PHENYLPYRUVIC ACID IN URINE

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Phenylpyruvic acid (PPA) is present in the urine of patients afflicted with oligophrenia phenylpyruvica (phenylketonuria). PPA is detected by first acidifying the urine and then adding ferric chloride reagent[1]. THE *et al.*[2] developed a quantitative method which also depends on the production of a green color with ferric chloride. This method gives more rapid and at least as accurate determinations as other methods reported in the literature.

In this determination, urine is diluted ten-fold with water and 1 ml of the solution is used; 5 ml of buffer, pH 2.2*, and 0.2 ml of a 10% ferric chloride solution are added. The urine is, therefore, finally diluted 50 times. According to the authors, possible precipitation of phosphates is prevented by the dilution and the buffer.

This method has been objected to because small amounts of PPA in the urine of phenylketonuric patients (due to the great dilution of the urine) cannot be accurately determined by reading off low extinctions. This is especially important for the regulation of patients diets. In order to be able to determine even these small amounts of PPA with sufficient accuracy, the method by THE *et al.* has been modified as follows.

METHOD

The series of determinations

Increasing amounts of urine (1–5 ml) were individually diluted to a final volume of 10 ml. It would be expected that the extinctions found in determining the amount of PPA in these urines would lie on a straight line passing through the origin (Beer's law). However, since the urines which are least diluted have the greater concentrations of inhibitory substances, their extinctions might deviate from those expected. But this appeared not to be the case and it can be concluded that the influence of inhibitory substances in a two-fold dilution of urine with water is of no importance.

In extinction measurements, nevertheless, the following points must be taken into account:

a. When a large quantity of urine is used its color may interfere.

b. The added ferric chloride colors the urine intensely yellow.

c. There may be substances present in the urine which react with ferric chloride to yield a (brown) color.

Thus, it would be desirable to use a control specimen by means of which the

* The buffer consists of 4.35 g glycine, 3.40 g NaCl, 4.20 ml 0.1 *N* HCl, made up to 1 l with distilled water.

above factors could be eliminated. Since this is impossible, in order to take the above factors into account, we divided the determinations into two phases:

I. The determination of the maximum extinction of the green color caused by the reaction of PPA with ferric chloride.

II. The determination of the extinction remaining after this green color had faded.

A (low) extinction (E_R) remains in normal urines (containing no PPA) when tested by the THE *et al.* method; this extinction appears directly after the addition of the reagents and remains constant for some time (see Fig. 1). If PPA is present in the urine, the green color which develops on addition of the reagents rapidly increases to a maximum (E_M) after about 1 min and then quite rapidly disappears. The rapidity of disappearance depends on the concentration of PPA. The residual extinction (E_R) remains after the green color caused by the PPA has completely vanished. The time necessary for this phenomenon varies from $\frac{1}{2}$- to 2 hours. The difference $E_M - E_R$ is equal to the corrected extinction of the PPA.

Water may be used as a blank, since the difference of two extinctions is measured.

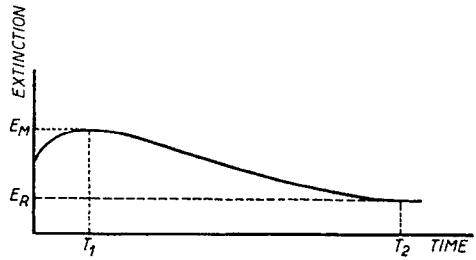

Fig. 1.

Measurements were made by means of a Beckman B spectrophotometer. A wavelength of 600 mμ was used; the value of E_R is a minimum at this wavelength.

The standard curve

Sodium-PPA (Hoffmann-LaRoche) with a purity of 96% was used. Aqueous solutions of increasing concentrations of sodium-PPA were made and the extinctions $E_M - E_R$ determined as above. Since pure aqueous solutions of PPA were used, the line passing through the E_M-points of the various solutions and the line passing through the points $E_M - E_R$ should be parallel to one another. The residual extinctions of all solutions are constant, hence the line passing through the E_R-points is parallel to the PPA-axis. The lines E_M and E_R intersect at one point lying on the extinction axis.

In Fig. 2 the determined standardization line (average of two determinations) is reproduced, while the values pertaining to Fig. 2 are recorded in Table I.

The straight line $E_M - E_R$ must *always* pass through the origin.

The most probable line which gives the values $E_M - E_R$ and passes through the origin, passes through the point $E_M - E_R = 0.663$ for 50 mg% PPA. The factor by which the found extinction must be multiplied in order to determine the amount of PPA is $50/0.663 = 75.5$.

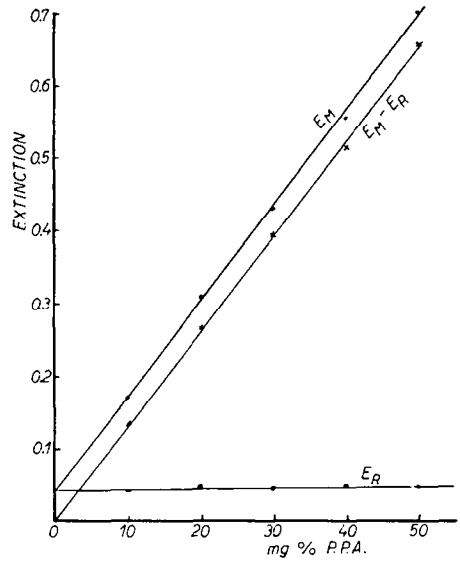

Fig. 2.

TABLE I

PURE AQUEOUS SOLUTIONS OF PHENYLPYRUVIC ACID

PPA mg%	E_M	E_R	$E_M - E_R$	*Extinction for 50 mg% PPA*
10	0.175	0.039	0.136	0.680
20	0.318	0.048	0.270	0.675
30	0.435	0.040	0.395	0.655
40	0.560	0.042	0.518	0.648
50	0.700	0.045	0.655	0.655
		Average 0.043		Average 0.663

APPLICATION

Measurements of the urine of patients with phenylketonuria

The results for pure aqueous PPA solutions do not hold true for urines containing PPA. In this case the lines which represent $E_M - E_R$ are not parallel because the inhibitory substances are proportional to the amounts of urine used. Likewise, the E_R-line should not lie parallel to the PPA axis. However, the lines E_M and E_R again join at a point on the extinction axis.

In Fig. 3 are shown the results obtained with the urine of a phenylketonuric patient (Sch.); this clearly illustrates the above mentioned facts. The lines E_M and E_R unite at point S. This extinction may be considered as the extinction caused by interfering substances in the absence of PPA. Although the concentration of the inhibitory substances cannot be expressed in absolute values, it can be expressed in mg per cent of PPA; in the present case, it equals 2.9 mg% PPA.

The amount of PPA in urine may be calculated from the following formula:

$$\text{mg\% PPA} = 75.5 \cdot \frac{10}{\text{ml of urine used}} \cdot (E_M - E_R)$$

From this example it is clear that the correction is needed whenever small quantities of PPA must be determined in urine. The influence of the inhibitory substances may be seen from the following.

Addition of PPA to urine of phenylketonuric patients

If the same quantity of PPA is added to each of a series of urine samples, and the same urines are analysed without addition of PPA, the following results may be expected.

If the line representing the difference of extinctions (with addition of PPA) is designated as $E_{MA} - E_{RA}$, and the line (without addition of PPA) as $E_M - E_R$, then the difference between the two represents a graphic area equal to the added amount of PPA. However, the PPA added is *not* quantitatively determined if the

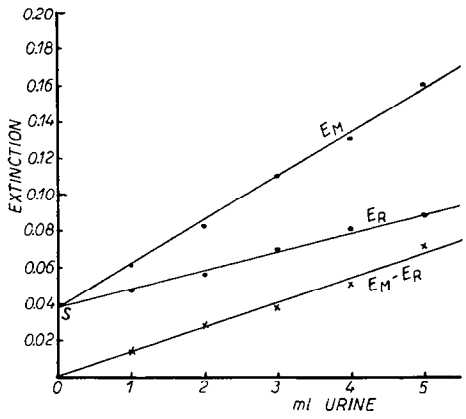

Fig. 3.

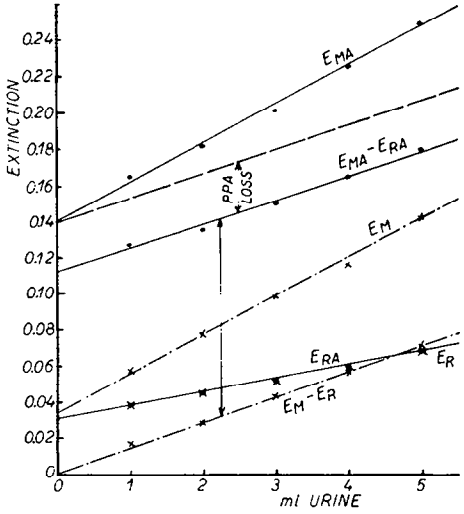

Fig. 4.

urine contains less than a certain amount of PPA. If this quantity is exceeded, the PPA *is* quantitatively determined. Apparently, the inhibitory substances present in the urine must first be saturated with PPA *or* be made inactive before the added PPA can be determined. However, this does not explain why the PPA already present in the urine is not destroyed by the inhibitory substances which are also present.

Patient Sch. was given a diet containing milk after his condition had improved so that practically no PPA was present in his urine. After a few days, he again excreted PPA in small amounts. The PPA concentration increased while addition of milk was made to the diet. In the initial period, when Sch. still excreted a small quantity of PPA, a series of determinations was undertaken. The series was repeated (with addition of pure PPA) when the patient had a much higher PPA excretion.

In Fig. 4 the results of such an experiment with and without the addition of PPA, are represented graphically; there are two series of lines (similar to those reported above).

The lines E_{RA} and E_R cover one another completely, as is to be expected. The calculated amount of PPA is equal to:

$$[(E_{MA} - E_{RA}) - (E_M - E_R)] \cdot (10/\text{amount of urine}) \cdot 75.5$$

Only 80% of the added quantity of PPA was found.

After the amount of PPA in Sch's urine had increased, nearly all the PPA was found quantitatively (Fig. 5). This is quite clearly seen by considering the differences between Fig. 4 and 5. In Fig. 4 the lines $E_{MA} - E_{RA}$ and E_{MA} do not intersect but they do intersect in Fig. 5.

It is thus clear that the method of using a series of determinations for the estimation of PPA in urine has advantages over the single determination method of THE *et al.* The correction for inhibitory substances must be acknowledged as a great improvement.

Although a single determination of PPA can be serviceable (if, for example, insufficient urine is available), it is more accurate to perform a series of determinations,

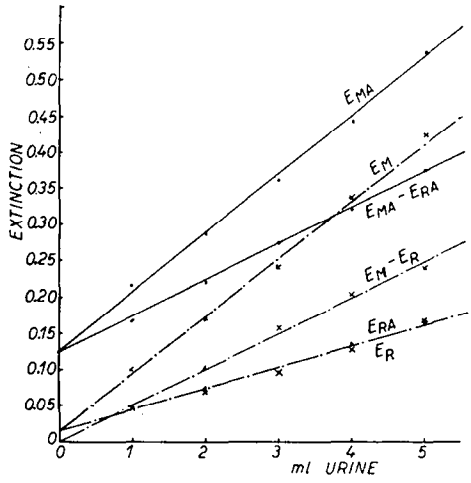

Fig. 5.

since an average of the results will give a truer value. The graph of the results indicates any faults which require correction.

The quantity of inhibitory substances is estimated approximately, and may be expressed in terms of PPA, by subtracting the E_R-values (obtained by calculation from the standard curve) from the E_R-values of the urines and multiplying the obtained extinctions by the factor for PPA.

Mixtures of PPA-containing urines

Mixtures were made of two urines containing different amounts of PPA, in the following manner.

	I	*II*	*III*	*IV*	*V*	*VI*	
Urine a	0	1	2	3	4	5	ml
Urine b	5	4	3	2	1	0	ml
Water	5	5	5	5	5	5	ml

1 ml of each of these mixtures was used for the PPA determination; this is analogous to the determination of PPA in urine diluted 1 : 1. Urines I and VI are the original unmixed urines. The extinctions of the urines would be expected to lie on the

line which unites the extinctions of the unmixed urines. The residual extinctions must lie also on the line. The results verified the expectations. In Fig. 6 are shown the results of mixing the urines of two patients Sch. and K. in the manner described; the residual extinctions are equal and the E_R-line runs horizontally. In Fig. 7, the results shown were obtained with portions of urine from patient Sch., taken on different days and

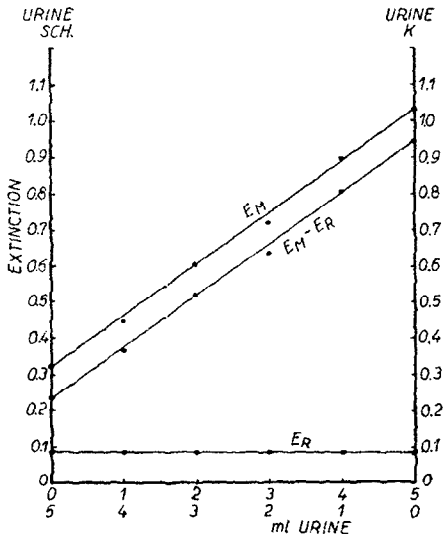

Fig. 6.

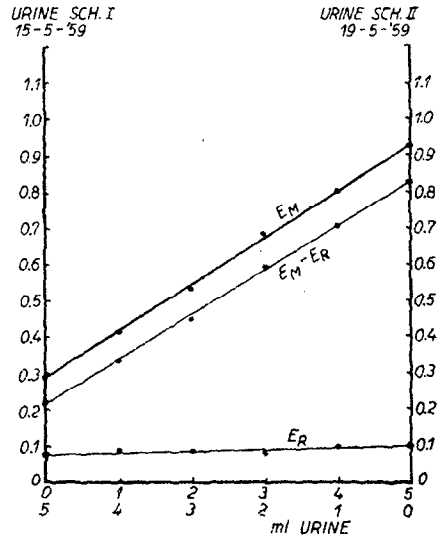

Fig. 7.

containing different amounts of PPA. The residual extinctions are not equal here and the line rises. The PPA extinction line again runs according to expectations.

SUMMARY

The method of THE, FLEURY AND VINK for the determination of phenylpyruvic acid (PPA) in urine is modified by measuring the extinction after the green colour with ferric chloride has faded, and subtracting this extinction from that found initially. More accurate values are obtained and low PPA values can be measured. The determination of PPA by taking increasing quantities of urine (up to 5 ml) is an advantage over a single PPA determination. The graphs give detailed information on the PPA-contents of urine and on the amount of inhibitory substances.

REFERENCES

[1] M. POLONOVSKY, P. DESGREZ AND F. DELBARRE, *Bull. soc. chim. biol.*, 29 (1947) 1049.
[2] T. P. THE, P. FLEURY AND C. L. J. VINK, *Clin. Chim. Acta*, 2 (1957) 424.