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Urinary creatinine concentration and urine color as indicators of specimen validity test

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Abstract

In this study, the concentration of urinary creatinine (Cr) and urine color were analyzed, and a correlation obtained, to objectively verify normal urine samples prior to forensic drug testing. Cr was analyzed via a colorimetric method based on the Jaffé reaction using a Cobas C-311 analyzer (Roche SA, Basel, Switzerland; Hitachi, Tokyo, Japan). The Cr concentration for urine specimen validity testing was measured to screen urine samples submitted after dilution or upon the addition of a foreign substance that interferes with drug testing. Urine samples containing < 20 mg/dL of Cr were classified as abnormal. The Korea Standard Color Analysis program was used for urine color analysis and correlations with Cr concentration analyzed. The color and Cr concentration of 271 urine samples were analyzed according to age and sex. The mean ± standard deviation of Cr concentration in males and females was 136.4 ± 66.2 mg/dL and 109.5 ± 71.1 mg/dL, respectively, with a statistically insignificant difference ($p = 0.4554$). Furthermore, the participants were categorized into young (19–34 years), middle-aged (35–49 years), and elderly (≥ 50 years) groups to compare Cr concentration; however, no significant difference was found ($p = 0.2143$–0.983). Strong variable correlations were identified between Cr and the characteristics of urine color. Despite various factors such as water consumption, health problems, and vitamins, urine color was found applicable for urine specimen validity testing. Future plans include the development of a smartphone camera application for use in urine color analysis to identify abnormal urine samples.

Keywords Urine drug testing, Creatinine, Urine color, Specimen validity, Adulteration

Introduction

Urine and hair are the most commonly used specimens in forensic drug testing. Urine has higher concentrations of the parent drug and its metabolites than hair because it is excreted after metabolism. Furthermore, urine samples are widely used in drug testing (Dolan K et al. 2004; Caplan and Goldberger 2001) because they are easy to collect. The sample collection process is one of the most critical factors prior to urine drug testing. To guarantee the integrity of the specimen and prevent any fraudulent acts for obtaining a false negative result, sample collection must be performed under direct supervision, with a tamper-proof collection container. In the United States (U.S.) and Europe, urine specimen validity testing (SVT) is mandatory for the prevention of tampering by dilution or the addition of foreign substances that interfere with forensic drug testing (Federal Register 2017; EWDTS 2022).

To contain the effects of dilution or concentration of urine samples, the drug released through urine must be quantified in less than 24 h; however, this guideline can cause practical difficulties in sample collection and lead to errors in the process (Chotayaporn et al. 2011; Leung 2007). Therefore, a method based on urine samples collected on the spot is used for facile sample collection in compliance with the 24 h drug quantity conditions, and the drug concentration measured from the specimen

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is reduced to creatinine (Cr) concentration for subsequent use (Cone et al. 2009; Arndt 2009).

The World Health Organization (WHO) states that the effective concentration range of urinary Cr is 30–300 mg/dL. A sample is defined as having been diluted if the urinary Cr concentration is 5.6–22.6 mg/dL in Europe and 5–20 mg/dL in the U.S (Federal Register 2017; EWDTS 2022). Using a stricter criterion than the aforementioned three criteria, a urine sample is considered abnormal and diluted when the urinary Cr concentration is 5–20 mg/dL.

Current urinary Cr analysis methods include the colorimetric method based on the Jaffé reaction, liquid chromatography-tandem mass spectrometry (LC–MS/MS), and isotope dilution mass spectrometry used as the standard analysis (Chung et al. 2008; Moore and Sharer 2017; Ou et al. 2015). In a prior study, a strong correlation ($r = 0.9897$) was found between the concentration of Cr measured using the colorimetric method and that measured through LC-MS/MS (Kwon et al. 2012; Luginbühl and Weinmann 2017). In this study, the urinary Cr concentration was measured using the colorimetric method, which shows a strong correlation with that measured using LC-MS/MS and is frequently used in general clinical testing.

Urine chromaticity is a practical indicator of the dilution of urine samples, although it cannot completely replace other SVT parameters such as specific gravity and pH. Diluted urine generally has a lighter color than normal urine. Conversely, highly concentrated urine is usually dark yellow or amber and may change according to the concentration of urochrome (Park and Shin 2013). Earlier water content identification methods in urine include an at-the-toilet-bowl method of self-assessing urine concentration via urine color, the use of a pocket-sized CMOS imaging-based analytical device, and a 3D origami microfluidic paper device (Wardenaar et al. 2021; Ye et al. 2017; Musile et al. 2023). In this study, a urine sample was collected in a transparent test tube and images were taken using a digital camera. The images were partially processed via image digital processing and the characteristics of urine color were determined using the Korea Standard Color Analysis program. Analyzing the correlations of chromaticity variables with the Cr concentration measured using the colorimetric method enables the development of a method to identify the water content in urine.

The purpose of this study is to measure the Cr concentration and color of urine to identify their correlation and determine whether a urine sample has been intentionally diluted. Thus, abnormal urine samples can be objectively verified prior to forensic drug testing.

Materials and methods

Reagents and apparatus

The Cr used in the preparation of the standard solution was purchased from Acros Organics (Geel, Belgium). Ultra-pure water was produced via a Millipore Milli-Q purification system (Millipore, Bedford, MA, USA). The synthetic urine (UriSub) used in the preparation of the quality control sample was obtained from CST Technologies, Inc. (Great Neck, NY, USA).

The standard stock solution was sequentially diluted with deionized water as needed, and the prepared standard solutions were stored at − 20 °C for subsequent use.

Urine samples

Though Cr is normally detected in human urine, there are constraints and limitations in conducting repeated measurements using human urine. Hence, quality control (QC) samples (20 mg/dL) were prepared by the addition of different aliquots of the working stock solution of the analyte to synthetic urine without Cr. Urine samples used in the Cr analysis were collected from 271 subjects. Some of these subjects were to be released from medical treatment and custody while others were complying with the community order that had been sent requesting for drug testing from probation offices in Seoul, Suwon, and other cities between January and February 2023. The age range of the subjects was 12–87 years, and the sex distribution was 185 males (68.3%) and 86 females (31.7%). The urine samples were stored at 4 °C prior to analysis.

Instrumentation

A Cobas C311 analyzer (Roche, Basel, Switzerland; Hitachi, Tokyo, Japan) was used for Cr analysis; the colorimetric method was based on the Jaffé reaction. The measurements were taken by adding the Cr Jaffé Gen.2 assay (CREJ2, Roche/Hitachi) reagent to the device. For quantification, a two-point calibration curve between 0.0 and 97.75 mg/dL was drawn. The manufacturer's guideline states that the lower limit of quantification (LLOQ) is 4.2 mg/dL, and the range of quantification is 4.2–622 mg/dL. For QC, samples of low concentration (Precipath PUC) at 51.3 mg/dL and high concentration (Precinorm PUC) at 93.5 mg/dL were used.

Sample preparation

Un-centrifuged urine (200 µL) was loaded in a polystyrene cup, and 10 µL was injected to the Cobas C311 analyzer for analysis.

QC samples

The standard Cr solution was added to synthetic urine to prepare QC samples ($n=5$) at 20 mg/dL to measure precision and accuracy. Repeated measurements were conducted for three days.

Stability test

Five measurements were taken for each of the seven urine samples to test the stability of urinary Cr. For short-term stability tests, measurements were taken after 2, 4, 6, and 8 h at room temperature in the lab before sample preparation and analysis. For long-term stability tests, measurements were taken after 7, 14, 20, and 30 d at 4 °C. The results were compared with those under the initial conditions, and the stability was determined to be acceptable if the deviation was < 15%.

Urine color analysis

For urine color analysis, images of the urine sample in a glass test tube were taken using a Cannon EOS 80D camera, with an 18–135 mm EF-S lens mount 1:3.5–5.6 IS USM at F9.0 aperture, a 1/125 shutter speed, an ISO 800 sensitivity, and a white balance (WB) 5000 K with G3 calibration, and 69 mm focal distance. To adjust the light intensity, a luminous plate (BL-2250P, Fomex) was set to 5400 K color temperature and applied from the top right and top left positions. White paper was placed on the wall background to control the light intensity. The Korea Standard Color Analysis program developed by the Korean Agency for Technology and Standards was used in the measurement and testing of urine color from some of the collected images. In this study, value and chroma, L*, a*, and b* values were determined using the Munsell color system-based Korea Standard Color System and International Commission on Illumination (CIE) color system. The Korea Standard Color System, revised based on the Munsell color system, regulates the selection and systematization of basic color terms. The Munsell color system defines each color as a sign representing the three parameters of color—hue, value, and chroma as follows: (hue × value) / chroma. The CIE color system was validated by the CIE in 1978, whereby L*, a*, and b* are measured (Han 2021). L* indicates the lightness (i.e., white, and black), a* indicates the redness and greenness, and b* indicates the yellowness and blueness.

Statistical analysis

Statistical analyses were performed to examine the differences in Cr concentrations according to age and sex. The measured values should exhibit homogeneity when used to verify the differences in the mean values between groups. When the measured values exhibit normal distribution, an independent t-test is used. However, the Wilcoxon rank sum test is used as a non-parametric method when normality cannot be assumed. The Shapiro–Wilk test was used to test normality (Bangtson and Goodkind 1982). R–4.2.2 (www.r-project.org) and RStudio–2022.12.0–353 (www.posit.co) software were used for the statistical analyses in this study.

Table 1 Method precision and accuracy for urinary creatinine

Compound	Nominal concentration (mg/dL)	Intra-day ($n=5$)		Inter-day ($n=15$)	
		Precision (% CV)	Accuracy (% bias)	Precision (% CV)	Accuracy (% bias)
Creatinine	20	0.9	6.9	1.5	6.8

Results and Discussion

Precision and accuracy

Table 1 lists the measurements of precision and accuracy of the analytical method. The intra-day and inter-day precision was 0.9% and 1.5%, respectively; the intra-day and inter-day accuracy was 6.9% and 6.8%, respectively. In both cases, the results were acceptable with < 15% deviation and ± 15% coefficient of variation.

Stability

The stability of Cr in urine was tested to determine the effects of time, temperature, and container type. For the short-term (8 h) and long-term (30 d) stability tests, urine samples from seven subjects were prepared as five samples for each measurement and the obtained ranges were −2.2 to − 2.3% and − 0.9 to − 1.1%, respectively (Fig. 1). Under typical analytical conditions for 8 h at room temperature and 30 d at 4 °C, no influencing factor was detected in the stability test of urinary Cr.

Urinary Cr concentration

The concentration of Cr was measured according to the methods of the analyzer and pretreatment set for the urine samples. A sample with 20–300 mg/dL Cr was defined as normal urine. The number of urine samples satisfying the criterion of Cr concentration was $n=243$. The measurements were 136.4 ± 66.2 mg/dL in males (n = 169) and 109.5 ± 71.1 mg/dL in females ($n=74$). The Cr concentration was compared after dividing the subjects on the basis of age into young (19–34 years, $n=72$), middle-aged (35–49 years, $n=91$), and elderly (≥ 50 years, $n=72$) groups. The urine samples of subjects under 19 years of age ($n=8$) were excluded from the age comparison analysis owing to their small number. The measurements were 149.9 ± 74.6 mg/dL, 123.1 ± 64.9 mg/dL, and 111.0 ± 64.7 mg/dL, respectively (Fig. 2). The

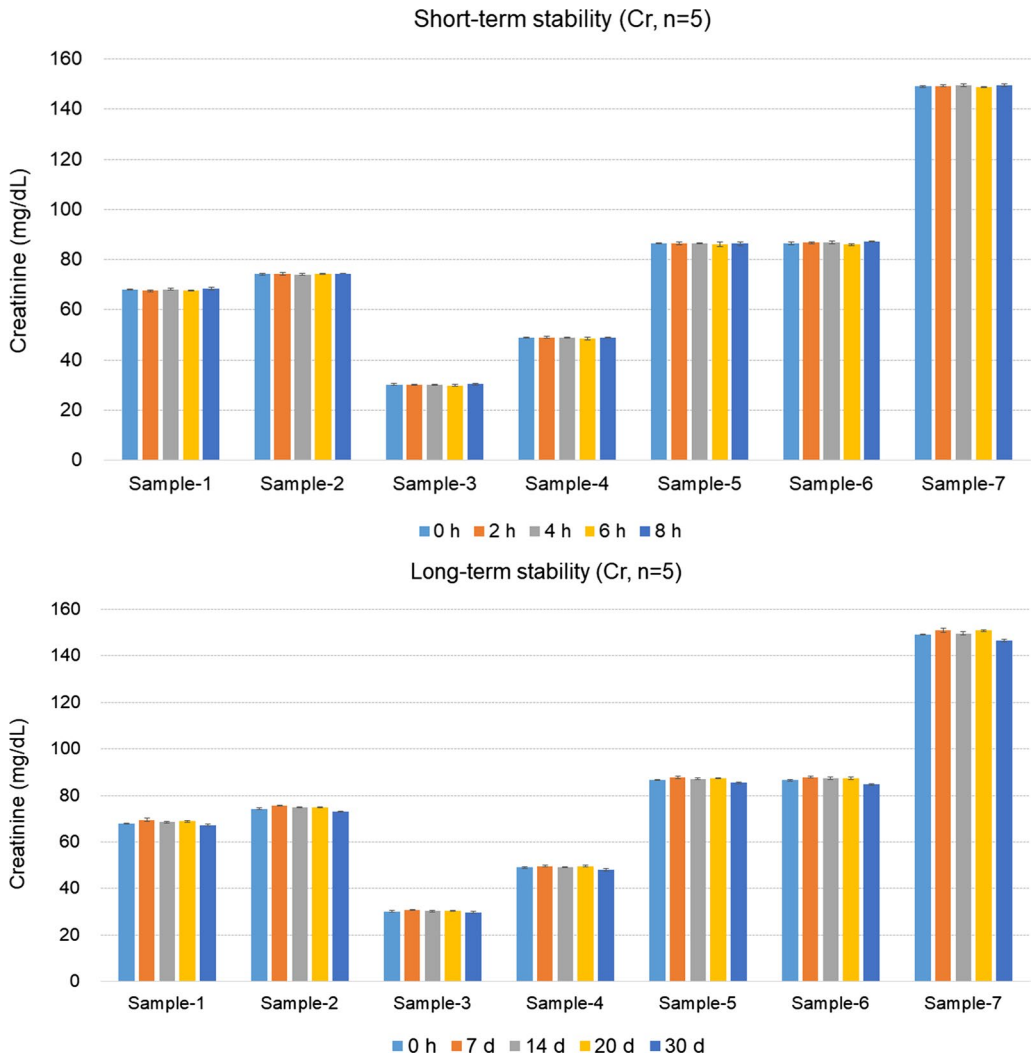

Fig. 1 Short- and long-term stability test results of urine creatinine ($n=5$, mean) at room temperature and 4 °C

results of this study show that the mean Cr concentration was higher in males than in females, and a decreasing trend was observed as the age of the subjects increased.

Comparison of urinary Cr concentration according to sex and age

In this study, the variation in Cr concentrations was compared based on sex (males and females) and age: young (19–34 years), middle-aged (35–49 years), and elderly (≥ 50 years) groups. The Shapiro–Wilk test showed that normality was not satisfied; therefore, the Wilcoxon rank sum test was performed on the measured values. Analyzing the samples according to sex indicated that $p=0.4554$ for Cr concentrations in the samples from 169 males and 74 females; therefore, no significant difference was found for the distribution of the measured values according to sex. Furthermore, analyzing the samples according to age indicated that $p=0.2143$, 0.2349, and 0.983 for the young (19–34 years), middle-aged (35–49 years), and elderly (≥ 50 years) groups, respectively; therefore, no significant difference was observed in the distribution of Cr concentrations according to age.

Correlations between urinary Cr and color space values

Among the urine samples, 243 out of 271 samples (89.7%) exhibited an effective Cr concentration within the normal range (20–300 mg/dL). The distribution of urinary Cr concentration for these samples is shown in Fig. 2. Table 2 summarizes the correlation coefficients obtained for the Cr concentration and chromaticity of the urine samples, and the distribution of these variables and the correlations with Cr are shown in Fig. 3. According to

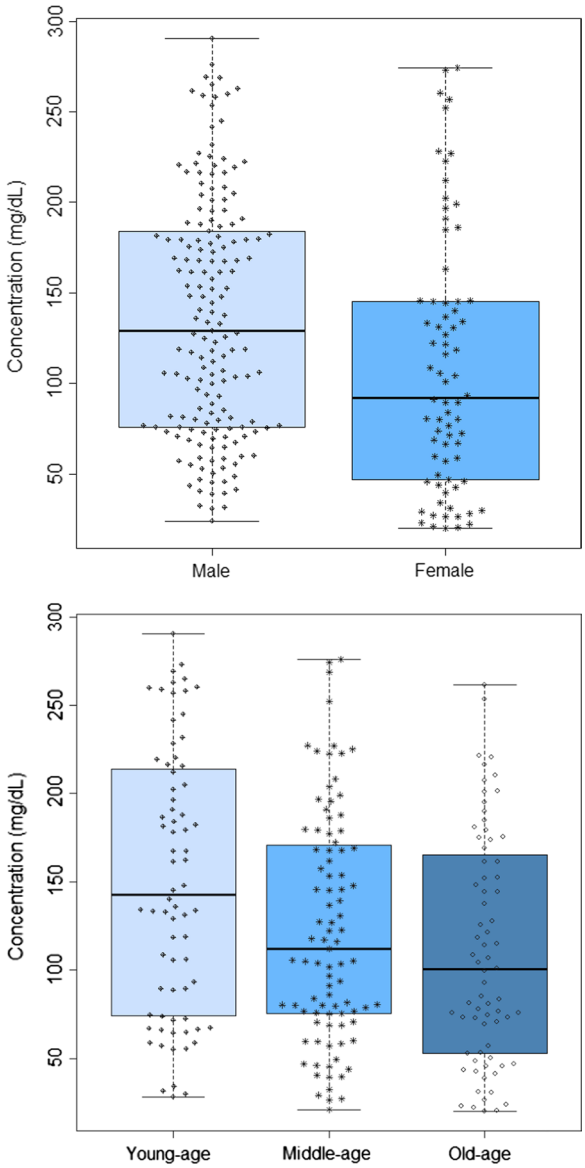

Fig. 2 Boxplot and frequency distribution of the concentration of creatinine in urine samples according to sex and age

the data in Table 2, chroma and b* exhibited strong correlations with Cr. The correlation between chroma and b* was 0.9881 at a high concentration, as shown in Fig. 3. In contrast, the chromaticity analysis of the urine samples (n = 16) with the urinary Cr concentration of < 20 mg/dL indicated that the value and chroma were both 0 for the samples with Cr concentration of < 10 mg/dL ($n = 5$), for which a* and b* were also 0. For all the samples with 10–20 mg/dL Cr concentration, the value and chroma were 9 and 1, respectively (Table 3).

In this study, urine chromaticity analysis could clearly distinguish between normal urine (creatinine concentration 20–300 mg/dL) and abnormal urine (creatinine concentration < 20 mg/dL). Urine chromaticity analysis is a qualitative method that can be readily used in the field without having to rely on more complex analytical equipment such as a spectrophotometer. However, accurate urine testing requires the spectrophotometric determination of Cr by the colorimetric method.

Conclusion

The Cr analysis method used in this study showed that the mean Cr concentration of the 271 urine samples tested was higher in males than in females and that the concentration decreased as age increased. The analysis of 243 urine samples within the normal range (20–300 mg/dL) of Cr concentration indicated that chroma and b* exhibited a strong correlation with Cr concentration. In contrast, the urine samples (n = 16) in the abnormal range (< 20 mg/dL) were clearly distinguished in the chromaticity analysis. Taking into consideration the various conditions that affect the determination of urine color, color assessment has been verified as useful method in urine SVT. The results of this study confirm that color assessment is a rapid and facile method for the identification of abnormal urine samples prior to forensic drug testing. Future plans include the development of a smartphone camera application to expand the scope of urine color assessments in urine SVT.

Table 2 Matrix of correlations

Variables	Cr	Value	Chroma	L*	a*	b*
Cr	1	−0.1159	0.7272	−0.5836	0.2842	0.7111
Value	−0.1159	1	−0.1361	0.3665	−0.3271	−0.0757
Chroma	0.7272	−0.1361	1	−0.7169	0.3125	0.9881
L*	−0.5836	0.3665	−0.7169	1	−0.5871	−0.6411
a*	0.2842	−0.3271	0.3125	−0.5871	1	0.1728
b*	0.7111	−0.0757	0.9881	−0.6411	0.1728	1

The CIELAB color space, also referred to as L*a*b* expresses color as three values: L* for perceptual lightness and a* and b* for the four unique colors of human vision. Thus each of L* a* b* can be viewed as a single variable for the CIELAB color space

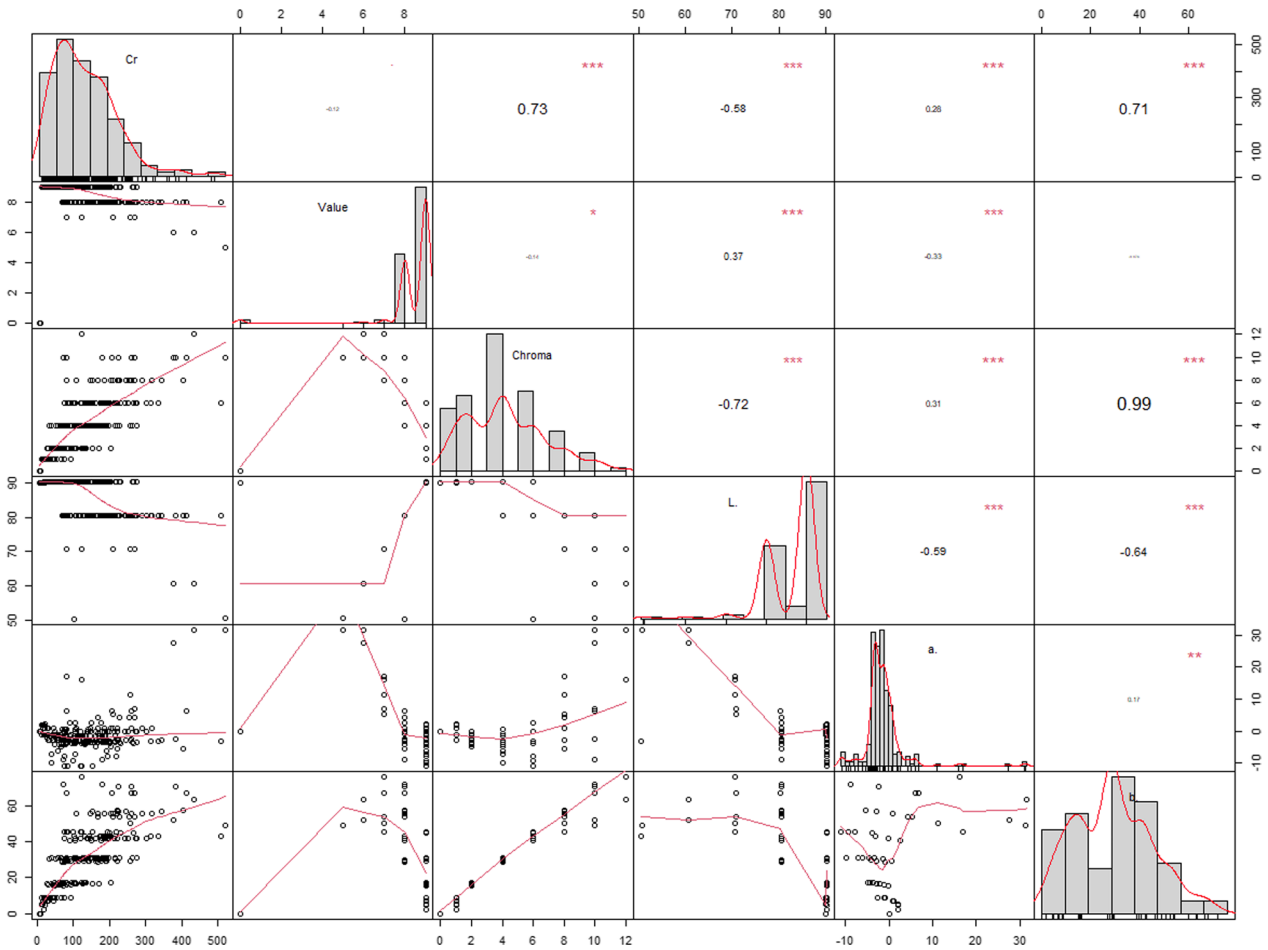

Fig. 3 Correlation chart and distribution diagram of urine color space values and creatinine concentration

Table 3 Color evaluation of sixteen urine samples with a creatinine concentration under the cutoff value of 20 mg/dL

Sample No	Creatinine concentration (mg/dL)	Korean Standard Color System		CIE L*a*b* color coordinate value			Urine color
		Value	Chroma	L*	a*	b*	
00036	6.3	0	0	90	0	0	
00164	7.5	0	0	90	0	0	
00140	8.3	0	0	90	0	0	
00064	9.2	0	0	90	0	0	

Table 3 (continued)

Sample No	Creatinine concentration (mg/dL)	Korean Standard Color System		CIE L*a*b* color coordinate value			Urine color
		Value	Chroma	L*	a*	b*	
00012	10.5	0	0	90	0	0	
00224	11.8	9	1	90	2	5	
00159	12.1	9	1	90	2	5	
00112	12.9	9	1	90	− 1	9	
00134	13.9	9	1	90	2	5	
00135	14.4	9	1	90	1	7	
00184	14.6	9	1	90	2	5	
00080	16.3	9	1	90	1	7	
00018	17.2	9	1	90	− 1	9	
00252	17.3	9	1	90	2	2	
00127	18.6	9	1	90	− 1	9	
00111	19.6	9	1	90	1	7	

Abbreviations

Cr	Creatinine
WHO	World Health Organization
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
QC	Quality control
CIE	International commission on illumination

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Author contributions
JHP contributed to conceptualization, investigation, formal analysis, validation and writing—original draft. NHK contributed to investigation and formal analysis. SYK contributed to methodology, writing—review and editing. BJK contributed to writing—review and editing. JYK contributed to conceptualization, visualization, writing—review and editing, and supervision. All authors have read and approved the final version of the manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests
There are no conflicts of interest to declare.

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