Fig. 5

Relation between manual and automatic values

nine serum samples by both the classical manual method of WARREN and by the proposed automatic method. The results are shown in table 1 and figure 5. As can be seen, the Autoanalyzer method gives consistently higher results by about 8%. This is most probably due to the incompleteness of the extraction of the coloured end product in the manual method.

Finally, we checked the effect of the most probable source of interference, namely the blood glucose. No interference could be detected by the addition of up to 10 times the normal amount of glucose.

This method has been in daily use in our laboratory for over a year and has been entirely satisfactory.

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A Micromethod for the Routine Estimation of Inorganic Phosphate in Serum and Urine

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A standard technique for the colorimetric determination of inorganic phosphate has been developed, based upon the work of ITAYA and UI, who found that Malachite Green at a low pH forms a complex with phosphomolybdate, which is measured at 660 nm. The present method is different in that: 1. a lower concentration of Malachite Green is used, resulting in an analytically more suitable determination. 2. another stabilizing reagent is used. The present method has been applied to serum as well as urine.

Eine Standardmethode für die kolorimetrische Bestimmung von anorganischem Phosphat wird angegeben. Sie beruht auf der Arbeit von ITAYA und UI, die fanden, daß Malachitgrün bei niedrigem pH einen Komplex mit Phosphomolybdat bildet, dessen Extinktion bei 660 nm gemessen wird. Die angegebene Methode unterscheidet sich von der von ITAYA und UI wie folgt: 1. Es wird eine geringere Malachitgrünkonzentration benutzt. Das ergibt eine analytische geeignetere Bestimmungsmethode. 2. Es wird ein anderes Stabilisierungsmittel verwendet.

Die Methode kann zur Phosphatbestimmung in Serum und Harn angewandt werden.

Recently ITAYA and UI (1) introduced a new micro-method for the colorimetric determination of inorganic phosphate. Their procedure depends on the fact that Malachite Green at a low pH forms a complex with phosphomolybdate with a marked shift of the absorption spectrum. The colour developed is measured at 660 nm. They reported determinations of percentage recovery with serum, and they compared the method with those of FISKE and SUBBAROW (2) and MARTIN and DOTY (3), but only a few experimental figures were given.

In the present paper we have examined some aspects of the work of ITAYA and UI. First we followed their standard procedure by pipetting 0.05 ml serum, standard or water into 4 ml 5% perchloric acid, adding 1 ml of the centrifugate to 5 ml colour-developing reagent,

immediately followed by 0.2 ml 1.5% Tween 20 solution. The optical density was measured at 660 nm after 10 min.

Later however, we modified their standard procedure by using a lower concentration of Malachite Green and a different stabilizing reagent. Our method gives precise and reproducible results when applied to serum as well as urine.

Method

Materials

Reagents

All reagents were analytical grade and included KH_2PO_4 (for the preparation of standards), 70% perchloric acid, $(\text{NH}_4)_6\text{MoO}_{24} \cdot 4\text{H}_2\text{O}$, Malachite Green and 37% HCl, all from Merck AG, Tween 20 (polyoxyethylene sorbitan monolaurate) from J. A. Boöm,

Meppel, Holland; and Sterox SE (a nonionic polyoxyethylene thioether) from Hartman-Leddon Cie, U. S.

Redistilled water was used throughout these experiments. Optical density was measured with the Vitatron UC 200 colorimeter. Absorption spectra were measured with the Unicam SP 500 spectrophotometer.

Colour-stabilizing reagent

According to ITAYA and UI the colour of the phosphomolybdate complex is rendered stable by adding Tween 20 at a final concentration of 0.05% immediately after the colour develops. The addition of detergent prior to the colorimetric reagent is not advisable because this retards formation of the complex.

We have confirmed these results. In addition, we have found the same retardation of complex formation when the colorimetric reagent and Tween 20 solution were added together in the required concentration to the sample containing phosphate. Because Tween 20 is not normally available in a clinical laboratory, we started experiments with a detergent which is often used in flame photometry, viz., sterox. 8 ml of a 2% sterox solution were added to 400 ml of colorimetric reagent, prepared according to ITAYA and UI. This gives a final concentration of 40 mg sterox per 100 ml. This mixture was now used as the colorimetric reagent and, of course, no Tween 20 was added.

A straight line was obtained for the concentration-range of 1 to 10 mg% phosphate; the absorption spectrum showed a maximum between 640 and 660 nm. The same results are obtained with Tween 20, but sterox can be added together with the colorimetric reagent, while Tween 20 cannot. From a routine point of view, this is more convenient than pipetting the detergent afterwards.

Concentration of Malachite Green

ITAYA and UI have studied the optical density, obtained by adding 1 μ g of inorganic phosphate to the acidified solution of dye and ammonium molybdate. They plotted the optical density against the final concentration of HCl at three different concentrations of the dye, viz., 0.5%, 0.2% and 0.05%. The authors showed that for all three concentrations of Malachite Green a final concentration of 1N HCl was suitable, but for their standard procedure they chose a final concentration of 0.12% Malachite Green (0.2% solution as reagent), which produces a blank optical density of about 0.180.

We have found that 0.045% is the lowest possible concentration of Malachite Green, that gives a linear standard curve with a sterox concentration of 40 mg/100 ml (Figs. 1 and 2). The blank optical density is 0.025 to 0.030. The absorption spectrum shows a maximum between 640 and 660 nm. (Fig. 3).

Fig. 1

Standard curve. The standard procedure was applied to the standard solutions containing 1 to 8 mg% (expressed as P_i) phosphate

Concentration of sterox and stability

We have made experiments with several concentrations of sterox in order to get both a linear standard curve and good stability. Stability is expressed as the difference in optical density (660 nm) of the same solution, measured after 10 and 30 min. With 40 mg sterox per 100 ml colorimetric reagent, standard solutions contain-

Fig. 2

Absorption of blank and standard solutions, plotted against different concentrations of Malachite Green.

Δ—Δ blank
□—□ 2 mg% standard solution
×—× 5 mg% standard solution
○—○ 8 mg% standard solution

Fig. 3

Absorption of 5 mg% P_i standard solution.

○—○ standard solution
×—× standard solution minus blank
Δ—Δ blank

ing 2 and 5 mg% phosphate remained entirely stable, while 8 mg% phosphate appeared somewhat less stable (mean difference in O. D. between 10 and 30 min. of 0.010).

Linear curves were also obtained with lower sterox concentrations, i. e. 10 and 20 mg/100 ml, but then the stability was reduced. Higher sterox concentrations, e. g. 80 mg/100 ml produce a higher blank optical density, whereas concentrations lower than 40 mg/100 ml do not further lower the blank optical density.

The standard technique

Colorimetric reagent

1 vol. of 4.2% $(NH_4)_6 \cdot MoO_{24} \cdot 4 H_2O$ in 4.5–5.0N HCl is mixed with 3 vol. of 0.045% Malachite Green. After 30 min. the mixture is filtered and the 2% sterox solution is added (8 ml per 400 ml colour-developing reagent, final concentration 40 mg/100 ml).

The reagent is stored at 4° C, but when used must be at room temperature. The reagent must be freshly prepared every week.

Procedure

0.05 ml serum, standard or water is pipetted into 4 ml 5% perchloric acid and the solution is mixed. After centrifuging 5 ml of the colorimetric reagent is added to 1 ml clear supernatant and the solution is mixed. The

optical density is measured at 660 nm after 10 min. It is essential, for the preparation of all reagents as well as for the procedure, that distilled water is used. Demineralized water may contain phosphorus, depending on the state of saturation of the resin column. The HCl must not be below 4.5N, for then the complex becomes unstable.

Comparison with the method of TAUSKY and SCHORR

We have compared the data obtained with the present method with data obtained from the same sera by the colorimetric method of TAUSKY and SCHORR (4), which employs molybdate and MOHR'S salt.

Figure 4 shows data obtained from 30 sera. Blanks, standards and sera were all measured in duplicate. It will be clearly seen that both methods give the same result. However, the present method uses only 0.05 ml serum per determination, in contrast with 0.5 ml serum for the other method.

Fig. 4

Comparison of the present method with the method of TAUSKY and SCHORR

Determination of phosphate in urine

For the determination of inorganic phosphate in human urine by the method of TAUSKY and SCHORR, the urine is acidified with HCl against Congo paper, and then diluted 1:100 with distilled water.

In the present method the urine is acidified in the same way, but is diluted 1:5. Sometimes, when a special diet low in phosphate has been prescribed, another dilution may be more suitable.

Standard technique for urine

The same colorimetric reagent is used as with serum. Procedure: 0.01 ml urine (1:5 diluted with distilled water), standard or water is pipetted into 4 ml 5% perchloric acid and the solution is mixed. To 1 ml of this solution, 5 ml colorimetric reagent is added and the optical density is measured at 660 nm after 10 min.

When much protein is present the solution must be centrifuged. The presence of large amounts of glucose does not affect the determination.

Comparison of the present method for urine with the TAUSKY-SCHORR method

Figure 5 shows the result of 18 urine determinations. Again it will be seen that both methods give the same results, except that the present method gives higher values for phosphate concentrations

Fig. 5

Comparison of the present method for urine with the method of TAUSKY and SCHORR

above 100 mg%. Phosphate concentrations from 5–200 mg% produce an optical density of 0.040 to 0.500 with our method, compared with 0.040 to 1.250 with the TAUSKY-SCHORR method. This means that, using the latter procedure, the urine must be diluted further when high amounts of inorganic phosphate are initially measured, which is equivalent to repeating the determination. This could not arise with the present method.

Discussion

It is obvious that the real advantage of the method of ITAYA and U_i, compared with the method of TAUSKY and SCHORR, is the much smaller amount of serum which is required for one determination. (0.05 ml instead of 0.5 ml.)

That was the main reason why we decided to examine critically some aspects of their work.

We have introduced a new standard technique, giving precise and highly reproducible results when applied to serum as well as urine. The present method differs firstly, in that a lower concentration of Malachite Green is used. A dye concentration of 0.045% gives the lowest possible blank value, resulting therefore in an analytically more suitable technique. Higher Malachite Green concentrations are not necessary to form the dye-phosphomolybdate complex; they only produce higher optical density values.

Another important advantage of our colorimetric reagent is that it does not stain the hands of the analysts engaged on this routine work.

The colour reagent of ITAYA and U_i, containing 0.2% Malachite Green, produces dark green spots on the skin which are not easily removed by washing and are still present after some hours. In the second place we used another stabilizing reagent, sterox, which is often used and therefore always available in a clinical laboratory. From a routine point of view it is important that sterox can be added to the colour-developing reagent, which then may be stored and used as such, while Tween 20 cannot. At the moment we are examining the possibility of determining the lipid-bound phosphorus of serum, starting with the protein precipitate obtained with perchloric acid.