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UV–vis spectroscopic study directly detecting inorganic phosphorus in urine and our reagent kit

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Abstract The determination of inorganic phosphorus in human urine is very important, since it has diagnostic value in some clinical cases. Here we apply a simple, sensitive and direct method to determine inorganic phosphorus in urine. This new ensemble is prepared by adding ytterbium chloride and pyrocatechol violet in a 2:1 molar ratio in an aqueous solution of 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid buffer at pH 7.0. The addition of the urine sample turned the blue ensemble yellow and altered the UV–vis absorption spectra. The ensemble exhibits excellent selectivity for inorganic phosphorus over other constituents of urine. We validate the accuracy of our method by the standard procedure (molybdenum blue assay for phosphate). The detection results are basically consistent with normal excretion of phosphate. Furthermore, we fabricated a new kind of inorganic phosphorus reagent kit, which enables us to inspect phosphate concentrations of urine with the naked eye. Fit for all kinds of various clinic uses, our reagent kit is a hopeful substitute for the molybdenum reagent kit.

Keywords UV–vis spectra · Pyrocatechol violet–2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid–Yb · Inorganic phosphorus · Urine · Reagent kit

Introduction

Phosphorus is involved in a number of important biomineralization processes such as bone formation as well as pathological processes such as the genesis of renal stones [1]. Consequently, its determination in biological fluids is an important task. For example, we have to determine the

inorganic phosphorus in human urine in the diagnosis of urolithiasis [2–4]. Several research workers have examined the factors predisposing to the formation of urinary and kidney stones [5–8]. Their results all proved that the higher inorganic phosphorus of urine is one of the causes for the formation of urinary and kidney stones. The conventional instrumental methods for detecting inorganic phosphorus in urine [9–12] have a common shortcoming, i.e., their linear calibration range is too narrow (ion chromatography is an exception; it has an excellent dynamic range). Thus, if the concentrations are too high to be measured directly, most of these methods adopt extensive manual dilution as sample pretreatment, consequently compromising the precision and accuracy. So it is a challenge to develop methods for directly detecting inorganic phosphorus in urine.

In our previous work, we have advanced a simple and sensitive probe for detecting phosphate. The ensemble was prepared by simply mixing YbCl_3 and pyrocatechol violet (PV) in a 2:1 molar ratio in an aqueous solution {10 mM, pH 7.0 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, HEPES, buffer} [13]. But in our earlier work, we did not carry out the experiment on how to apply our ensemble in detecting phosphate in biological fluids such as urine. In this paper, we apply the system to directly, quantitatively, physiologically and pathologically detect the concentration of phosphate [14]. More importantly, we fabricated a kind of inorganic phosphorus reagent kit which enables us to inspect the concentration of urine with the naked eye. Owing to its prominent advantages, it is suitable for clinical use in all types of hospitals and can serve as a hopeful substitute for the molybdenum reagent kit.

Experimental

Chemical and reagents

The chemicals used were of analytical-reagent grade. PV was purchased from Shanghai and sodium monhydrogenphosphate was purchased from Beijing. Ytterbium oxide was a product of Rare Earth Graduate School of China.

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HEPES was purchased from Sigma. All solutions were made up with deionized water. HEPES buffer solutions were obtained by adding NaOH 0.1 M solution into 10 mM aqueous HEPES using a Beckman Φ 50 pH meter. Ytterbium chloride was prepared from ytterbium oxide and 37% hydrochloric acid. Ytterbium ion solution was prepared by dissolving ytterbium chloride in water. Urine samples were collected from healthy volunteers within a period of 24 h and stored at 4°C prior to analysis.

Apparatus

pH determinations were performed using a Beckman Φ 50 pH meter. UV-vis spectra were recorded with an HP8453 spectrophotometer. PO-120 quartz cuvettes (10 mm in diameter) and Finnpette Digitals were both purchased from Shanghai.

Measurement procedure

Using the PV-HEPES-Yb³⁺ ensemble, we detected inorganic phosphorus in urine samples. The procedures were as follows. In 10 mM, pH 7.0 HEPES buffer containing 50 μ M PV and 100 μ M Yb³⁺ (a blue solution), the urine sample from one healthy volunteer was gradually titrated into the solution. At the same time the changes in the absorption peaks of solution in the UV-vis spectrum were recorded. When no more changes in the absorption peaks of the system took place, titration came to a halt. Then we could calculate the inorganic phosphorus concentration in urine.

Results and discussion

UV-vis spectra

Figure 1a shows the UV-vis spectra obtained when titrating the urine from an adult into the 10 mM, pH 7.0 HEPES buffer solution containing 100 μ M YbCl₃ and 50 μ M PV. With the addition of urine, the absorption peak at 623 nm decreased, while the peak at 444 nm increased. When the total volume of added urine reached 20 μ L,

titration ended. The concentration of inorganic phosphorus excreted was 10 mmol/L. Similarly, Fig. 1b shows UV-vis spectra of urine from a child about 1 year old. The concentration of inorganic phosphorus excreted from the child was 50 mmol/L.

Selectivity over other constituents

In a previous paper, we addressed the selectivity of the system. We knew that the ensemble exhibited excellent selectivity towards phosphate anions over other common anions, including Cl⁻, SO₄²⁻, CH₃COO⁻, HCO₃⁻ and ClO₄⁻ (Fig. 2). Fig. 2a shows that adding other anions to the PV-HEPES-Yb³⁺ system did not bring about any changes in color or in the UV-vis spectrum of the system. Fig. 2b shows that the existence of other anions (1 M) had no effect on the detection of phosphate anions. The selectivity of the ensemble towards anions was better than that reported by Lee et al. [15]. Our system has a stronger ability of recognizing phosphate ions in aqueous solvents than the synthetic receptor and peptide receptor reported in Ref. [16]. To our knowledge, efficient analytical methods for detecting phosphate anions with good selectivity over other common anions in aqueous solution are rare [17]. In addition, we have already got its selectivities relative to ATP, ADP and AMP, i.e., phosphate ions \approx ATP \gg ADP \gg AMP [13]. Our system has almost the same selectivities to phosphate ions and ATP; however, it is well-known that excretive urine contains hardly any ATP.

Urine contains many other organic and inorganic compounds, such as creatinine, sugar, calcium, oxalate, divalent species and transition ion chelators, besides the aforementioned ordinary anions. Do these compositions show some responsibility for the changes in the UV-vis spectra and color? We took the HEPES buffer as a blank, and then added 10 μ L urine to it. The result shows that there is no UV-vis absorbance in the range of from 350 to 1,000 nm, suggesting that there are no other absorbance peaks coming from other compounds of the urine in the range of detection in the UV-vis spectra. Thus, we may conclude that the changes in the absorbance peak in this range resulted completely from the measurement processes and there was no cumulation or disturbance.

Fig. 1 **a** UV-vis spectra: the urine from an adult was added to 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (10 mM, 2 mL) buffer containing pyrocatechol violet (PV) (50 μ M) and Yb³⁺ (100 μ M) with $V_{\text{urine}}=0-20$ μ L; **b** the urine from a child was added to HEPES (10 mM, 2 mL) buffer containing PV (50 μ M) and Yb³⁺ (100 μ M) with $V_{\text{urine}}=0-4$ μ L

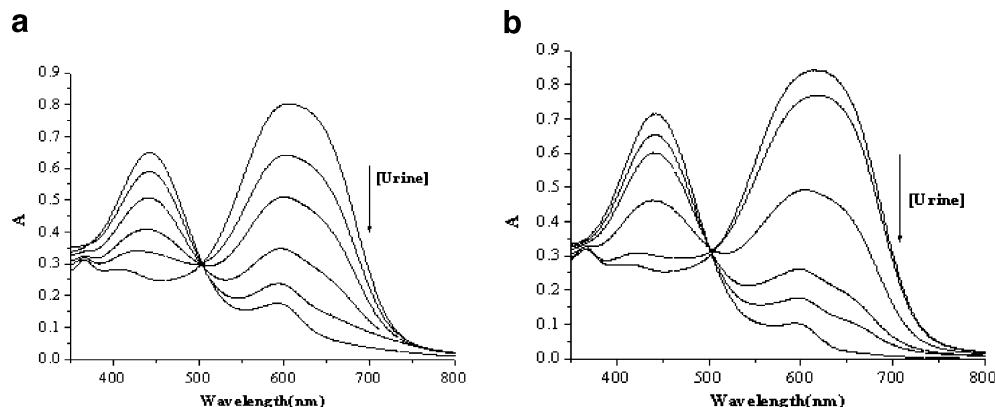
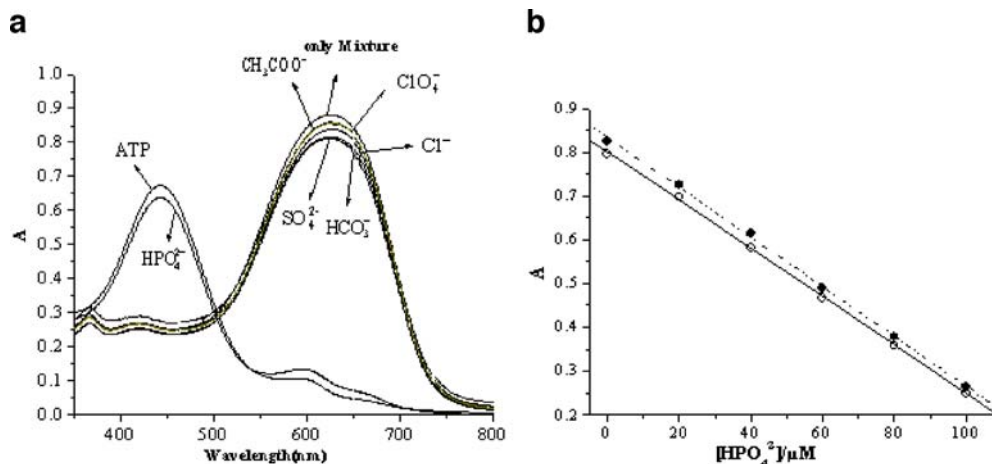


Fig. 2 **a** UV-vis spectra of the $[\text{Yb}_2(\text{PV})]$ mixture (50 μM in a HEPES buffer solution) with various anions added, HPO_4^{2-} and ATP (100 μM); **b** absorbance (at 623 nm) against concentration of HPO_4^{2-} in HEPES solution (pH 7.0) in the absence (blank symbols and solid line) and presence of other anions (solid symbols and broken line) (Cl^- , AcO^- , HCO_3^- , ClO_4^- and SO_4^{2-})



To prove that the universal existence of divalent species or transition ion chelators in urine incurs no disturbance to exclude the possibility of interference from iron or other cations in the measurement, the following experiments were carried out.

Firstly, as soon as the excessive [2 mM YbCl_3 was added into the [2 mM HPO_4^{2-} solution ($V_{\text{YbCl}_3}/V_{\text{HPO}_4^{2-}}=1.02:1$), precipitation occurred, a clear solution was gained through centrifugation and decantation processes; the solution (from 0 to 300 μL) was then added into 2 mL 10 mM HEPES buffer containing 50 μM PV and 100 μM Yb^{3+} , and no changes in absorption peak intensity and color were observed, i.e., no phosphate was detected in the solution, thus suggesting that the Yb^{3+} could completely remove HPO_4^{2-} from solution by forming sediment. Similarly, we added the excessive YbCl_3 into the collected urine samples whose content of HPO_4^{2-} was presumably quantitated with our methods. After the mixture had been treated in accordance with the aforementioned procedures, a great deal of the disposed urine sample (0–300 μL) was added into the 2 mL 10 mM HEPES buffer containing 50 μM PV and 100 μM Yb^{3+} , and changes were observed in absorption peak intensity and system color. The experiments excluded the possibility of interference from other ions with the measurement.

In addition, we added an adequate amount of untreated urine sample into 2 mL 10 mM HEPES buffer only containing 50 μM PV, and no change in either the UV-vis spectra or the system color was observed. The system was still yellow. The experiments excluded the possibility of interference from iron or other cations with the measurement.

Linearity and detection limits

Most instrumental methods available for the determination of phosphate in clinical samples have a common drawback; that is, their linear calibration range is too narrow. In our experiment, we plotted the curve with absorbance values at 623 nm against concentrations /0–70 mM/ of urine added to the PV-HEPES- Yb^{3+} system. We found our measurement obeyed the Beer-Lambert absorption law very well within

the urine concentration range of 0–70 mM. Linear regression with least-squares fitting yielded a correlation coefficient of 0.99976 (Fig. 3). The lower detection limit of our method is around 10^{-4} M.

Validation

In order to validate the accuracy of the method, we detected urine samples by the standard procedure (molybdenum blue assay for phosphate) and obtained equivalent results with our measurement. Fig. 4 and Table 1 give the results (spectra) for three adults obtained with the two kinds of detection methods.

Finally, the recovery experiments were performed: The results are compiled in Table 2. The results indicated the accuracy of the method, as expressed by the calculated recovery values, was satisfactory.

Analysis of results

In our experiment, we collected urine from a few healthy adults and children. We gradually added undiluted urine

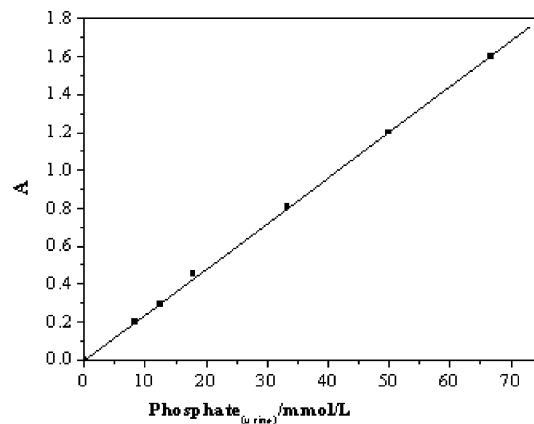


Fig. 3 The working curve for urine measurement with the absorbance value against various concentrations of urine (0–70 mM)

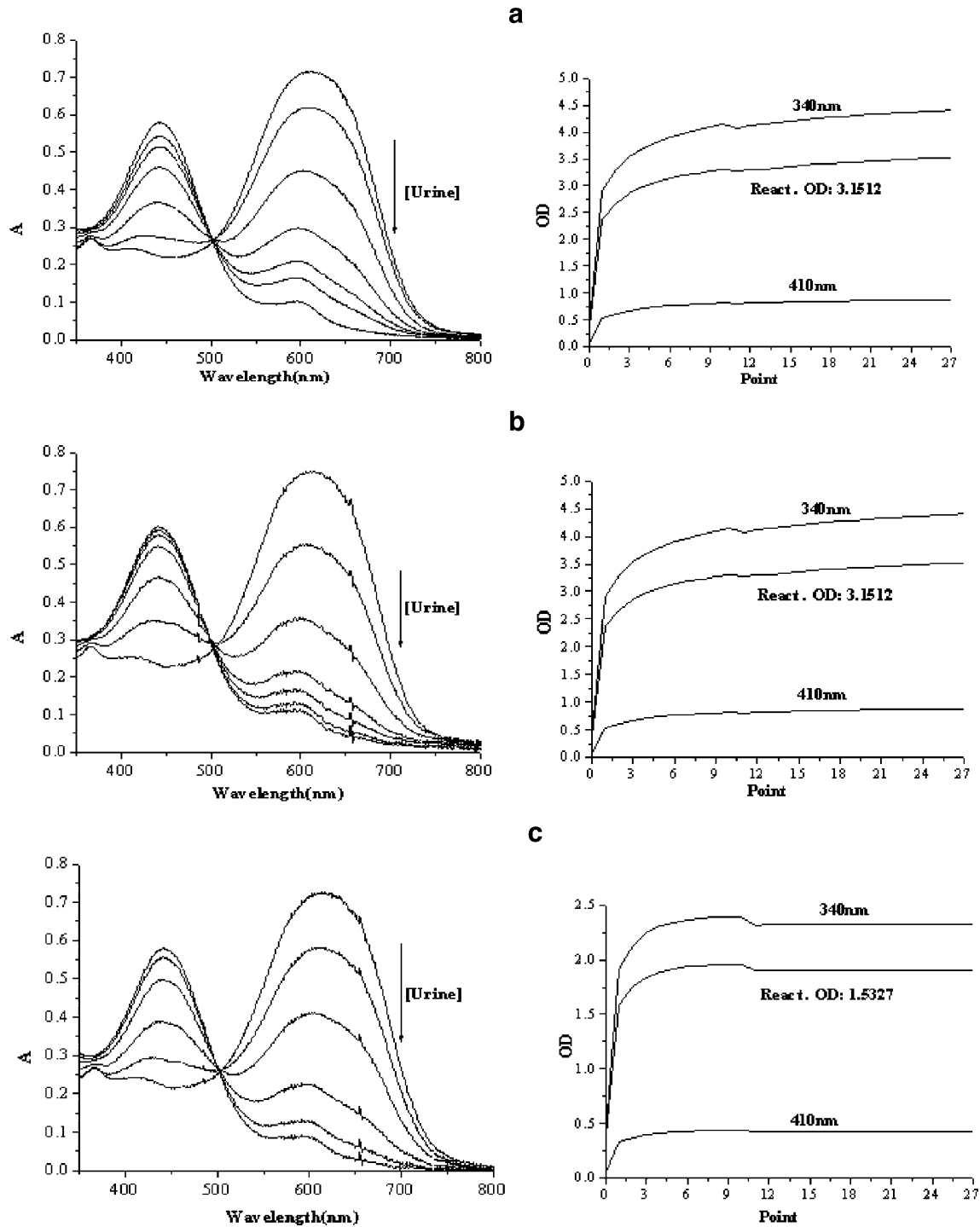


Fig. 4 Left: UV-vis spectra of urine sample from the three same adults. Right: The results from molybdenum blue assay for phosphate

Table 1 Results of our method compared with those of the molybdenum blue assay method for phosphate

Sample	Phosphate (mmol/L) from molybdenum blue assay	Phosphate (mmol/L) from our method
1	17.73	17.85
2	17.54	17.54
3	8.27	8.33

sample from one adult into the buffer system containing 100 μM YbCl_3 and 50 μM PV. The absorption peak at 444 nm in the UV-vis spectra increased no longer and the color of the solution changed back to yellow when the volume of urine added reached 20 μL (Fig. 1a). Here, we found that the concentration of inorganic phosphate excreted is 10 mmol/L. The concentrations for other adults were within the range 8.33–25.0 mmol/L. The result was basically consistent with normal excretion of phosphate

Table 2 Recovery of phosphate in urine

Sample	Added phosphate (mg/L)	Found phosphate (mg/L)	Recovery (%)
1	–	1,371	
	500	1,857	99.0
2	–	1,195	
	500	1,687	99.3
3	–	9,59	
	500	1,451	99.2

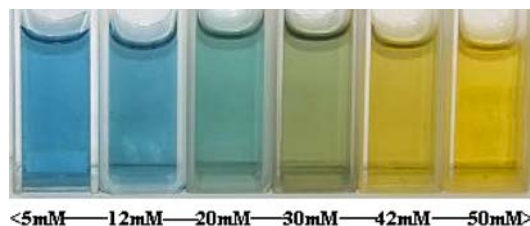
(12.9–42.0 mmol/L) [18, 19]. When the volume of undiluted child urine amounted to 4 μ L in the system no change was observed in UV–vis spectra and color (Fig. 1b). That is to say, the concentration of phosphate in the urine of the children was 50 mmol/L. Table 3 lists detailed results of some samples.

The reasons for higher phosphate concentration in the urine of the children are manifold. One is that the children are at the stage of blooming bone growth compared with adults. Secondly, the alkaline phosphatase in the body of children is active and the metabolism of children is fast. Of course, the phosphate in the urine also depends on the diet.

The human body regulates phosphate levels in the blood through the controlled release of parathyroid hormone (PTH) [2] from the parathyroid gland and calcitonin from the thyroid gland. In particular, PTH keeps phosphate levels from becoming too high by stimulating the excretion of phosphate in urine. In healthy individuals and those not on phosphate-binding medications, urine phosphate simply reflects phosphate intake. Increasing dietary intake of potassium has been reported to increase serum phosphate concentrations apparently by decreasing renal excretion of phosphate [20]. Persistent deviation from the normal concentration of phosphate is indicative of thyroid malfunction (hyperparathyroidism or hypoparathyroidism), urolithiasis, disorders of the phosphorus and calcium metabolism, vitamin D deficiency or intoxication, poor kidney function, and Fanconi syndrome. There is also evidence of a correlation between phosphaturia and phosphate concentration in the serum [21]. In addition, increasing urinary content of phosphate may provide a useful means to assess response to phosphate supplements in the premature infant [22].

Table 3 Phosphate concentrations of some urine samples from adults and children

Sample	Added V_{urine} (iL)	Phosphate (mmol/L)
Adult ₁	24	8.33
Adult ₂	11.2	17.85
Adult ₃	11.4	17.54
Adult ₄	16	12.5
Child ₁	6	33.33
Child ₂	4	50.00
Child ₃	3	66.66

**Fig. 5** Criterion rank

Inorganic phosphorus reagent kit

As is well known, molybdenum blue assay for phosphate has serious shortcomings: molybdate reduction is affected by slight changes in pH, the rate of complex formation is markedly influenced by protein concentration and the acid pH required leads to hydrolysis of organic phosphate [23], which results in overestimation of P_i concentration. Let it be noted that widespread molybdenum blue assay for phosphate including ours all resort to large apparatus. So the determination of phosphate of human urine is not to be carried out in such places as small hospitals, clinics or at home. In order to overcome the limitation, we can use our inorganic phosphorus reagent kit including a chromatic diagram and R_1, R_2, R_3 reagents to inspect the concentrations of urine at any moment with the naked eye. This method can be used abroad in all types of hospitals and at home.

We may prepare a criterion color rank by adding HPO_4^{2-} (20, 40, 60, 80, 100 μ M) into HEPES containing 50 μ M PV and 100 μ M Yb^{3+} pH=7.0, 10 mM buffer respectively (Fig. 5) and obtain a chromatic diagram of blue, light blue, blue-green, yellow-green, light yellow and yellow from left to right. The colors correspond to concentration ranges below 5, 5–12, 12–20, 20–30, 30–42, and 42–50 mM. We confect pH 7.0 10 mM HEPES buffer marked R_1 , confect 2 mM PV marked R_2 , confect 2 mM $YbCl_3$ marked R_3 .

We use the inorganic phosphorus reagent kit to inspect the concentration range of phosphate from human urine in the following steps: (1) add the three reagents into a glass tube at a volume ratio $R_1/R_2/R_3$ of 40:1:2 to get a blue system; (2) add a urine sample with a volume of 1/25 of R_3 into the tube and shake it for 20–30 s, and compare it with the criterion rank to get the concentration range of phosphate from urine sample. For example, we added some adult urine into the aforementioned system and got the color shown as Fig. 6; thus, we knew the concentration range of urine phosphate from the adult was 12–20 mM.

Fig. 6 The concentration range of phosphate from adult urine

Our inorganic phosphorus reagent kit possesses the following advantages over the molybdenum reagent kit: (1) the detection object is an undiluted sample so pretreatment is saved, which is very convenient for clinical uses; (2) our sample volume for determination is small and the determination time is short; (3) the determination resorts to use of the naked eye and does not depend upon large apparatus, and as a consequence the detection process is simple and the effect is distinct; (4) our equipments and reagents are all cheap, and are fit for all kinds or types hospital and places.

Conclusion

To sum up, we developed a sensitive, rapid and direct method for detecting urine phosphate both spectrophotometrically and visually as well as a quantitative assay of phosphate. Our method is suitable for performing direct determinations of phosphate in urine without any pretreatment and any interference. Moreover, we fabricated a novel inorganic phosphorus reagent kit for use everywhere without resorting to large apparatus. Now more and more people are suffering from lithiasis as a result of better living standards. Timely inspection of urine phosphate is one of the clinical means of diagnosis. For example, increasing urinary content of phosphate may provide a shortcut to assess the response to phosphate supplements in the premature infant [22]. So our kit, as a hopeful substitute for the molybdenum reagent kit, will find extensive use in hospitals and clinics.

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