

## Research Article

# Establishment of a Method for Measuring Antioxidant Capacity in Urine, Based on Oxidation Reduction Potential and Redox Couple $I_2/KI$

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**Objectives.** To establish a new method for determination of antioxidant capacity of human urine based on the redox couple  $I_2/KI$  and to evaluate the redox status of healthy and diseased individuals. **Methods.** The method was based on the linear relationship between oxidation reduction potential (ORP) and logarithm of concentration ratio of  $I_2/KI$ . ORP of a solution with a known concentration ratio of  $I_2/KI$  will change when reacted with urine. To determine the accuracy of the method, both vitamin C and urine were reacted separately with  $I_2/KI$  solution. The new method was compared with the traditional method of iodine titration and then used to measure the antioxidant capacity of urine samples from 30 diabetic patients and 30 healthy subjects. **Results.** A linear relationship was found between logarithm of concentration ratio of  $I_2/KI$  and ORP ( $R^2 = 0.998$ ). Both vitamin C and urine concentration showed a linear relationship with ORP ( $R^2 = 0.994$  and  $0.986$ , resp.). The precision of the method was in the acceptable range and results of two methods had a linear correlation ( $R^2 = 0.987$ ). Differences in ORP values between diabetic group and control group were statistically significant ( $P < 0.05$ ). **Conclusions.** A new method for measuring the antioxidant capacity of clinical urine has been established.

## 1. Introduction

Free radicals were first described in the early twentieth century and now, as a result of in-depth research into the effects of free radicals on biological systems, it is known that reactive oxygen and nitrogen radicals attack DNA, proteins, lipids, and other biological macromolecules, inducing changes in structure and function and eventually leading to physiological and pathological changes [1]. It is now clear that free radicals are linked to many physiological phenomena and diseases [2], including aging [3, 4], tumors [5] and mutations, inflammation, cardiovascular and cerebrovascular disease [6, 7], atherosclerosis, diabetes, and autoimmune diseases, together with neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [8, 9]. To protect against damage, the body has antioxidant systems to clear free radicals and keep generation and removal in a state of

dynamic equilibrium [10]. Under pathological conditions, the redox balance will be shifted in the direction of oxidation or reduction, resulting in oxidative stress or reductive stress. Both of these states of imbalance can lead to physiological and pathological changes [11] and evaluation of redox state is thus important for the study of disease mechanisms and for monitoring clinical treatment [12].

Methods used to measure antioxidant capacity include [13] (1) determination of scavenging ability and inhibitory effects on reactive oxygen and nitrogen radicals [14], (2) measurement of reducing power (e.g., ability to reduce  $Fe^{3+}$ ), (3) quantitative determination of special small molecule antioxidants, (4) measurement of antioxidant enzyme activities, (5) cyclic voltammetry, and (6) determination of the oxidation/reduction ratios of systems such as GSH/GSSG and NADH/NAD. Because of the complicated nature of antioxidant systems and because the specific mechanism

of the antioxidant component is often not known, all of these methods have limitations and do not reflect the total antioxidant capacity of the system. Most of these methods are typically used to determine the antioxidant capacity of serum and plasma and are rarely used for the determination of urine specimens. The determination of total antioxidant capacity is a more reliable and comprehensive way to evaluate oxidative stress and monitor antioxidant therapy. Measurement of total antioxidant capacity better reflects the body's redox state [15] than determination methods that measure only a single antioxidant substance or a small number of antioxidants.

The oxidation reduction potential (ORP) reflects the ratio of oxidizing substances to reducing substances in solution. Measurement of ORP is, in principle, more reliable than other methods for determining the antioxidant capacity of the body and is the best method for assessing the ORP of urine. ORP is a thermodynamic parameter and, because of the complicated nature of biological systems, it is not appropriate to simply use the thermodynamic conditions of the Nernst equation to calculate ORP. Using the relative ratios of the concentrations of oxidized and reduced species in the redox couples GSH/GSSG and NADH/NAD (Schafer and Buettner) allows the Nernst equation to be used for determining the potential of various redox pairs in biological systems [16]. In this case, the ORPs of the redox pairs reflect the redox state of the biological system. Electrochemical methods currently used for the determination of total antioxidant capacity include the direct ORP method [17], the depolarization ORP method [18], and cyclic voltammetry [19]. When the direct ORP method was used with urine samples, a long time was needed to obtain a stable reading of ORP because of the limitations of the electrochemical properties of the electrode and the complexity of the redox system in urine.

Modification of the direct ORP method by introduction of a fixed concentration of an exogenous redox pair [iodine/potassium iodide ( $I_2/KI$ )] allows better determination of the ORP of urine samples. Oxidizing or reducing substances in urine affect the ORP value of the added redox pair and the change in ORP value reflects the redox status of the urine and the total antioxidant capacity of the urine. Urine contains the end products of metabolism that are excreted when blood passes through the kidney and its main components are low molecular weight oxidant and antioxidant substances [20]. Compared with serum, the composition of urine (mainly urea, inorganic salts, uric acid, creatinine, and urinary bile [21]) is relatively simple, making it more suitable for determination of ORP. Urine is also an easily accessible specimen and can be used to dynamically and continually monitor human antioxidant capacity. Analysis of urine specimens is presently used to monitor a number of diseases, including diabetes [22–25]. Our new method for measurement of ORP using urine samples has the following characteristics: (1) readings can be obtained in 120 s and the method is reproducible, reliable, and sensitive and (2) all substances that induce redox reactions with iodine or iodide ions cause changes in the measurement of potential, more fully reflecting the antioxidant capacity of urine. Using urine rather than blood for clinical monitoring of oxidative stress and antioxidant therapy is less traumatic for the patient and

greatly reduces the risk of cross infection. In the present study, we used our newly developed method to measure the ORP of a diabetic group and a normal group to evaluate the redox state of the two populations. Our new method could be used for evaluating redox state and antioxidant therapy in the clinic.

## 2. Materials and Methods

**2.1. Establishment of  $I_2/KI$  Redox Couple Method.** The equipment (Type ORP-431 measurement instrument, Shanghai DAPU Instrument Co., LTD. China) and electrode (ORP composite electrode) were calibrated before measurement. In the zero ORP correction, insert the electrode into buffer solution (pH = 7.00) saturated with quinhydrone. Record ORP value as  $E_1$ , which should be  $86 \pm 15$  mV ( $25^\circ\text{C}$ ). Immerse the test end of the electrode in distilled water for washing, and then insert the electrode into buffer solution (pH = 4.00) saturated with quinhydrone. Record ORP value as  $E_2$  and make sure that the difference between  $E_1$  and  $E_2$  ( $E_2 - E_1$ ) is greater than 170 mV ( $25^\circ\text{C}$ ), which is in a correction range. Then plot the standard curve of ORP values versus concentration ratio of the solution after correction.

The electrode solution was a solution of  $I_2$  (0.1 M) and KI (0.4 M) in deionized double distilled water. Electrode solution (200  $\mu\text{L}$ ) and urine (500  $\mu\text{L}$ ) were mixed with deionized double distilled water (2300  $\mu\text{L}$ ). The mixed solution was placed in an opaque cuvette and kept in the dark at room temperature for 1 h. ORP values were recorded at 120 s. Electrode solution without urine was used as the control.

**2.1.1. Comparison of Time Required to Obtain Stable ORP Value Using Direct Method (Direct Insertion of Electrode into Untreated Urine) with Time Required Using  $I_2/KI$  Method.** The ORP was measured by directly inserting the ORP combination electrode into a sample (3 mL) of mixed urine obtained from five healthy individuals. The ORP values were determined at the following time points: 10, 20, 30, 40, 50, and 60 s; 2, 4, 6, 8, 10, 20, 30, 40, and 60 min; 2, 3, 4, 5, 6, 7, and 8 h.

$I_2/KI$  electrode solution (200  $\mu\text{L}$ ) and mixed urine sample (500  $\mu\text{L}$ ) were mixed with deionized double distilled water (2300  $\mu\text{L}$ ). The mixed solution was placed in an opaque cuvette and kept in the dark at room temperature for 1 h. The ORP values were determined at the following time points: 30, 60, 90, and 120 s. Electrode solution without urine was used as the control.

**2.1.2. Linear Relationship between ORP and Different Concentration Ratios of  $I_2/KI$  Solution.** The linear relationship between the concentration ratio of  $I_2/KI$  solution and the ORP value was obtained through Nernst equation.  $I_2/KI$  electrode solutions with concentration ratios of 1:4 (0.1 M  $I_2$  and 0.4 M KI), 1:8 (0.1 M  $I_2$  and 0.8 M KI), 1:12 (0.1 M  $I_2$  and 1.2 M KI), 1:16 (0.1 M  $I_2$  and 1.6 M KI), and 1:20 (0.1 M  $I_2$  and 2 M KI) were used to verify the linear relationship.  $I_2/KI$  (200  $\mu\text{L}$ ) solution was mixed with deionized double distilled water (2800  $\mu\text{L}$ ). The mixed solution was kept in the dark at room temperature for 1 h. The ORP values were recorded at 120 s.

### 2.1.3. Optimization of Concentration Ratio of I<sub>2</sub>/KI Solutions.

In order to determine an optimal concentration ratio for the I<sub>2</sub>/KI electrode solution, solutions with different concentration ratios were treated with mixed urine and compared with the control group that had no added urine. Differences in ORP at different concentration ratios were measured. Electrode solutions with different concentration ratios (Section 2.1.2, 200 μL) and mixed urine sample (500 μL) were mixed with deionized double distilled water (2300 μL). The solution was kept in the dark at room temperature for 1 h. The ORP values were recorded at 120 s. Electrode solutions without urine were used as controls.

**2.1.4. Determination of Linearity.** Because of the complex components of urine, the known reducing agent vitamin C was also used in the oxidation/reduction reaction with the I<sub>2</sub>/KI electrode solution. Vitamin C solutions of different concentrations (0.1, 0.08, 0.06, 0.04, and 0.02 M) were used to evaluate the linearity of the relationship between the concentration of vitamin C and the ORP. Electrode solution (200 μL) and vitamin C solution (500 μL) were mixed with deionized double distilled water (2300 μL). The mixed solution was kept in the dark at room temperature for 1 h. The ORP values were recorded at 120 s.

Different concentrations of a mixed urine sample from five healthy individuals (100%, 80%, 60%, 40%, and 20% of the initial concentration) were also used to evaluate the linearity of the relationship. Electrode solution (200 μL) and mixed urine (500 μL) were mixed with deionized double distilled water (2300 μL). The mixed solution was kept in the dark at room temperature for 1 h. The ORP values were recorded at 120 s.

**2.1.5. Determination of Precision.** Experiments were conducted in accordance with the guidelines set out in Document EP5-A2 from the National Committee for Clinical Laboratory Standards (NCCLS) ("Evaluation of Precision Performance of Quantitative Measurement Methods"; approved guideline, second edition). Each run was carried out in duplicate, with two runs each day for a total of twenty days. The time interval between two runs was greater than 2 h. The experiments were carried out at two concentrations. A mixed urine sample from five healthy individuals was used as the high concentration group (group H). The mixed urine sample was diluted by 50% for use as the low concentration (group L). I<sub>2</sub>/KI electrode solution (200 μL) and mixed urine sample (500 μL) were mixed with deionized double distilled water (2300 μL). The mixed solutions were kept in the dark at room temperature for 1 h. The ORP values were recorded at 120 s. Electrode solution without urine was used as the control (group C). Average values ( $\bar{x}$ ), standard deviations (SD), and coefficients of variation (CV) of the ORP values were calculated to evaluate the repeatability of the I<sub>2</sub>/KI redox potential method.

**2.1.6. Comparison of Iodine Titration and I<sub>2</sub>/KI Redox Potential Methods.** The more classical approach of iodine titration can be used to determine the quantity of iodine that reacts with a given volume of urine. If similar values are obtained

using the iodine titration method and the I<sub>2</sub>/KI redox potential method, the redox potential method is reliable.

**Iodine Titration.** Mixed urine samples from five healthy individuals were used at concentrations of 100%, 80%, 60%, 40%, and 20% of the initial concentration. Urine was added dropwise from a burette into a stirred mixture of iodine solution (200 μL) containing I<sub>2</sub> (0.1 M) and KI (0.4 M) solution and starch solution (5 g/L, 5 mL) in a conical flask. The volume of urine consumed when the iodine solution in the conical flask remained colorless for >30 s was measured. A total of 25 urine samples were measured and experiments at each concentration were carried out in duplicate.

The volume of urine consumed by 200 μL iodine was used to calculate the quantity of iodine that would react with 500 μL urine according to the following formula:

$$0.1 \text{ M} \times 0.2 \times 10^{-3} \text{ L/V (urine)} \\ = n \text{ (the number of mole of I}_2 \text{ consumed) / 500 } \mu\text{L.} \quad (1)$$

The quantity of iodine that would react with 500 μL urine was

$$n \text{ (the number of mole of I}_2 \text{ consumed)} \\ = 0.1 \times 0.2 \times 10^{-3} \times 500/\text{V (urine)}. \quad (2)$$

**I<sub>2</sub>/KI Redox Potential Method.** The same concentrations of mixed urine that were used for the iodine titration were used in this experiment. I<sub>2</sub>/KI electrode solution (200 μL) and mixed urine samples (500 μL) were mixed with deionized double distilled water (2300 μL), giving a total volume of 3 mL. The mixed solutions were kept in the dark at room temperature for 1 h. The ORP values were recorded at 120 s. The urine consumption of 25 samples was measured and experiments at each concentration were carried out in duplicate. A mixture of electrode solution (200 μL) and deionized double distilled water (2800 μL) was used as the control.

According to the Nernst equation, the potential of the electrode can be calculated using the equation:

$$\varphi = \varphi^\theta + \frac{0.0592}{2} \times \lg \frac{1}{c(\text{I}^-)^2}. \quad (3)$$

The electrode potential of the control group is then

$$\varphi_0 = \varphi^\theta + \frac{0.0592}{2} \times \lg \frac{1}{c_0^2} \quad (4)$$

set control group  $c(\text{I}^-) = c_0$

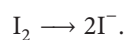
and the electrode potential of the experimental group is

$$\varphi_1 = \varphi^\theta + \frac{0.0592}{2} \times \lg \frac{1}{c_1^2} \quad (5)$$

set experimental group  $c(\text{I}^-) = c_1$ .

Formula (4) and formula (5) provide

$$\Delta\varphi = 0.0592 \times \lg \frac{c_1}{c_0} \quad c_1 = 10^{(\Delta\varphi/0.0592)} \times c_0 \quad (6)$$



The change in  $c(I^-)$  before and after the reaction between iodine and urine can be used to calculate the quantity of iodine consumed by the 500  $\mu\text{L}$  urine:

$$n \text{ (the number of mole of } I_2 \text{ consumed)} = \frac{(c_1 - c_0)}{2 \times 3 \times 10^{-3}} \quad (7)$$

## 2.2. Determination of ORP Value of Urine from

### *Diabetes Mellitus (DM) Patients Using $I_2/KI$ Redox Potential Method*

**2.2.1. Source of Specimens.** Clinical specimens were collected from the First Affiliated Hospital of Dalian Medical University. Random urine specimens were obtained from DM patients (first morning urine of new untreated inpatients with an empty stomach) and from healthy individuals undergoing physical examinations. Thirty DM patients (15 males and 15 females, mean age  $52.43 \pm 11.01$  years) were included in the experimental group. Thirty healthy individuals (15 males and 15 females, mean age  $51.42 \pm 11.07$  years) were used as the control group. The experimental group and the control group were matched by age and sex and recruited on the same day.

Urine specimens were stored at  $-20^\circ\text{C}$  after collection and thawed to room temperature before the experiment. The study was approved by the Ethical Committee of Dalian Medical University.

**2.2.2. Method.** Urine specimens from the diabetic group and the control group (500  $\mu\text{L}$ ) and  $I_2/KI$  electrode solution (200  $\mu\text{L}$ ) were mixed with deionized double distilled water (2300  $\mu\text{L}$ ). The solutions were kept in the dark at room temperature for 1 h. The ORP value was recorded at 120 s. Each measurement was carried out in duplicate.

**2.3. Statistical Analysis.** The statistical software package Statistical Product and Service Solutions 13.0 (which is abbreviated as SPSS 13.0 and whose manufacturer is SPSS Inc., Chicago, USA) was used to evaluate the results. The average value for each parallel sample in Section 2.1.6 was calculated and the statistical correlation of the two methods was analyzed. Average values and standard deviations ( $\bar{x} \pm s$ ) were calculated for ORP values from the clinical specimens in Section 2.2.2. The Student's  $t$ -test was used in comparison between groups, using  $\alpha = 0.05$  as the test level. A  $P$  value  $< 0.05$  (two tailed) was considered to be significant.

## 3. Results

**3.1. Comparison of Time Required to Obtain Stable ORP Value by Direct Method (Direct Insertion of Electrode into Original Urine Sample) and  $I_2/KI$  Method.** Changes in the values of ORP over time measured using the direct method are shown in Figure 1(a). The ORP value falls very noticeably during the first hour. The rate of decrease slows gradually after the first hour but the ORP value is still falling after 8 h. A long time is thus needed to obtain a stable reading.

Changes in the values of ORP over time measured using the  $I_2/KI$  method are shown in Figure 1(b). ORP values for

TABLE 1: The determination of precision for  $I_2/KI$  redox potential method.

Group	$\bar{\chi}$ (mV)	In-run		Between-day	
		SD	CV (%)	SD	CV (%)
Control	415.93	0.79	0.19	1.79	0.43
Low	407.89	0.45	0.11	1.59	0.39
High	398.58	0.80	0.20	1.71	0.43

Control, electrode solution with no urine; low, electrode solution with 50% concentration urine; high, electrode solution with 100% concentration urine.

both the control group and the group in which urine had been added to the electrode solution were stable within 120 s, indicating that the  $I_2/KI$  method has appropriate stability.

**3.2. Linear Relationship between ORP Values and Different Concentration Ratios of  $I_2/KI$  Solution.** Using SPSS 13.0 software, when the logarithm of the concentration ratio of the  $I_2/KI$  electrode solution was set as the  $x$ -axis and the ORP value was set as the  $y$ -axis, there was a linear relationship between the logarithm of the concentration ratio of the  $I_2/KI$  electrode solution and the ORP value ( $R^2 = 0.998$ ). The curve fitting equation was  $Y = 18.354X + 424.88$  (Figure 2).

**3.3. Optimization of Concentration Ratio of  $I_2/KI$  Solution.** ORP changes using electrode solutions with different concentration ratios were measured to identify an optimal concentration ratio for the reaction with urine. A 1 : 4 ratio of  $I_2/KI$  for the electrode solution gave the maximum difference (16 mV, Figure 3) and most clearly reflected the influence of urine antioxidant capacity on ORP value.

**3.4. Determination of Linearity.** Using SPSS 13.0 software, when the concentration of diluted vitamin C was set as the  $x$ -axis and the ORP value was set as the  $y$ -axis, there was a linear relationship between the concentration of vitamin C and the ORP value ( $R^2 = 0.994$ ). The curve fitting equation was  $Y = -0.254X + 413.381$  (Figure 4).

When the concentration of diluted mixed urine was set as the  $x$ -axis and the ORP value was set as the  $y$ -axis, there was a linear relationship between the concentration of mixed urine and the ORP value ( $R^2 = 0.986$ ). The curve fitting equation was  $Y = -0.157X + 413.19$  (Figure 5).

**3.5. Determination of Precision.** ORP values in the low concentration group, the high concentration group, and the control group are shown in Table 1. The precision of the method can be obtained from the experimental data.

**3.6. Comparison of Iodine Titration and  $I_2/KI$  Redox Potential Methods.** The quantities of iodine consumed by a mixed urine sample (500  $\mu\text{L}$ ) using the iodine titration method and the  $I_2/KI$  redox potential method are shown in Table 2.

The statistical correlation of  $n$  (the number of mole of  $I_2$  consumed) obtained using the two methods was analyzed using SPSS 13.0 software. When  $n_1$  (iodine titration method) was set as the  $x$ -axis and  $n_2$  ( $I_2/KI$  redox potential method) was set as the  $y$ -axis, a linear relationship was observed ( $R^2 = 0.987$ ). The slope was close to 1 and the intercept was close to



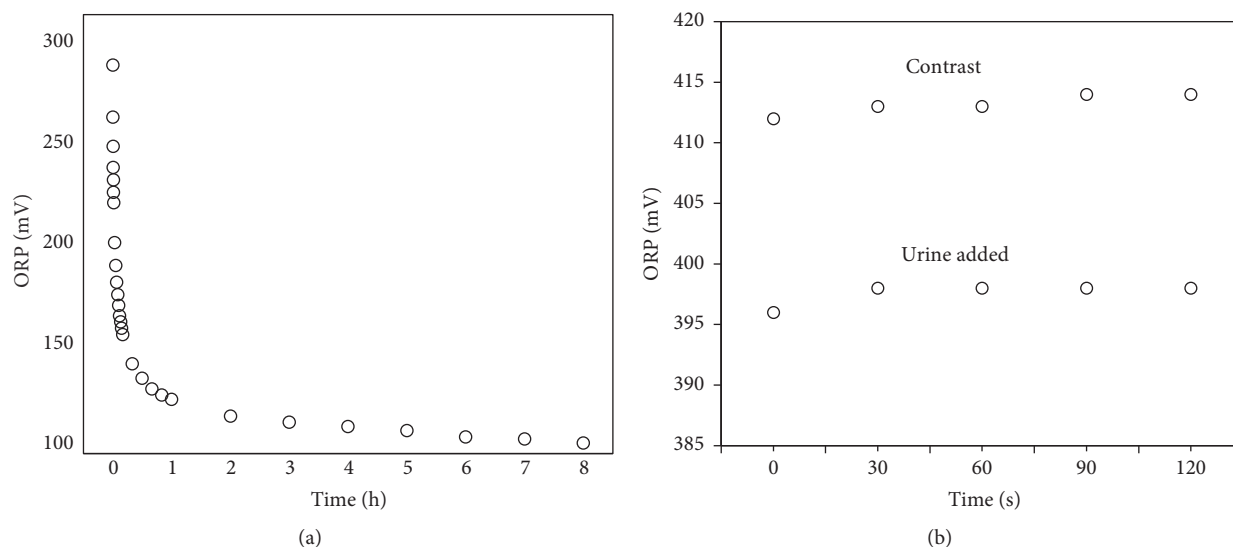


FIGURE 1: Changes in the values of ORP over time by the direct method (a) and by the  $I_2/KI$  electrode method (b).

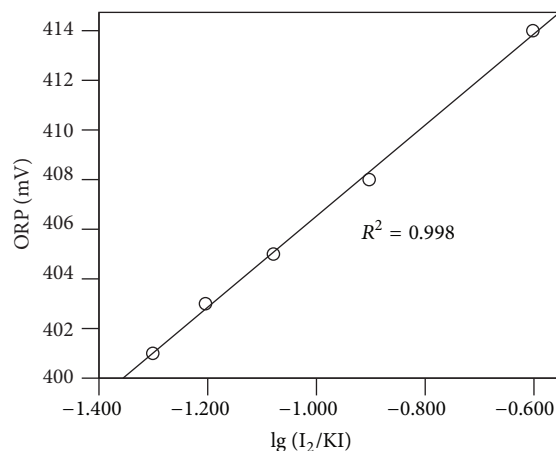


FIGURE 2: Liner relationship between the ORP value and the logarithm of the concentration ratio of  $I_2/KI$ . (The ratios of  $I_2/KI$  are 1:4, 1:8, 1:12, 1:16, and 1:20, resp.).

TABLE 2: Comparison between two methods for the quantities of iodine consumed by urine (mmol).

Methods	Concentration of urine				
	20%	40%	60%	80%	100%
Iodine titration	0.0074	0.0120	0.0200	0.0240	0.0327
$I_2/KI$ redox potential	0.0068	0.0148	0.0216	0.0266	0.0345

0 (Figure 6). The curve fitting equation was  $Y = 1.0625X + 0.0004$ .

**3.7. Determination of ORP Value of Urine from Clinical DM Patients Using  $I_2/KI$  Redox Potential Method.** The average ORP value for DM patients was higher than that for healthy individuals (Table 3). The difference between the two groups was considered to be significant at the test level of  $\alpha = 0.05$ .

## 4. Discussion

ORP reflects the ratio of oxidizing and reducing substances in solution and can be calculated using the Nernst equation under conditions of redox balance. Introduction of a redox couple ( $I_2/KI$ ) with a known concentration ratio provided a good linear relationship between ORP and the logarithm of the concentration ratio of  $I_2/KI$  ( $R^2 = 0.998$ ) (Figure 2), thus establishing the experimental method. A 1:4 ratio of  $I_2/KI$  for the electrode solution gave the maximum difference (16 mV, Figure 3) and most clearly reflected the influence of urine antioxidant capacity on ORP value. If the concentration ratio of  $I_2/KI$  increased to 1/3 or 1/2, the exceeded iodine would make the measurement unstable, which would eventually bring interference to the measurement. So we chose 1/4 as the optimal concentration ratio of  $I_2/KI$ . Because of the complex composition of human urine, it is difficult to directly obtain a stable reading of ORP in urine in a short period of

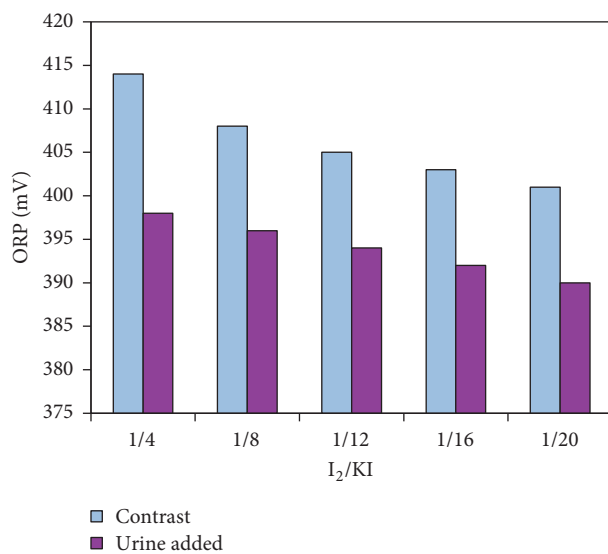


FIGURE 3: ORP changes reacted with urine using electrode solutions with different concentration ratios.

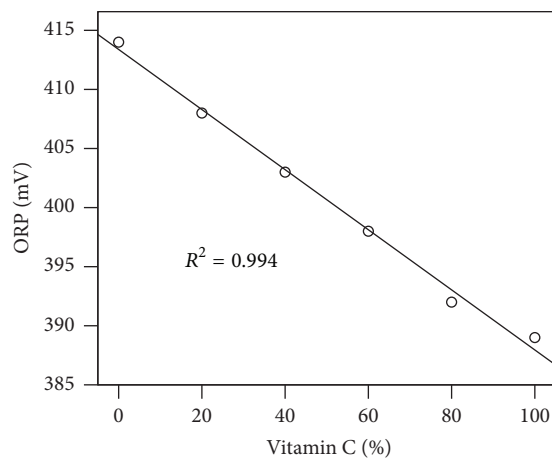


FIGURE 4: Liner relationship between the ORP value and the concentration of vitamin C.

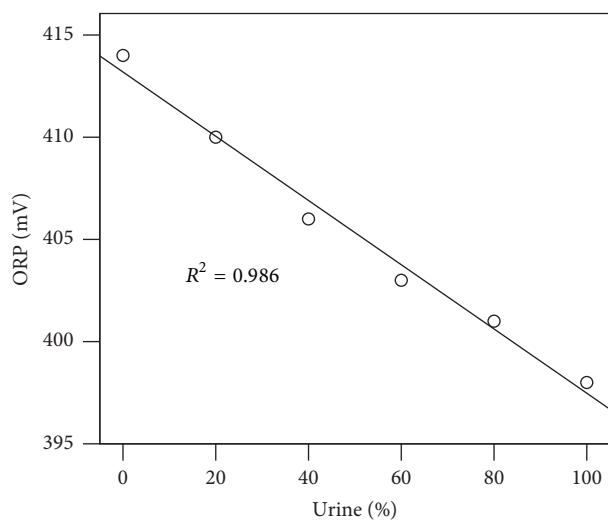


FIGURE 5: Liner relationship between the ORP value and the concentration of mixed urine.

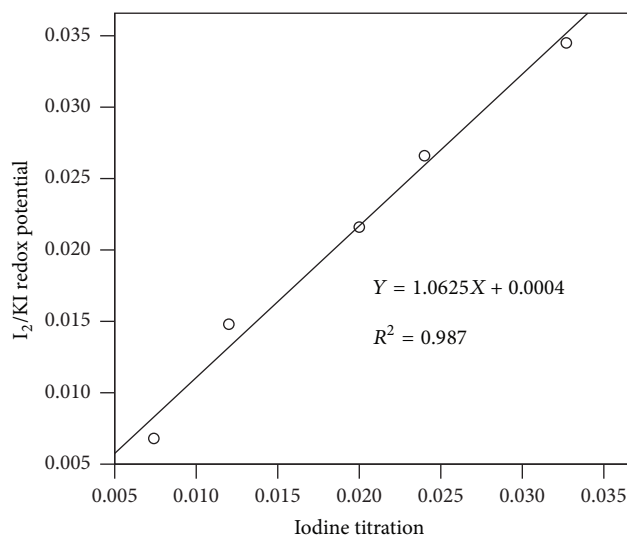


FIGURE 6: Linear relationship between  $n$  ( $I_2$  consumption) calculated by iodine titration method and that by  $I_2/KI$  redox potential method.

TABLE 3: The ORP value of urine of 30 diabetes mellitus and 30 healthy subjects.

Groups	Average value	Standard deviation	$t$	$P$
Diabetes mellitus	401.82	5.932	4.970	<0.001
Healthy subjects	397.90	5.539		

time (Figure 1(a)). After reaction of  $I_2/KI$  electrode solution (1:4 concentration ratio) with urine, however, a stable ORP reading could be obtained in 120 s (Figure 1(b)). The ORP value obtained when urine was added to the electrode solution was significantly lower than the ORP value of the control group (Figure 1(b)). This is because, in the Nernst equation, the oxidizing substance  $I_2$  is in nonionic form, its concentration is 1, as stipulated by convention, and  $I_2$  is converted to  $I^-$  after reaction with reducing substances in urine. As the concentration of  $I^-$  increases, the concentration ratio of  $I_2/KI$  decreases, leading to lower ORP values. We initially speculated that antioxidants in urine would react preferentially with the iodine solution. Using an invariant volume of electrode solution, we found that the ORP reading suddenly became unstable when the urine volume exceeded a certain amount. This may happen because antioxidants in the urine have consumed all of the available  $I_2$  and a stable reading cannot be obtained from a solution containing only KI with no  $I_2$ . As long as the measured ORP value was stable within 120 s, the antioxidants in urine were completely oxidized by  $I_2$ , indicating that the measured ORP value reflected the total antioxidant capacity of the urine specimen. Lower ORP values indicate the presence of more antioxidant substances in the urine and greater antioxidant capacity.

When the known reducing substance vitamin C reacted with the  $I_2/KI$  electrode solution, a good linear relationship was seen between the concentration of vitamin C and the ORP value ( $R^2 = 0.994$ ) (Figure 4). In the experiment with different concentrations of urine, the concentration of urine

and the ORP value also showed a linear relationship ( $R^2 = 0.986$ ) (Figure 5), indicating that the experimental method was suitable for determination of the antioxidant capacity of urine. In the precision experiments, the in-run, between-run, and between-day CV values were all within the acceptable range.

The traditional iodine titration method was used for comparison with the  $I_2/KI$  electron potential method because reducing substances in urine react completely with iodine in both methods. After calculation of the amount of iodine consumed by the same sample using both methods, correlation analysis showed that the results of two methods were statistically correlated ( $R^2 = 0.987$ ) (Figure 6). The traditional titration method, however, was more complicated and time-consuming to perform, needed larger amounts of urine, and necessitated washing and drying the burette after each determination. The electrode potential method, on the one hand, was very sensitive and accurate, was relatively simple to carry out, and needed only 2 min for each specimen. Because the starch added in iodine titration method could be reacted with substances in urine samples, the results would be interfered. The electrode potential method, on the other hand, has no added starch and truly reflects the antioxidant capacity of the urine sample. Our newly developed  $I_2/KI$  electrode potential method, based on the Nernst equation, can thus reduce interference and give accurate results quickly, making it very suitable for clinical applications.

The experiment was carried out under neutral condition; no buffer material was added to adjust the pH of the urine, ensuring that the activity of antioxidant substances in the urine was under nature status. Since the measurement of ORP is affected by temperature, the reaction and test temperatures were strictly maintained at 25°C. The normal human body temperature of 37°C was not used for two reasons. Firstly, since the reaction is nonenzymatic, controlling the temperature at 37°C was of little importance and secondly, the increased temperature caused evaporation of  $I_2$ , leading

to errors in the measurement. Our newly developed method can provide a stable ORP reading in 120 s, needs only small amounts of urine, and is more rapid and simple to operate than the traditional direct method for determination of ORP.

Many studies have shown that the antioxidant capacity of serum from DM patients is significantly reduced compared with that of healthy individuals [26, 27]. It can be inferred, therefore, that the antioxidant capacity of urine from diabetics will be lower than that of urine from healthy individuals. We determined the antioxidant capacity of urine from 30 DM patients and 30 healthy volunteers using the new method. The ORP of the DM group was  $401.82 \pm 5.932$  mV and that of the control group was  $397.90 \pm 5.539$  mV. Applying the Student's *t* test, the difference between the ORP value of the DM patients and that of the healthy control subjects was shown to be statistically significant ( $P < 0.05$  at the test level of  $\alpha = 0.05$ ). More antioxidants were thus discharged in the urine of healthy individuals than in that of diabetics. This finding is consistent with literature reports [26] and showed that the newly established method can distinguish between the two groups. The new method is thus suitable for evaluation of the antioxidant capacity of urine.

An altered oxidation/reduction balance is associated with many pathological and physiological conditions, including senescence, tumors and mutations, inflammation, cardiovascular and cerebrovascular diseases, diabetes mellitus, autoimmune diseases, and neurodegenerative degenerative diseases such as Parkinson's disease and Alzheimer's disease. Determination of the antioxidant capacity of urine is a convenient method for evaluation of the redox state of the human body that removes the trauma of blood sampling and avoids cross infection.

## 5. Summary

Briefly, compared with the traditional way, the new method we proposed just in this paper for determining the antioxidant capacity of human urine has three advantages: better stability, less possible interference, and better simplicity, rapidity, and sensitivity. So, it is an alternative to determine the antioxidant capacity of urine and can be helpful in evaluating redox status of some diseases in the clinical practice.

## Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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