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Fluorometric Determination of Urinary Phosphate with 3-Hydroxy-3′,4′-dimethoxyflavone-magnesium System

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An attempt was made to develop a sensitive and selective method for the determination of phosphate utilizing the quenching of fluorescence of 3-hydroxy-3′,4′-dimethoxyflavone-magnesium chelate by phosphate ion, after selective extraction of phosphate as phosphomolybdic acid from other interfering ions. A new fluorometric method was thereby established for the determination of phosphate which is useful for urinary phosphate assay.

Most of the methods employed in general for the determination of phosphate are based on the absorption spectrophotometry of phosphomolybdic acid produced by reaction with molybdate or of a blue color which results when phosphomolybdate is treated with a reducing agent in aqueous or organic medium. These methods have been reviewed by Wadelin and Mellon.[2] Recently, more sensitive and selective methods, such as amplification method,[3] fluorometry,[4] atomic absorption spectrophotometry,[5] and enzymic method,[6] have been reported for the determination of micro-amounts of phosphate. Of these methods, the most sensitive may be the enzymic method which is based on the conversion of glycogen to glucose 6-phosphate in the presence of inorganic phosphate, activated phosphorylase, and phosphoglucomutase. Glucose 6-phosphate then reacts with the oxidized form of triphosphopyridine nucleotide or nicotinamide adenosine diphosphate (NAD) to yield their reduced forms whose fluorescence is measured. However, this method uses expensive reagents. It has been known that phosphate ion interferes with fluorometric determination of metal ions. Land and Edmonds[4] tried to use this phenomenon to determination of phosphate ion, and various metal chelate systems were investigated, such as aluminum-morin, glalium-quercetin, aluminum-flavonol, gallium-morin, and zirconium-flavonol, among which aluminum-morin system was reported to be better than the others. However, a satisfactory method to remove a number of interfering cations and anions was not achieved.

In the preceding paper,[7] we reported that 3-hydroxy-3′,4′-dimethoxyflavone combines with magnesium ion in N,N-dimethylformamide (DMF) containing 5% of ammonium buffer of pH 10.70 to form a strongly fluorescent chelate which is useful for the determination of micro-amounts of magnesium, and that some kinds of anions, such as phosphate, citrate, oxalate, or fluoride, markedly fade the fluorescence of the magnesium chelate. We tried to develop a sensitive and selective method for the determination of phosphate utilizing this quenching phenomenon after the selective extraction of phosphomolybdic acid.

1) Location: *Mitahora, Gifu, 502, Japan.*
2) C. Wadelin and M.G. Mellon, *Anal. Chem.*, **25**, 1668 (1955).
3) V. Djurkin, G.F. Kirkbright, and T.S. West, *Analyst*, **91**, 700 (1966).
4) D.B. Land and S.M. Edmonds, *Microchim. Acta*, **1966**, 1013.
5) G.F. Kirkbright, A.W. Smith, and T.S. West, *Analyst*, **92**, 411 (1967).
6) D.W. Schulz, J.V. Passonneau, and O.H. Lowry, *Anal. Biochem.*, **19**, 300 (1967).
7) T. Hayashi, S. Kawai, and T. Ohno, *Chem. Pharm. Bull.* (Tokyo), **21**, 1147 (1973).

Apparatus and Reagent

The fluorescence intensity was measured with a Shimadzu RF-501 spectrofluorometer. The slits were arranged to have an excitation beam of 15 nm and a fluorecsence beam of 2 nm. Measurements of pH were made with a Hitachi-Horiba M-5 pH-meter.

Ammonium Molybdate Solution——Ammonium molybdate solution was prepared by dissolving 2.7 g of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (reagent grade) in 250 ml of redistilled water.

Standard Phosphate Solution——Phosphate solution of 100 μg PO_4^{3-}/ml was prepared by dissolving 37.7 mg of $Na_2HPO_4\cdot 12H_2O$ (reagent grade) in 100 ml of redistilled water. Working solution was prepared by diluting it with redistilled water for use.

3-Hydroxy-3′,4′-dimethoxyflavone solution, magnesium nitrate solution, and 0.5M ammonium buffer solution (pH 10.70) were prepared in the same ways as described in the preceding paper. Redistilled water was used in all the experiments. Isobutyl acetate was used after distillation.

Result and Discussion

Quenching Mechanism

In the preceding paper,[7] we reported that the strong fluorescence of 3-hydroxy-3′,4′-dimethoxyflavone-magnesium chelate faded by addition of phosphate. In order to investigate this phenomenon, spectrophotometric study was carried out. Spectra (A to G) in Fig. 1 show two maxima at 360 and 430 nm in the visible region, and an isosbestic point at 389 nm. As phosphate content increases, the spectral pattern approaches that of the solution of 3-hydroxy-3′,4′-dimethoxyflavone (H). This fact indicates that the quenching by phosphate is due to the elimination of magnesium from 3-hydroxy-3′,4′-dimethoxyflavone-magnesium chelate.

Standing Time

A mixture of 10 ml of the equivalent mixture of 2.5×10^{-4}M solution of 3-hydroxy-3′,4′-dimethoxyflavone and 2.0 μg Mg^{2+}/ml solution of magnesium nitrate, and 5.0 ml of 0.5M ammonium buffer solution containing 10 μg of phosphate was diluted to 100 ml with DMF. Variation of fluorescence intensity with time was measured every 5 min from 10 min after

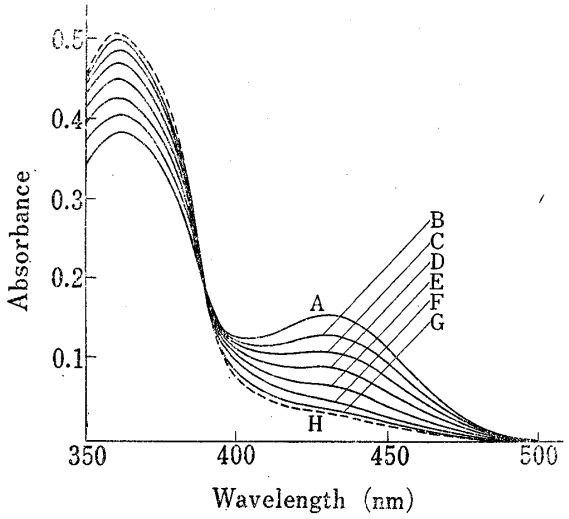

Fig. 1. Absorption Spectra

A–G: Reagent soln. 1.0 ml, $Mg(NO_3)_2$ soln. 1.0 ml, ammonium buffer soln. containing various amounts of PO_4^{3-} (A: 0 μg, B: 1 μg, C: 2 μg, D: 3 μg, E: 4 μg, F: 5 μg, G: 10 μg) 0.5 ml, DMF to make 10 ml.
H: Reagent soln. 1.0 ml, ammonium buffer soln. 0.5 ml, DMF to make 10 ml.

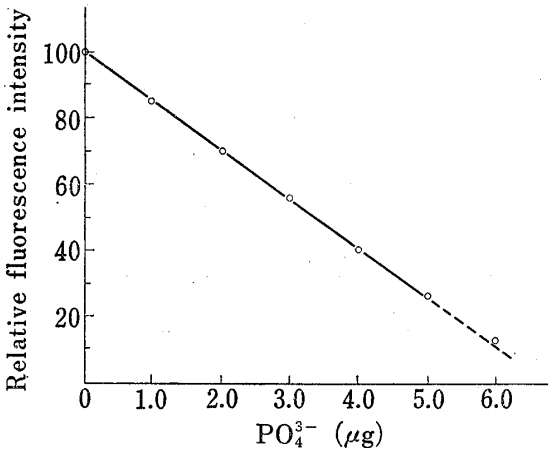

Fig. 2. Calibration Curve

mixing. The fluorescence intensity was nearly constant at least between 10 and 70 min after mixing.

Procedure I: Procedure for the Determination of Phosphate in Model Solution

From the results described above the following optimum condition was established as the standard procedure for the fluorometric determination of phosphate in a model solution.

Two milliliters of the equivalent mixture of 2.5×10^{-4}M solution of 3-hydroxy-3′,4′-dimethoxyflavone and 2.0 μg Mg^{2+}/ml solution of magnesium nitrate, and 0.5 ml of 0.5M ammonium buffer solution containing phosphate are placed in a 10-ml volumetric flask and the mixture is diluted to the mark with DMF. The fluorescence intensity is determined at 497 nm with excitation at 445 nm. Fig. 2 shows the calibration curve. There is a linear relationship between the fluorescence intensity and concentration of phosphate in the range of 0 to 5 μg of phosphate. The linearity of this calibration curve is better than that in the case of aluminum-morin system reported by Land and Edmonds.[4]

Separation of Phosphate Ion from Interfering Ions

Effect of several ions on the fluorescence intensity was tested with a solution containing 2.0 μg of phosphate according to Procedure I and the results are summarized in Table I. There was a strong interference by fluoride, oxalate, citrate, trichloroacetate, and tartarate ions which seems to be due to the elimination of magnesium from the magnesium chelate of 3-hydroxy-3′,4′-dimethoxyflavone as seen in the case of phosphate ion. For the technique to be specific, these interfering ions must be separated from a sample solution before proceeding with the quantitative assay. Therefore, a prior separation of phosphate as phosphomolybdic acid by solvent extraction with isobutyl acetate was carried out. It is well known that phosphate is converted to phosphomolybdic acid by reaction with molybdate, and the acid can be selectively extracted into some kinds of organic solvent. Most methods for the trace analysis of phosphate are based on these properties. It is also well known that phosphomolybdic acid is broken down into phosphate and molybdate by alkali.

In the present fluorometric method, as alkali for decomposition of phosphomolybdic acid, ammonium buffer of pH 10.70 was used and, as can be seen from Table I, co-existance of

Table I. Spectrofluorometric Determination of Phosphate in the Presence of Various Salts

Salt	R.F.I.			
	1 mg	100 μg	10 μg	1 μg
None	66.0	66.0	66.0	66.0
NaCl	6.85			
KBr	66.0			
KI	58.5	66.8		
KF	0	0	0	58.0
Na_2SO_4	0	54.2	66.7	67.0
Na_2SO_3	1.7	56.5	66.5	
NH_4SCN	62.0	67.0		
Na_2CO_3	6.5	66.5		
NaOAc	38.0	57.0	66.7	
$(NH_4)_2C_2O_4\cdot2H_2O$		0	28.5	55.0
Potassium citrate	0	0	27.0	54.0
$Na_2B_4O_7\cdot10H_2O$	34.5	66.3		
CCl_3COOH	0	0	20.0	59.0
$Na_2WO_4\cdot2H_2O$	1.2	57.3	69.0	
$(NH_4)_6Mo_7O_{24}\cdot4H_2O$	53.0	66.7		

PO_4^{3-}: 2.0 μg

molybdate produced by decomposition does not affect the fluorometric determination of phosphate. From preliminary experiments, the extraction method reported by Kirkbright and his co-workers[5] was adopted.

Decomposition of Phosphomolybdic Acid

In a 300 ml-separatory funnel, 70 ml of ammonium molybdate solution, 70 ml of redistilled water, 14 ml of HCl (reagent grade), and 7 ml of aqueous phosphate solution containing 42 μg of phosphate were placed, the solution was mixed, and allowed to stand for 5 min. Then, 70 ml of isobutyl acetate was added, the mixture was shaken for 1 min, and allowed to separate. The lower aqueous phase was discarded, the organic phase was washed with 70 ml of 2M HCl, and the aqueous phase was discarded. Eight milliliters of the isobutyl acetate phase was placed in each of seven flasks, evaporated to dryness by the aid of a rotary evaporator, and the residue was dried in a cabinet dryer for 30 min at about 70°. When cooled, 1.0 ml of 0.5M ammonium buffer solution was added to the peach-like flask and shaken for various periods of time. With 0.5 ml of this solution, variation of fluorescence intensity with shaking time was measured according to Procedure I. The results are shown in Fig. 3. Curve B is considerably less variable than curve A. It is not evident why the use of glass beads lessens the variability of the results.

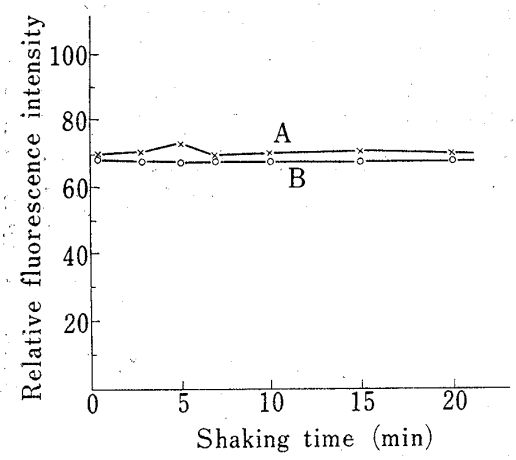

Fig. 3. Effect of Shaking Time on Fluorescence Intensity

B: Results obtained from the same experiment as A but addition of two glass beads (ϕ 4 mm) in a peach-like flask before evaporating isobutyl acetate.

Table II. Recovery of Phosphate from Urine

Amt. added (μg)	Found (μg)	Recovery (%)
4.00	3.99	99.8
	4.02	100.5
	3.81	95.3
	3.99	99.8
	4.04	101.0

coefficient of variation : 2.0 %

Procedure II: Procedure for the Determination of Urinary Phosphate

From the results described above, the following optimum conditions were established as the standard procedure for the fluorometric determination of urinary phosphate.

In a 50 ml-separatory funnel, 10 ml of ammonium molybdate solution, 10 ml of redistilled water, 2 ml of conc. HCl, and 1.0 ml of a sample solution are added, the solution is mixed, and allowed to stand for 5 min. Then, 10 ml of isobutyl acetate is added, the mixture is shaken for 1 min, and allowed to separate. The lower aqueous phase is discarded, the organic phase is washed with 10 ml of 2M HCl, and the aqueous phase is discarded. Eight milliliters of the isobutyl acetate phase is placed in a 10 ml peach-like flask, in which two glass beads (ϕ 4 mm) are placed, and isobutyl acetate is evaporated by a rotary evaporator. The residue is dried in a cabinet dryer for 30 min at about 70°, cooled to room temperature, and 1.0 ml of 0.5M ammonium buffer solution is added to the peach-like flask which is shaken for 10 min. One half ml of this solution and 2.0 ml of the equivalent mixture of 2.5×10^{-4}M solution of 3-hydroxy-3′,4′-dimethoxyflavone and 2.0 μg Mg^{2+}/ml solution of magnesium nitrate are placed

in a 10 ml-volumetric flask and the mixture is diluted to 10 ml with DMF. After mixing, the fluorescence intensity is measured at 497 nm with excitation at 445 nm.

In this procedure, there was found to be a linear relationship between the decrease of fluorescence intensity and concentration of phosphate in the range of 0 to 12 μg of phosphate.

Urinary phosphate was determined according to the Procedure II and the results obtained through the entire procedure from control urine samples spiked with 4.00 μg of phosphate per ml showed a good recovery as shown in Table II.

Table III. Spectrofluorometric Determination of Phosphate in the Presence of Various Ions

Cation (0.1 mg)	R.F.I.	Anion (1.0 mg)	R.F.I.
None	64.0	none	64.0
Al^{3+}	64.5	NaCl	63.0
Be^{2+}	59.0	KBr	63.5
Ca^{2+}	64.2	KI	65.5
Cd^{2+}	63.5	KF	62.0
Cu^{2+}	64.5	Na_2SO_4	65.0
Fe^{3+}	66.0	NH_4SCN	62.5
Mg^{2+}	65.2	Na_2CO_3	64.8
Mn^{2+}	64.3	NaOAc	63.5
Ni^{2+}	63.7	$(NH_4)_2C_2O_4 \cdot H_2O$	63.3
Pb^{2+}	65.5	Na_2SO_3	64.0
Sr^{2+}	64.5	potassium citrate	66.0
Th^{4+}	64.5	$Na_2B_4O_7 \cdot 10H_2O$	65.5
Zn^{2+}	65.0	$Na_2WO_4 \cdot 2H_2O$	64.5
Zr^{4+}	90.0	CCl_3COOH	63.5

PO_4^{3-}: 6.0 μg

Effect of several ions on this fluorometry was tested with a sample solution containing 6.0 μg of phosphate and the results are summarized in Table III. Marked interference was seen only with beryllium and zirconium ions, but these metal ions are not present in ordinary urine. Therefore, this method is useful for the clinical determination of trace amounts of phosphate in urine.