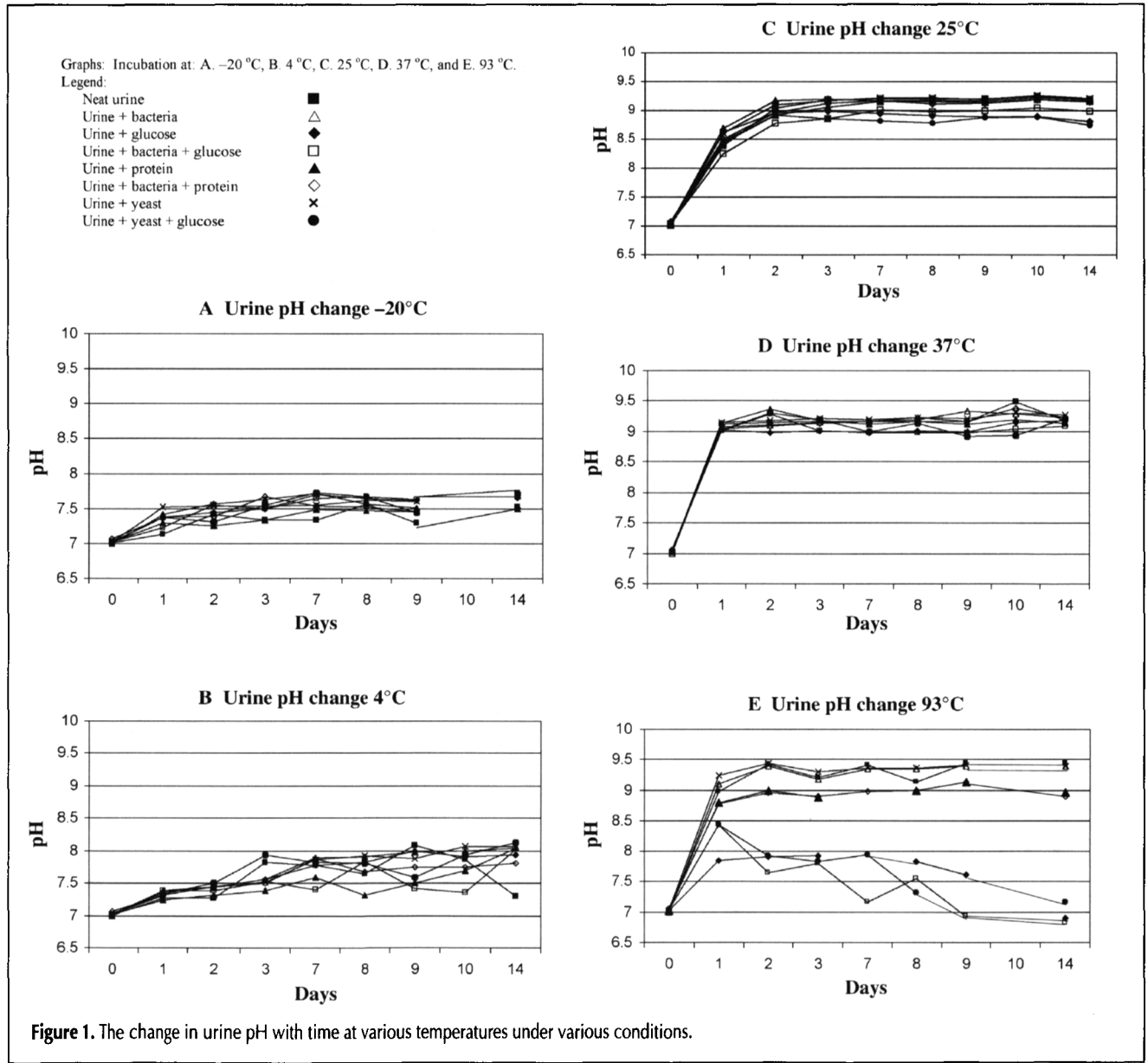
Urine pH reference interval verification

To verify the clinical reference interval of 4.5–8 (average 5–6) for random (untimed) urine pH, data statistics were obtained from three different sources: a hospital laboratory, a clinical reference laboratory, and a medical examiner’s office

Table II. Summary Statistics for Random Urine pH Reference Interval Verification					
Laboratory	n	Mean	Range	Median	Mode
Hospital	2206	6.1	5.0–8.5*	5.9	5.0
Reference	9122	6.3	5.0–8.5*	6.0	6.0
Forensic	108	6.1	4.6–8.5	5.9	5.8

* The reportable range for the urine dipstick method was 5.0–9.0 with a reportable interval of 0.5 pH units.

(7). The summary statistics are presented in Table II. As a point of interest, the data from the hospital laboratory, when plotted as a histogram (data not shown), did not exhibit the expected Gaussian distribution. Instead, the 0.5 pH unit bin percentages gradually declined from the high of pH 5.0, with the majority of the data clustered in the pH 5–6 range. The data from the reference laboratory, which represented specimens that had a delay in analysis of up to 24 h, did exhibit a Gaussian distribution with a tail skewed to higher pH values. Like the reference laboratory pH data, the postmortem pH values, having a delay in collection from the time of death to sampling, when plotted as a histogram, exhibited a skewed Gaussian distribution with a peak between pH 5.5 and 6.0. Thus, specimens from the reference laboratory, where the majority of the specimen pH values fell in the range of pH 5.5–7.0, and the post-mortem laboratory where the majority of the pH values were clustered between 5.0 and 6.5, exhibited a shift towards increased pH values with time when compared to the clinical laboratory data. Whether the urine pH statistics were from the



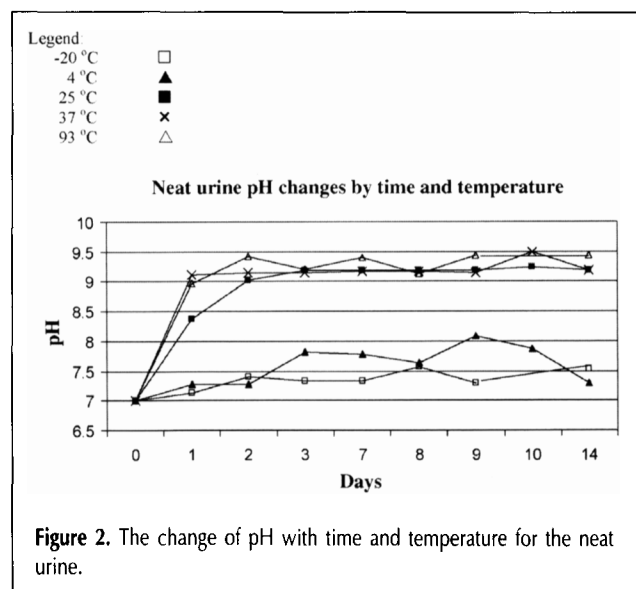
hospital laboratory, the clinical reference laboratory, or the medical examiner's office, none of the 11,436 specimens tested had a pH > 8.5. In fact, 3 of 108 (2.8%) specimens from the postmortem laboratory, 45 of 9122 (0.5%) specimens from the reference laboratory, and 28 of 2206 (1.3%) specimens from the clinical laboratory were between pH 8.0 and 8.5 [total 76 of 11,436 (0.7%)]. In the postmortem data where pH values < 5.0 were measurable, no values < 4.5 were found. The lowest measured pH was 4.6.

In vitro alkalinization

In the in vitro alkalinization study, the maximum pH value reached, whether by the addition of sodium bicarbonate solution or crystals, was 7.9. The maximum pH reached was not influenced by the initial pH value of the urine specimen.

pH changes by time, temperature, and composition

Those urine aliquots stored at -20°C were relatively stable, exhibiting at most a +0.7 increase in pH over the two-week incubation period. As the temperature increased, the pH of the urine aliquots also increased. At 4°C, the pH gradually increased with time to reach a high of pH 8.1 at 14 days. At 25°C, the pH jumped dramatically for all conditions by day 1 of incubation. By day 2, the pH for the 25°C incubation aliquots had leveled off to a pH of about 9.2 for the majority of the conditions. The pH in those samples containing glucose never exceeded pH 9.0, with the highest pH values ranging from 8.7 to 9.0 at 25°C. At 37°C, peak pH values > 9.0 were achieved by day 1. Those specimens containing glucose and incubated at 93°C showed an initial increase in pH at day 1 (pH 7.8–8.4) followed by decreasing pH values with time. Urine spiked with glucose and incubated at 93°C darkened in color, varying from honey to black, with some of these specimens developing a precipitate. Specimens in the other conditions incubated at 93°C achieved pH values ranging from 8.8 to 9.2 by day 1 and leveled off for the duration of the incubation. Graphical representation of the pH changes with time is presented in Figure 1. As a point of interest, the inclusion of microorganisms (bacteria and yeast) and protein and glucose at pathological levels did not



have any significant effect on the changing pH values, excluding the pH decreasing effect of glucose at 93°C after day 1, as evident from the graph that depicts the changes in the neat urine at various temperatures (Figure 2).

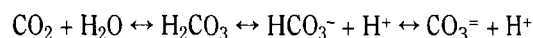
Changes in urine creatinine, urea, uric acid, osmolality, and specific gravity by temperature

Urine specific gravity was stable at each incubation temperature. The other analytes showed varying stability by temperature. Urine urea was stable only at -20°C, uric acid at -20 and 4°C, and creatinine at -20, 4, and 25°C. Urine osmolality values were stable at -20 and 4°C, increased at 25 and 93°C, and decreased at 37°C (Figure 3).

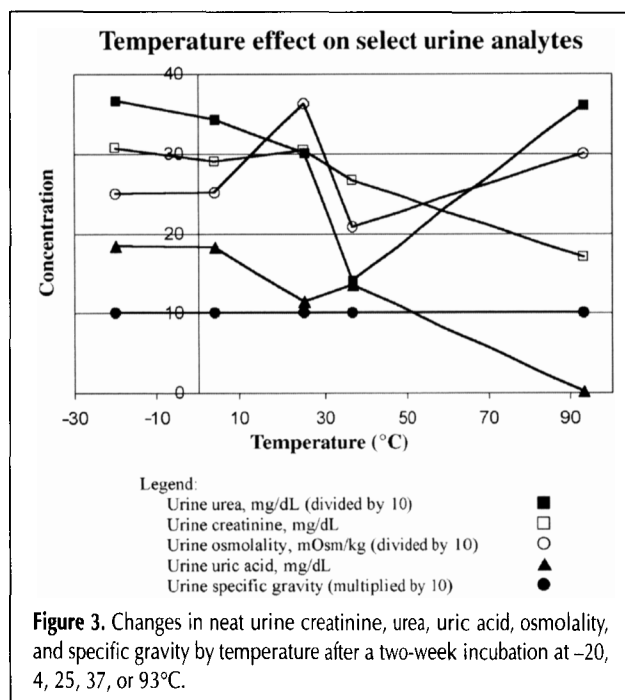
Discussion

Review

Whole blood pH is maintained within an extremely narrow pH range of 7.35–7.45 at the expense of urinary pH. Because urine is able to achieve a hydrogen ion concentration 1000× greater than blood, its range of pH is 4.5–8 (average pH 5–6) (7,8). The lungs and kidneys are primarily responsible for regulating the body's acid-base content, and thus the blood pH range that is compatible with human life. The kidneys, which produce urine, function in acid-base balance primarily through the selective regulation of plasma bicarbonate by affecting the equilibrium of the following reaction sequence (8):



By secreting and excreting hydrogen ions in the form of ammonium ions, phosphates, sulfates, and weak organic acids and reabsorbing bicarbonate, the renal tubules maintain blood pH (8).



The pH of urine is dependent on the time of day, the prandial state, diet, health status, and medications (9). Various pre-analytical variables that may affect urine pH values are given in Table III (10). Urinary pH exhibits a diurnal variation with decreased pH values at night and in the early morning (most acidic towards midnight) followed by increasing pH values upon awakening (11). Urine tends to become alkaline immediately after a meal because of a phenomenon known as the alkaline tide and gradually becomes acidic between meals (12).

A high protein diet is associated with acidic urine, and a vegetarian diet typically produces more alkaline urine because of bicarbonate formation from fruits, especially citrus, and vegetables.

Often, the urine specimen becomes contaminated preanalytically with bacteria during collection. Both the male and female urethras are colonized with microorganisms; thus, urine collected by conventional voiding is often bacterially contaminated (11). Midstream random urine collections, whether

Table III. Preanalytical Variables, Disease States, and Drugs that Can Cause Physiological Changes in Urine pH (10)

Decreased pH			Increased pH		
Preanalytical variable	Disease	Drug	Preanalytical variable	Disease	Drug
Cadmium exposure	Acquired adrenal insensitivity	Acetazolamide	Aldosterone	Acute renal failure	Acetaminophen
Diurnal variation	Acute poststreptococcal glomerulonephritis	Amiloride	Bed rest	Adrenal cortical hyperfunction	Acetazolamide
Heparin		Aminoglycosides	Citrate	Adrenal cortical hypofunction	Amiloride
Lead exposure	Amyloidosis	Ammonium chloride	Meals		Amphotericin B
Mercury exposure	Carbonic anhydrase II deficiency	Ascorbic acid	Storage		Aspirin
Methionine	Chronic obstructive pulmonary disease	Cholestyramine	Toluene	Amyloidosis	Carbenoxolone
	Cystinosis	Converting enzyme inhibitors	Vanadium	Autoimmune thyroiditis	Cefdinir
	Desmolase deficiency	Corticotropin		Balkan nephropathy	Cimetidine
	Diabetes mellitus	Coumarin		Chronic active hepatitis	Diclofenac
	Methenamine	Diazoxide		Chronic pyelonephritis	Diflunisal
	Diabetic nephropathy	Metolazone		Cryoglobulinemia	Etodolac
	Diarrhea	Niacin		Cystinosis	Fenoprofen
	Distal renal tubular acidosis (type IV)	Spironolactone		Diabetes mellitus	Flurbiprofen
	Familial methyl oxidase deficiency	Streptozocin		Distal renal tubular acidosis	Ibuprofen
	Galactosemia			Fabry's disease	Ifosfamide
	Glycogen storage disease I			Fibrosing alveolitis	Indomethacin
	Gout			Hepatolenticular degeneration (Wilson's disease)	Ketoprofen
	Hereditary fructose intolerance			Hereditary fructose intolerance	Lithium
	3- β -Hydroxydehydrogenase deficiency			Hypercalciuria, idiopathic	Mafenide
	21-Hydroxylase deficiency			Hypergammaglobulinemia	Mefenamic acid
	Lactosuria			Hyperparathyroidism	Naproxen
	Light chain multiple myeloma			Marfan's syndrome	Niacinamide
	Lowe's syndrome			Medullary sponge kidney	Ofloxacin
	Lupus nephritis			Metabolic alkalosis	Orthophosphate
	Malabsorption			Polyarteritis nodosa	Parathyroid extract
	Medullary cystic disease			Primary biliary cirrhosis	Ranitidine
	Metabolic acidosis			Proximal renal tubular acidosis (type II)	Triamterene
	Metachromatic leukodystrophy			Renal transplant rejection	Sodium bicarbonate
	Multiple myeloma			Sickle cell disease	
	Nephropathy, obstructive			Sjögren's syndrome	
	Pseudohypoaldosteronism			Urinary tract obstruction	
	Renal transplant rejection			Vitamin D intoxication	
	Renal transplantation			Voltage-dependent distal renal tubular acidosis (type 1)	
	Renal vein thrombosis				
	Renovascular hypertension				
	Salt-wasting nephritis				
	Sjögren's syndrome				
	Transient mineralocorticoid deficiency of infancy				
	Tubulointerstitial disease				
	Tyrosinemia				
	Vitamin D deficiency				
	Vitamin D resistance				
	Wilson's disease				

with or without prior cleansing of external genitalia, have bacterial contamination rates of up to 30% (13,14). The problem can be aggravated in women because of further contamination from intestinal flora if the collection is not done carefully (11). Left standing, the pH of a bacterial-contaminated, unpreserved urine specimen will continue to increase. Bacterial contamination of urine with microorganisms that split urea may yield urinary pH values > 8.0 because of bacterial decomposition of urea to ammonia. Urine specimens collected at autopsy by bladder puncture are considered sterile and free of bacterial contamination unless the bladder itself is harboring bacteria (11).

The disease states that can affect urine pH are listed in Table III (10). Common causes of alkaline urine include respiratory and metabolic alkalosis, prolonged vomiting, urinary tract infection with urea-splitting bacteria, and meals (15). Common causes of acidic urine include respiratory and metabolic acidosis, dietary cranberries, diabetes mellitus, starvation, and severe diarrhea (15). In acid-base disorders, the urine pH is an indicator of renal compensation in an attempt to return blood pH to normal. An individual with normal renal function and with either respiratory or metabolic acidosis is expected to excrete acidic urine while a patient with either respiratory or metabolic alkalosis should produce alkaline urine as a means of compensation (12). Any renal tubular disorder, such as renal tubular acidosis, that adversely affects secretion and reabsorption of acids and bases will alter urine pH (12).

Medications that influence urine pH are found in Table III (10). Commonly, urine pH changes are idiopathically caused by treatments for urinary tract infection and calculi formation (15). In certain clinical situations, urine is deliberately manipulated to produce an alkaline pH to aid renal clearance of certain drugs, especially in cases of drug overdose (16).

Care must be taken in interpreting pH results determined by dipsticks because of methodological limitations. In the forensic laboratory, urine dipsticks are used for screening purposes only, and these screening pH results are not reported. In the clinical laboratory, determination of urine pH is commonly done by reagent dipsticks imbedded with indicator dyes. These reagent strips rely on the urine's hydrogen ion concentration to cause a color change in the indicator dyes. Because no one indicator dye will cover the entire random urinary pH reference interval, most reagent strips utilize two indicators, methyl red and bromthymol blue (BTB) (7). Methyl red responds to pH concentrations in the range from 4.2 to 6.2, producing a color change from red to yellow (7). BTB is responsive in the pH range of 6 to 7.6, producing a corresponding color change from yellow to blue (7). When combined into one reagent pad, the double indicator color changes from orange at pH 5 to yellow then green and finally blue at pH 9 (7). These indicator dyes are usually not very analytically sensitive, providing results within ± 0.5 –1.0 pH unit accuracy, and have a reportable pH range between 5 and 9.0 (15).

There are no known interferences associated with urine dipstick pH measurements. Analytical error can be caused by reagent bleeding into adjacent pads on the clinical multi-analyte dipsticks, especially from a very acidic protein reagent pad (9). In addition, timing of the reading of the reagent pad

color is important since the color of the reagent block changes with time (17). For dipstick pH measurements, there is a tendency for pH to increase with time, especially in the pH 6–8 range (18). For dipstick measurements that are read manually, there is a subjective element to the color interpretation, mainly in the pH 5–6 range (18).

Bias has been noted between urine pH determined by dipstick and the gold standard pH meter method (19). Specimens with a pH > 6.5 by the dipstick method oftentimes had a lower pH as determined by the pH meter, whereas those with a pH \leq 5.5 by dipstick had a higher true pH (19).

Urine pH reference interval verification

There are no medical conditions, either normal or abnormal, that can produce a pH > 9 in freshly excreted urine. In a study of 2600 patients screened by routine outpatient urinalysis with urine pH determined by dipstick, 53 patients were determined to have alkaline urine pH results, with only one given a value of pH 9.0 by dipstick (20). Random urine specimens from cannabinoid users ($n = 38$) yielded initial pH values ranging from 5.1 to 8.8 (21). In our study to verify the clinical reference interval for random urine specimens, none of the 11,436 specimens tested had a pH > 8.5, and only 0.7% were pH > 8 and < 8.5. The lowest measured pH was 4.6. Thus, the clinical reference interval of 4.5–8 for a random urine pH was verified and, conversely, a non-fresh, improperly preserved, or tampered random urine specimen with a pH \geq 9 is suspect (7).

In vivo alkalization

In vivo alkalization of urine is performed in certain drug overdose situations (16). In cases of salicylate poisoning, sodium bicarbonate is administered until the urine pH is in the range of 7.5–8.5 (16). In a study of induced metabolic alkalosis through sodium bicarbonate administration, in vivo urine pH averaged 7.56 ± 0.03 (22). These recommendations and findings are consistent with our in vitro maximum sodium bicarbonate result of pH 7.9.

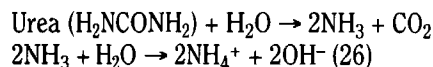
pH changes by time, temperature, and composition

Temperature and pH have important effects on enzymatic and spontaneous chemical reactions (23). As temperatures decrease, both enzymatic and spontaneous chemical reactions are significantly slowed (23). Changing the pH has a tremendous impact on enzymes, which typically have a narrow pH range for catalytic activity (23). In addition, pH determines protein binding and the stability of pH-sensitive molecules (23). Thus, it is important to understand the preanalytical effects of post-void urine temperature and pH changes and their relationship to the analyte conditions present at sample collection.

Urine contains urea and uric acid, non-protein nitrogenous waste compounds, which are common constituents of urine. The stability of these two molecules is affected by bacterial contamination. Uric acid is degraded by microbial uricase activity to allantoin, hydrogen peroxide, and carbon dioxide while urea is labile to bacterial urease activity with ammonia and carbon dioxide produced as products (24,25). To prevent degradation, it is recommended that urine for uric acid and urea determinations be stored at 4°C to inhibit bacterial growth

(24,25). Optimally, urine for urea analysis is best preserved at pH values < 5.0 (24).

To assess the extent of alkalization caused by a urea-splitting bacterium, urine was spiked with the gram-negative *Pseudomonas aeruginosa*. This specific bacterium species was selected because of its ability to produce urease, an enzyme that converts urea to ammonia, and its identification as a common pathogen in contaminated urine specimens (26,27). A urea-splitting microorganism is able to produce alkaline urine through the following mechanism:



Rhodotorula minuta is a common pathogen in yeast infections. Both the bacteria and yeast were spiked in the urine to a concentration of 49,000 colonies/mL, a value considered as substantial microbiological growth (27). In our study, the expected increase in pH from bacterial urease activity was not evident. Similarly, a yeast contamination effect on pH could not be discerned in light of the predominant temperature effect.

To simulate excessive amounts of sugar and protein in urine and their effects on urine pH, D-glucose and human albumin were each added to a concentration of 2000 mg/dL, a concentration equivalent to 4+ on the urine dipstick. Neither protein nor glucose appeared to affect urine pH except for the protective effect of glucose against excessive urine alkalization at increased temperatures.

As indicated by the results of this study, increased incubation temperatures were associated with development of urine with pH values > 9.0. This study investigated the extremes of temperature that may be encountered during specimen transportation, including excessive cold and heat. Internal temperatures of closed vehicles on hot days can exceed 65°C, and temperatures on exposed surfaces, such as dashboards, can reach 93°C (28–31).

In a rat study, fresh urine was collected from two animals after an overnight fast (9). At void, the pH values of the two urines were 6.2 and 7.1 (9). After incubation at 25 and 37°C, the pH values increased to 8.7 and 8.9 to 9.0, respectively, after 24 h (9). When these alkaline urine specimens were evaporated to dryness and resuspended in water to the initial volume, the resultant pH was less than the fresh urine pH (9). The authors surmised that the increase in pH with time was due to the loss of CO₂ and the evaporation process resulted in the loss of ammonia, causing the return to acidic pH values (9). In our study design, urine aliquots were tightly capped with minimal air space within the vial. Though the vial contents were transferred to test tubes that would accommodate the diameter of the pH electrode, pH measurements were taken immediately upon transfer. Thus, the loss of CO₂ may not explain the observed increase in urine pH, but production of ammonia does. In conclusion, study findings demonstrate that increased temperature is directly correlated with increases in pH.

Changes in urine creatinine, urea, uric acid, osmolality, and specific gravity by temperature

The major non-protein nitrogenous urine analytes—creati-

nine, urea, and uric acid—were examined for their stability in neat urine at various temperatures. Degradations of these compounds could result in altered urine pH values. Uric acid and creatinine degraded with increasing temperatures. The pattern seen with urea at first appears illogical since at 93°C urea is stable, in contrast to its progressive degradation at the lower temperatures. One explanation for this pattern is that the urine pool was contaminated by urea-splitting bacteria that are destroyed at 93°C.

Analytes that are indicative of urine concentration, specific gravity and osmolality, were also examined. While urine specific gravity is stable, osmolality varies inconsistently with temperature, mirroring at warmer temperatures the pattern exhibited by urine urea. This is to be expected because urea is a major contributor to urine osmolality. The increase in osmolality at 93°C reflects the urea stability.

In addition, urine creatinine and specific gravity, like pH, are used for specimen validity testing. A urine specimen is classified as substituted when the creatinine is < 2 mg/dL and the specific gravity is ≤ 1.0010 or ≥ 1.0200 (32). A urine specimen is considered dilute when the creatinine is ≥ 2 mg/dL but < 20 mg/dL and the specific gravity is > 1.0010 but < 1.0030 (32). This study found that specific gravity is relatively stable when incubated at temperatures that could be encountered during transport. The range of measured specific gravities was 1.005 to 1.008 with a –20°C control specific gravity result of 1.006. Urine creatinine was unstable at higher temperatures and achieved concentrations < 20 mg/dL (lowest: 16.8 mg/dL) at 93°C. At none of the investigated temperatures were results for urine creatinine concentration or specific gravity obtained that met the reporting criteria of a substituted or dilute specimen.

Effect of pH on stability of drugs of abuse in urine

Previous studies have examined the stability of drugs of abuse in urine at various temperatures and/or pH values. As a special project task under SAMHSA contract number 277-2003-00044, 12 forensic laboratories certified by the National Laboratory Certification Program analyzed aliquots of drug-free human urine spiked with amphetamine, methamphetamine, benzoylecgonine, codeine, morphine, 6-acetylmorphine (6-AM), 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid (THCA), and phencyclidine (PCP) to concentrations 1.5 to 2 times the initial test cut-off (33). These aliquots of spiked urine were adjusted to pH ranges of 5–5.5 (acidic control urine), 8–9, 9–9.5, and 9.5–10 (33). The manufactured urine specimens were shipped by overnight courier without cold packs on the day of preparation, stored at room temperature upon receipt by the laboratories, and analyzed on days 1 and 3 post-preparation for urine pH determined by pH meter and confirmatory drug concentrations by gas chromatography–mass spectrometry (33). Urine pH was stable, showing little to no change between analysis days 1 and 3 (33). Amphetamine, methamphetamine, codeine, morphine, and THCA showed no loss in drug concentration in the basic urines when compared to the acidic urine control over the three-day study period (33). Benzoylecgonine, PCP, and 6-AM decreased significantly in concentration when compared to the acidic

urine control. These decreases in drug concentration increased with increasing urine alkalinity and with increasing time (33). Thus, within the invalid pH range, loss of drug analytes has been confirmed, specifically for benzoylecgonine and 6-AM because of hydrolysis and PCP because of decreased solubility (33).

One study looking at workplace drugs found urine spiked with drugs, including morphine, codeine, cocaine, and amphetamine, and incubated for two weeks either frozen, at room temperature, or at 37°C still yielded acceptable results for qualitative testing (34). Bacterial growth was evident in some of the urine aliquots incubated at room temperature and 37°C (34). In addition, some of the urine aliquots stored at 37°C became alkaline with time (34). In another study of drug stability in 236 physiological urine specimens stored at -20°C for one year, THCA, amphetamine, methamphetamine, morphine, codeine, cocaine, benzoylecgonine, and PCP showed minimal concentration changes except for an average 37% decrease for cocaine (35).

In a study examining the stability of free and glucuronidated THCA in authentic urine specimens incubated at -20, 4, 20, and 40°C, the glucuronide was unstable at temperatures $\geq 4^\circ\text{C}$, resulting in decreased glucuronide and increased free THCA concentrations (21). Overall, increased temperatures ultimately caused a loss in the total THCA concentration (21). In the same study, the stability of free and glucuronidated THCA at various pH values, ranging from 5.0 to 8.0, was investigated in spiked urine (21). Free THCA is most stable at pH 5.0, with recovery decreasing with increasing pH and time (21). The glucuronidated metabolite deteriorated to free THCA at all pH values tested, with instability increasing with increasing pH and time (21).

Amphetamine in urine exhibited a decreased recovery when stored at either -20 or 4°C for 10 months but degraded dramatically when stored at 25–35°C (36). Urine specimens containing amphetamine heated at 60, 70, and 100°C at either pH 5.1 or 7.6 for 1 h had decreased recoveries for all heating conditions (37).

Free and conjugated morphine were stable in urine at -20 and 4°C for 10 days with decreased recoveries at these temperatures after 10 months (36,38). At 18–22 and 37°C, free and conjugated morphine were stable for 10 days but degraded dramatically when stored at 25–35°C for 10 months (36,38). The reported stability of 6-AM in urine varied by study. In urine stored at -20°C, reported stability was a minimum of two years (39). In another study, the degradation of 6-AM was 7 and 9% after 7 and 14 days, respectively, at -20°C (40). At 4 and 18–22°C, urine 6-AM was reported stable for 10 days and a slight degradation occurred at 37°C (38). Another study found a 10% loss in urine 6-AM at 20°C after 4 h and a 100% loss after two weeks (40). 6-AM, when exposed to pH 9 conditions, exhibited negligible hydrolysis, but hydrolysis was complete when highly alkaline conditions were combined with increased temperatures (40).

Urine acetylcodeine, stored at either -20 or 4°C at either pH 4.7 or 8.0, was stable for 23 weeks (41). When stored at room temperature at pH 8.0 for 5 weeks, acetylcodeine completely hydrolyzed (41). When incubated at pH 4.7 at room tempera-

ture, only 58% of acetylcodeine was recovered (41).

In another study, drug-free urine was spiked with cocaine HCl and incubated at 25°C for 72 h at various pH values (pH 5, 7, 8, and 9) (42). At pH values of 5 and 7, only 0.6–0.7% of the cocaine hydrolyzed (42). Under alkaline conditions (pH 8 and 9), cocaine was unstable, undergoing spontaneous hydrolysis to benzoylecgonine and to the ecgonine methylester (42). After 72 h, the detected concentrations of benzoylecgonine and ecgonine methylester at pH 8 were 34% and 36%, respectively, and at pH 9 were 43% and 43%, respectively, of the initial amount of cocaine added (42). Urine specimens containing benzoylecgonine heated at 60, 70, and 100°C at either pH 5.1 or 7.6 for 1 h had decreased recoveries for at all heating conditions (37).

Conclusions

The results of this study have important implications for workplace urine drug testing. Urine pH and creatinine concentration are used as criteria in specimen validity testing. Both are unstable at higher temperatures, with urine pH increasing and creatinine concentrations decreasing. When the decreasing urine creatinine concentration was combined with the stable specific gravity measurements, none of the incubation temperatures produced results that would be classified as either substituted or dilute. This study demonstrated that increased incubation temperatures could produce urine pH values > 9 , with pH 9.5 being the highest pH value attained in the study. We now more clearly understand that the pH of urine specimens collected for federal workplace drug testing programs can potentially be affected by the time and temperature of transport and storage prior to analysis at the laboratory. Urine pH > 9 may affect the stability of some drugs of abuse target analytes and decrease their concentration if present in the donor's specimen. It is important to note that none of the conditions examined in this study produced a specimen with a pH > 9.5 , thus limiting the impact of the pH elevation on concentrations of drugs of abuse.

The final review of laboratory-reported results and discussion of the results with the specimen donor by the MRO are essential because a laboratory result reported as invalid does not automatically identify specimen tampering. In the Medical Review Officer (MRO) Manual for Federal Workplace Drug Testing Programs effective November 2004 (43), the MRO is instructed to contact the donor to determine if he or she has an explanation for the invalid result. When the donor has no alternative medical explanation for the elevated pH and the specimen has a value between 9.0 and 9.5, it is important for the MRO to consider the length of time between specimen collection and testing at the laboratory. Extended transport time and increased seasonal or environmental temperatures may affect the pH of that donor's urine specimen. Because urine pH values > 9 are achievable with increased temperatures in as little as two days, specimens classified as invalid should be considered in the context of this study.

This paper is intended to supplement information used by

MROs in evaluating alternative, non-medical explanations for federal workplace drug test results reported as invalid.

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