Spectrophotometric Determination of Glucosamine and Its Analogous Amino Sugars with *o*-Hydroxyhydroquinonephthalein and Palladium(II)

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A simple and highly sensitive spectrophotometric method for the determination of glucosamine and its analogous amino sugars was established based on fading of the palladium(II)-*o*-hydroxyhydroquinonephthalein-hexadecyltrimethylammonium complex. In the determination of glucosamine, Beer's law is obeyed in the range of 0.02 – 0.18 $\mu\text{g ml}^{-1}$, with an effective molar absorptivity at 630 nm and the relative standard deviation being $8.4 \times 10^5 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ and 1.08% ($n = 10$). This method is about 70-times more sensitive than the Elson-Morgan method. The method was successfully applied to the assay of glucosamine in actual samples.

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Glucosamine, which is a component of proteoglycan, is found in and around the cells of the cartilage in people's joints, and is currently used for the treatment of degenerative joint disease in small animals, and represents a novel approach for the treatment of degenerative conditions. Also, glucosamine is considered to be a dietary supplement, and as such the content uniformity as well as the glucosamine products are rarely determined. Methods for analyzing glucosamine include spectrophotometry,¹⁻⁸ spectrofluorometry,^{9,10} liquid chromatography¹¹⁻¹³ and gas chromatography.^{14,15}

On the other hand, glucosamine and its analogous amino sugars, such as mannosamine and galactosamine, have several donor atoms capable of metal complex formation and binding^{16,17} with copper(II), zinc(II), nickel(II), iron(III), cobalt(III) and so on. We have reported¹⁸⁻²⁰ on simple and sensitive methods for various organic compounds based on the fading of a colored dye-metal complex. In the color reaction between *o*-hydroxyhydroquinonephthalein (QP) and palladium(II) (Pd(II)), we noticed that the color development of the QP-Pd(II) complex was interfered with by small amounts of amino sugars, such as glucosamine. We, therefore, speculated that a method which would utilize fading of a QP-Pd(II) complex in the presence of an amino sugars would serve as a simple and highly sensitive determination of amino sugars. Here, we report on a spectrophotometric method for the determination of glucosamine and its analogous amino sugars based on the fading of a QP-Pd(II) colored complex.

Experimental

Reagents and apparatus

A stock solution ($1.0 \times 10^{-2} \text{ M}$, $1 \text{ M} = 1 \text{ mol dm}^{-3}$) of

D-glucosamine hydrochloride (Wako Pure Chem. Co. Ltd.) in water, and the working solution was prepared by suitable dilution of this stock solution as required. A solution ($5.0 \times 10^{-4} \text{ M}$) of palladium(II) was prepared from a stock solution (Wako Pure Chem. Co. Ltd., $1000 \mu\text{g l}^{-1}$) by dilution with water. A solution of QP, which had been synthesized according to a method described in the literature,²¹ was prepared in a $1.0 \times 10^{-3} \text{ M}$ methanol solution containing one drop of hydrochloric acid. A $1.0 \times 10^{-2} \text{ M}$ aqueous solution of hexadecyltrimethylammonium chloride (HTAC, Tokyo Kasei Kogyo Co.) was prepared by dissolving HTAC purified by recrystallization in water. A buffer solution of pH 7.5 was made by mixing appropriate amounts of a 0.1 M potassium dihydrogenphosphate solution and a 0.1 M disodium hydrogenphosphate solution. Reagent-grade chemicals were used throughout. Pure water was prepared by purifying deionized water with Milli-Q Labo system just before use.

A Shimadzu spectrophotometer (Model UV-160) with 1.0-cm matched silica cells was used for an absorbance measurement. The pH measurements were made with a Horiba (F-11) pH meter in combination with a calomel glass electrode.

Standard procedure for the determination of glucosamine

The following components were mixed in a 10-ml volumetric flask: a solution containing 0.2 – 1.8 μg of glucosamine, 0.6 ml of a $5.0 \times 10^{-4} \text{ M}$ Pd(II) solution, 1.5 ml of a $1.0 \times 10^{-2} \text{ M}$ HTAC solution, 2.5 ml of the buffer solution and 0.8 ml of a $5.0 \times 10^{-4} \text{ M}$ QP solution. The mixture was diluted to 10 ml with water, transferred into a test tube, mixed well and kept at 50°C for 30 min. After the solution had been cooled in water to room temperature, the difference in the absorbance (ΔA) between the resultant solution and a reagent blank solution prepared under the same conditions was measured at 630 nm against water.

Results and Discussion

Optimization of reaction variables

In order to examine the utility of a method for the

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Fig. 1 Absorption spectra of QP-Pd(II) and QP solutions in the presence of glucosamine. $[Pd(II)] = 3.0 \times 10^{-5}$ M; $[QP] = 4.0 \times 10^{-5}$ M; $[CTAC] = 1.5 \times 10^{-3}$ M; pH = 8.0. Curves 1, 2, 3, 4 and 5 for QP-Pd(II) solutions (glucosamine concentration (M): curve 1 = 0; curve 2 = 1.0×10^{-6} ; curve 3 = 2.0×10^{-6} ; curve 4 = 3.0×10^{-6} ; curve 5 = 4.0×10^{-6}). Curve 6, QP solution. Reference, water.

determination of glucosamine based on fading of a dye-metal colored complex, the color reactions between various dyes and metal ions were studied by comparing the situations in the presence of glucosamine. The effect of metal ions on the reaction system was investigated. Only palladium(II) was effective among various metal ions tested: palladium(II), copper(II), zinc(II), nickel(II), cobalt(II), iron(III), manganese(II) and molybdenum(VI). Next, the dyes²² were studied by using QP, *o*-sulfophenylfluorone (SPF), salicylfluorone (SF), phenylfluorone (PF), *p*-carboxyphenylfluorone (PCPF), Pyrogallol Red, Pyrocatechol Violet, Xylenol Orange and 4-(2-pyridylazo)resorcinol. These dyes could be arranged in the following order with respect to the sensitivity: $QP > SF > SPF > PF > PCPF \gg$ the others ≈ 0 .

The effect of the pH on the reaction was examined by using various buffer solutions, such as 0.1 M potassium dihydrogenphosphate/0.1 M disodium hydrogenphosphate, 0.1 M citric acid/0.1 M disodium hydrogenphosphate, 0.1 M potassium dihydrogenphosphate/0.05 M borax, 0.1 M hydrochloric acid/0.1 M tris(hydroxymethyl)aminomethane and 0.1 M hydrochloric acid/0.1 M sodium 5,5-diethylbarbiturate solutions. The best pH range was from 6.8 to 8.2 by using 2.5 ml of 0.1 M potassium dihydrogenphosphate/0.1 M disodium hydrogenphosphate buffer solution.

The effect of the amounts of QP and Pd(II) on ΔA was examined while maintaining a fixed final concentration of glucosamine (0.90 μg). The results indicated that the molar ratio of QP to Pd(II) should be greater than equimolar. Accordingly, all further work was carried out with 3.0×10^{-5} M Pd(II) and 4.0×10^{-5} M QP solutions in relation to the determination limit of glucosamine and the absorbance of a QP-Pd(II) solution.

In order to stabilize the color development and to enhance the sensitivity, the effects of different surfactants were examined: cationic (HTAC, hexadecylpyridinium chloride, zephiramine, stearyltrimethylammounium chloride), nonionic (Tween 20, Triton X-405, polyvinylalcohol, Brij 35), anionic (sodium dodecylsulfate, sodium di(2-ethylhexyl)sulfosuccinate) and amphoteric (Amphitol 24B, Swanol AM-101). The use of

Table 1 Sensitivities and reproducibilities of the proposed method

Substance	$\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$	RSD ^a , %
Glucosamine	8.4×10^5	1.08
Mannosamine	5.7×10^5	1.48
Galactosamine	5.2×10^5	1.36
UDP- <i>N</i> -Acetyl-D-glucosamine	4.0×10^4	— ^b
Streptomycin	2.5×10^5	— ^b

a. Mean of 10 determinations.

b. Undetermined.

HTAC was the most effective in terms of sensitivity.

The color development in this reaction system did not occur instantaneously at room temperature. Thus, the effects of the incubation temperature and time were examined at 40, 50 and 60°C. A maximum and constant ΔA value was obtained by heating at 50°C for 30 min, followed by cooling in water to room temperature. The ΔA value remained constant for at least 3 h after the solution had been cooled to room temperature.

Figure 1 shows the absorption spectra of QP-Pd(II) in the presence or absence of glucosamine and QP solutions.

Calibration curve

A calibration curve for glucosamine was constructed by the standard procedure. A good linear relationship was observed over 0.02 – 0.18 $\mu\text{g ml}^{-1}$ of glucosamine. The effective molar absorptivity (ϵ) was calculated from the slope of the calibration graph to be $8.4 \times 10^5 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$. The relative standard deviation (RSD) for ten runs of 0.90 μg of glucosamine was 1.08%. The calibration curves for other amino sugars and related compounds were also constructed under the optimum conditions. As shown in Table 1, the sensitivities of mannosamine and galactosamine were somewhat low compared with that of glucosamine. The reaction between proteoglycans, such as hyaluronic acid and chondroitin sulfate, and the QP-Pd(II) solution were not studied in the present work. This method is about 70-times more sensitive than the Elson-Morgan method,^{1,2} and gives excellent reproducibilities.

Interference of foreign substances

The interference from various foreign substances on the determination of glucosamine was examined. Inorganic ions, such as sodium, potassium, ammonium, calcium, magnesium, chloride, bromide, nitrate, sulfate, and phosphate, did not noticeably affect the accuracy of the determination of glucosamine, even when these ions were present in large excess amounts compared with that of glucosamine. A fairly large error appeared in the presence of copper(II), iron(III), zinc(II) and glucuronic acid. Organic substances, such as glucose, glycine, caffeine, urea, ascorbic acid and methylcellulose, interfered very little. The results are summarized in Table 2.

Application

The proposed method was applied to the determination of glucosamine in commercial dietary supplements (tablets). The contents of the tablets were accurately weighed and ground in a mortar to a fine powder. The requisite volume of powder was weighed, transferred into a 100-ml volumetric flask, diluted to the mark with water and filtered. An appropriate amount of the sample solution was taken and assayed according to the standard procedure. The accuracy of the proposed method was checked by a thorough replicate analysis of each spiked sample. Neat standards were prepared under the same conditions. The

Table 2 Effect of foreign substances on the assay of glucosamine

Substance	Added (Substance/ glucosamine)	ΔA at 630 nm	Recovery, %
NaCl	1.0×10^2	0.432	100.0
K ₂ SO ₄	1.0×10^2	0.432	100.0
CH ₃ COONa	1.0×10^2	0.432	100.0
NH ₄ Cl	1.0×10^2	0.432	100.0
NaNO ₃	1.0×10^2	0.432	100.0
KBr	1.0×10^2	0.432	100.0
Sodium citrate	1.0×10^2	0.432	100.0
Mg(II), chloride	1.0×10^2	0.432	100.0
Ca(II), chloride	1.0×10^2	0.432	100.0
Cu(II), nitrate	1.0	0.506	117.1
Fe(III), sulfate	1.0	0.450	104.2
Zn(II), chloride	2.0	0.455	105.3
Glucuronic acid	1.0	0.461	106.7
Pyridoxine-HCl	2.0×10	0.432	100.0
Glycine	2.0×10	0.432	100.0
Ascorbic acid	1.0×10^2	0.432	100.0
Caffeine	1.0×10^2	0.432	100.0
Glucose	1.0×10^2	0.432	100.0
Lactose	1.0×10^2	0.432	100.0
Urea	1.0×10^2	0.432	100.0
Methylcellulose	1.0 mg	0.432	100.0

[glucosamine] = 5.0×10^{-7} M; [Pd(II)] = 3.0×10^{-5} M; [QP] = 4.0×10^{-5} M; [HTAC] = 1.5×10^{-3} M; pH, 8.5; reference, water.

recoveries of added quantity were about 102 – 103%. This indicates that the proposed method gives accurate results. These results are given in Table 3.

Composition of the colored complex and reaction mechanism

In order to clarify the reaction mechanism, the composition of the colored complex was studied by Job's method of continuous variation and the molar ratio method. The Pd(II)-to-QP ratio and the [Pd(II)/QP-to-HTAC] ratio were 1:1 and 1:1, respectively. On the other hand, the glucosamine-to-Pd(II) ratio was 1:1 in the presence and absence of QP. It was found that the reaction rate between glucosamine and Pd(II) was much faster than between QP and Pd(II), by tracing the absorption spectrum of the Pd(II) solution. The experimental results imply that glucosamine, which has ligands containing oxygen and nitrogen atom as donor atoms, reacted with Pd(II) to give the glucosamine-Pd(II) complex in the first step, followed by the formation of QP-Pd(II)-HTAC [1:1:1] complex.

In conclusion, a simple and highly sensitive spectrophotometric method for glucosamine and its analogous amino sugars was established with QP and Pd(II) in a HTAC micellar medium. This procedure is based on a decrease in the absorbance of the QP-Pd(II)-HTAC ternary complex solution in the presence of amino sugars, such as glucosamine. The reaction mechanism was examined in this study, and an amino sugar-Pd(II) complex was formed as the result of a competitive reaction between an amino sugar and QP to Pd(II) in the first step. A residual Pd(II) subsequently reacted with QP in the presence of HTAC to give the ternary complex of Pd(II)-QP-HTAC [1:1:1]. The sensitivity ($\epsilon = 8.4 \times 10^5 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$) of the proposed method was much more sensitive than that of other spectrophotometric methods. The proposed method, owing to no need for solvent extraction, should be useful for a simple and highly sensitive determination of glucosamine in real samples.

Table 3 Application of this method to determine glucosamine in a dietary supplement

Sample ^a	Content/mg ^a		RSD ^b , %	Recovery ^{b,c} , %
	Nominal amount	Present method		
A	375.1	365.5	1.20	102.2
B	500	502.4	1.25	102.8

a. Sample A (glucosamine hydrochloride): Tablet (per 1 Tab.) also contained dextrin, cellulose, methylcellulose, stearic acid, glycerol and silicon dioxide, stidine. Sample B (glucosamine sulfate): Tablet (per 1 Tab.) also contained ascorbic acid, methylsulfonyl methane, calcium and chondroitin sulfate.

b. Mean of 5 determinations.

c. Glucosamine taken, 0.45 μg .

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