High Performance Liquid Chromatography of Phosphomolybdate and Silicomolybdate: Simultaneous Determination of Phosphate and Silicate

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A method for the simultaneous determination of phosphate and silicate was established by high performance liquid chromatography as their molybdocomplexes. The excellent separation of them was performed on a column packed with Styragel 60 Å (Waters Associates, Inc.) using an aqueous 55%(v/v) acetonitrile solution containing 0.75 M sulfuric acid and 0.02 M tetrabutylammonium hydrogensulfate as an eluent. The calibration curves for phosphate and silicate were linear in the range 0.03–10 µgP/ml and 0.01–5.0 µgSi/ml, respectively. The method was applied to the determination of phosphate and silicate in human urine and plasma.

Keywords High performance liquid chromatography, phosphate, silicate, phosphomolybdate, silicomolybdate

Phosphorus and silicon are essential elements for organisms and exist in part as phosphate and silicate which may play very important roles biologically.^{1–4} Therefore, phosphate and silicate in biological materials have been determined by colorimetry depending on the formation and reduction of molybdocomplexes.^{5–10} These molybdenum blue methods are convenient for routine work and used in the clinical laboratories, but they are not so sensitive and accurate because of the formation of a few unstable colored products.

Besides molybdenum blue methods, molybdenum yellow methods^{11–13} have been applied to the determination of phosphate and silicate in environmental samples. However, in the determination of phosphate and silicate in biological materials, these methods are apt to be interfered by the components having strong absorbance in UV-region.

This paper describes the separation of phosphomolybdate (P-Mo) and silicomolybdate (Si-Mo) by high performance liquid chromatography (HPLC) and its application to the simultaneous determination of phosphate and silicate in biological materials such as human urine and plasma.

Experimental

Reagents

Acetonitrile and methanol for HPLC were purchased from Wako Pure Chemicals. Tetrabutylammonium hydrogensulfate (TBA) was purchased from Tokyo Kasei Kogyo Co., Ltd. All other chemicals were of reagent grade and reagent solutions were prepared by dissolving in deionized and distilled water. Styragel 60

Å and Styragel 100 Å were purchased from Waters Associates, Inc. Bio-Beads SX-2 and TSK gel LS-111 were obtained from Bio-Rad Laboratories and Toyo Soda Co. Ltd. The standard solution of phosphate was prepared from potassium phosphate(monobasic) by dissolving in distilled water. The standard solution of silicate (1000 µg/ml as Si) for absorption spectrophotometry (Wako Pure chemicals) was used by diluting the solution with distilled water before use.

The reagent solution of 1.0%(w/v) ammonium molybdate was prepared by dissolving 1.0 g of ammonium molybdate in 100 ml of 0.2 M sulfuric acid. The solution was stored in a plastic bottle at room temperature in the dark.

Apparatus and chromatographic conditions

The high performance liquid chromatographic system used consisted of a reciprocating pump (PSU-2.5, Seishin Seiyaku Co. Ltd., Tokyo), a loop injector (VMU-5, Seishin Seiyaku Co. Ltd., Tokyo), UV-detector (UVILOG-7, Oyo-Bunko Kiki Co. Ltd., Tokyo) and recorder (SS-250F, Sekonic, Tokyo). A pyrex glass tube (5 mm I.D.×200 mm) was packed with Styragel 60 Å. P-Mo and Si-Mo were detected at 310 nm which is their absorption maxima.

The eluent, 55%(v/v) CH₃CN–0.75 M H₂SO₄–20 mM TBA, was prepared as follows: 6.79 g of tetrabutylammonium hydrogensulfate was dissolved in 250 ml of 3.0 M sulfuric acid and then 550 ml of acetonitrile was added. The solution was made up to 1 l with deionized distilled water.

Assay procedure

Molybdocomplexes of phosphate and silicate were formed by adding 500 µl of 0.2 M sulfuric acid

containing 1.0% ammonium molybdate to 500 μ l of sample solution. After 30 min, 1.0 ml of aqueous 66%(v/v) acetonitrile solution containing 0.6 M sulfuric acid was added and the solution was submitted to HPLC.

Analysis of plasma. Five hundred microliter of plasma was placed in a centrifuge tube and 2 ml of chloroform and 500 μ l of 10%(w/v) trichloroacetic acid(TCA) were added. After shaking vigorously, it was centrifuged at $2000\times g$ for 5 min. The supernatant (500 μ l) was transferred to a plastic tube, followed by the addition of 10 μ l of 10% bromine in ethanol and molybdocomplexes were prepared by the procedure described above.

Analysis of urine. Urine sample (500 μ l) diluted 25 times with distilled water was placed in a plastic tube and molybdocomplexes were prepared in the same manner as for plasma samples.

Molybdenum blue methods

Determination of phosphate in human urine was done according to the procedure of the molybdenum blue method achieved by Fiske and SubbaRow⁵, and silicate in human urine according to the procedure of the molybdenum blue method of Baumann.⁹

Results and Discussion

Separation of molybdocomplexes

One of the major problems concerned with the chromatographic separation of P-Mo, Si-Mo and excess reagent, molybdate, originates from the dissociability of molybdocomplexes in the eluent. Especially, P-Mo dissociates reversibly and Si-Mo is present almost irreversibly. This is the reason why miscible organic solvent with water has to be used as a component of the eluent. Figure 1 shows that P-Mo is

Fig. 1 Effect of organic solvents on the stabilization of phosphomolybdate. The solution containing P-Mo (100 μ g/ml as P) and 0.4% molybdate was diluted 100 times with the aqueous solution containing 0.45 M H_2SO_4 and organic solvent (MeOH($\square$), EtOH($\blacktriangle$), PrOH($\triangle$), CH_3CN ($\bullet$) and dioxane($\circ$)). P-Mo was detected at 330 nm at which the absorbance of molybdate (blank) was low.

scarcely dissociated in the eluent which contains methanol, ethanol, propanol, dioxane or acetonitrile.

Separation of these complexes was investigated by ion pair-reversed phase high performance liquid chromatography (IP-RPHPLC) using styrene-divinylbenzene(DVB) copolymers such as Amberlite XAD-2, Bio-Beads SX-2, TSK gel LS-111, Styragel 60 $\text{\AA}$ and Styragel 100 $\text{\AA}$. Organic solvents examined were methanol, ethanol, propanol, dioxane and acetonitrile. The separation of these complexes was achieved successfully by IP-RPHPLC system using Styragel 60 $\text{\AA}$ as a packing material and 55%(v/v) CH_3CN -0.75 M H_2SO_4 -20 mM TBA as the eluent. A typical chromatogram is shown in Fig. 2.

Though both Si-Mo and P-Mo in general form slightly soluble ternary complexes with quarternary ammonium, ternary complexes with TBA are soluble in aqueous acetonitrile solution containing more than 50%(v/v). When an aqueous sample containing molybdocomplexes was introduced into flow stream of the eluent by a loop sampler, ternary complexes of molybdocomplexes formed with TBA were precipitated due to the decrease of acetonitrile content at interface between the eluent and an aqueous sample. This is the reason why acetonitrile was added to the aqueous solution containing molybdocomplexes.

Effect of the acetonitrile content

In IP-RPHPLC used styrene-DVB copolymer as a packing material, the acetonitrile content affects the capacity factor(k') for molybdocomplexes. The capacity factor of P-Mo decreased significantly with the increase of acetonitrile content in the eluent, but those of molybdate and Si-Mo scarcely decreased. At a lower content than 50%(v/v), P-Mo and Si-Mo formed

Fig. 2 Separation of phosphomolybdate and silicomolybdate by HPLC. 1, Molybdate; 2, Si-Mo; 3, P-Mo. Column, styragel 60 $\text{\AA}$ (5 mm I.D. $\times$ 200 mm); eluent, 55% (v/v) CH_3CN -0.75 M H_2SO_4 -20 mM TBA; flow rate, 1.0 ml/min; sample size, 50 μ l; detection, 310 nm.

slightly soluble ternary complexes with TBA.

Effect of the concentration of sulfuric acid

The concentration of sulfuric acid affects the capacity factor for molybdocomplexes. The capacity factor of P-Mo decreased significantly with the increase of concentration of sulfuric acid, but those of Si-Mo and molybdate were scarcely affected by the concentration of sulfuric acid.

Effect of the concentration of TBA

The capacity factor of molybdocomplexes is shown in Fig. 3 as a function of the concentration of the TBA in aqueous 55%(v/v) acetonitrile solution containing 0.75 M sulfuric acid as an eluent. Capacity factors of P-Mo and Si-Mo increased with the increase in the concentration of TBA as an ion pair reagent, and that of P-Mo reached a maximum at 20 mM TBA. In the concentration higher than 20 mM TBA, the elution profile of the three species was not reproducible.

Fig. 3 Capacity factors of phosphomolybdate and silicomolybdate vs. TBA concentration. ■, P-Mo; □, Si-Mo; eluent, 55% (v/v) CH₃CN-0.75 M H₂SO₄-TBA. Other chromatographic conditions are as in Fig. 2.

Table 1 Effect of foreign substances on the determination of phosphate and silicate

Substance		Recovery, %	
		Si	P
—	—	100	100
NaCl	10 mg/ml	98.3	102
KI	10 mg/ml	87.1	12.7
NaF	10 mg/ml	0.0	0.0
CaCl ₂	10 mg/ml	102	96.4
H ₂ O ₂	10 mg/ml	0.0	0.0
Br ₂	10 mg/ml	102	100
Uric acid	0.2 mg/ml	89.4	28.1
Heparin	0.1 mg/ml	101	99.7
H ₂ SiO ₃	10 µg/ml as Si	—	100
H ₂ PO ₄	10 µg/ml as P	98.4	—
H ₄ P ₂ O ₇	10 µg/ml as P	100	101

P-Mo(1.0 µgP/ml) and Si-Mo(1.0 µgSi/ml) were formed in 0.1 M H₂SO₄ aqueous solution containing 0.4% ammonium molybdate and foreign substance.

Influence of foreign substances

Table 1 shows that the present method is interfered by a few foreign substances. Reducing substances such as uric acid and potassium iodate interfered with the present method by reducing the P-Mo. The reduced P-Mo was eluted earlier than P-Mo under the conditions of HPLC given in Fig. 2. Hydrogen peroxide couldn't be used for the prevention of interference from reducing substances, because it oxidized molybdate to yellow peroxymolybdate¹⁴, which did not form molybdocomplexes with phosphate and silicate. Then, bromine was used as an oxidizing agent because it did not oxidize the molybdate.

Sodium fluoride used in general as an anticoagulant interfered with the present method, but heparin did not interfere.

Precision

In the operating conditions described above, the calibration curve for silicate was linear in the range 0.01–5.0 µgSi/ml and that for phosphate was linear in the range 0.03–10 µgP/ml. By using the procedure described above, the limits of detection for phosphate and silicate were 0.01 µgP and 0.005 µgSi/ml (*S/N*=2), respectively. When 0.5 µg/ml of phosphate and silicate were determined ten times by the recommended procedure, relative standard deviations(RSD) were 3.18 and 3.64%, respectively.

Analysis of urine and plasma

Bromine was added excessively to diluted urine and deproteinized plasma for the oxidization of reducing materials as uric acid. Phosphate and silicate in the pretreated samples were determined according to the

Table 2 Recovery tests of silicate and phosphate from human urine and plasma

Added/µg ml ⁻¹		Found/µg ml ⁻¹		Recovery, %	
		Si	P	Si	P
Urine					
—	—	12.5	320	—	—
25.0	200	38.3	520	103	100
50.0	400	62.4	728	99.8	102
Plasma					
—	—	1.21	21.4	—	—
1.0	4.0	2.24	25.5	103	103
2.0	8.0	3.16	29.9	97.4	106

Table 3. Determination of silicate and phosphate in human urine and plasma

Subject	Urine/µg ml ⁻¹		Plasma/µg ml ⁻¹	
	Si	P	Si	P
A	13.2	618	0.44	27.6
B	14.6	470	0.44	38.4
C	30.3	618	1.57	25.4
D	15.9	540	0.96	34.1
E	18.2	420	1.21	31.7

Fig. 4 Relationship between molybdenum blue methods and present method for determination of phosphate and silicate in human urine samples.

method described in the experimental section. The analytical results of recovery tests from urine and plasma are shown in Table 2. The recoveries of phosphate varied from 100 to 106% and those of silicate from 97.4 to 103%.

Analytical results of phosphate and silicate in human urine and plasma are shown in Table 3.

The concentrations of silicate and phosphate in normal human urine measured by the present method (x) were compared with those obtained by the molybdenum blue methods^{5,9} (y). We found significant correlations: $r=0.990$, $y=0.883x+3.92$ for silicate; $r=0.983$, $y=0.910x+84.4$ for phosphate (Fig. 4). At low levels, values obtained by molybdenum blue methods were larger than those obtained by the present method for both phosphate and silicate.

It should be emphasized that the present method is more accurate than molybdenum blue methods, because the reaction product is single and stable in acidic aqueous solution.

Moreover, we will try to study physiological roles for silicate and phosphate in bone calcification processes by the use of this method.

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