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A New Rapid and Convenient Method for Microdetermination of Hydroxyproline Using Flow Injection Analysis

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Abstract

A more rapid and convenient microdetermination of hydroxyproline (Hyp) has been established by modification of the conventional KISO method using flow injection analysis. This paper goes into details. This flow method is based on the color change reaction between pyrrole, the oxidation and decarboxylation product of Hyp, and Ehrlich's reagent. Toluene extraction is not necessary, and the used organic solvent is ethylene glycol monomethyl ether (methyl cellosolve) only. A good linear relationship was obtained between the concentration of Hyp (from 0 to 80 μg/ml) and absorbance.

Since hydroxyproline (Hyp) is found in the collagen of the connective tissue of nearly all animals, Hyp [1-5] has been measured extensively in studies of collagen formation and metabolism [6,7], as in medical investigations of wound healing [8,9]. However, these assays require relatively large amount of samples and are often troubled by several inconvenient points, such as low reproducibility and time consumption

because of the rather complicated procedures. A less complex and more convenient procedure, which would yield satisfactory results, has long been desired for the measurement of the imino acid contained, often in microquantities, in connective tissue protein. A batch method, the KISO method [10,11], was established for the microdetermination of Hyp with rather good reproducibility. It has been used mainly in biochemical research on wound healing and also in several other fields of medical science [11-23]. The increasing needs in current clinical studies have encouraged us to develop a new modification to overcome some of the difficulties in the batch method.

The flow injection analysis (FIA) [24-29], which was developed by Ruzicka and Hansen in 1975, is one of the continuous flow methods: a discrete sample is injected into a carrier stream which is pumped in set flow rates into the fine tube, and the sample is detected through the detector downstream after the reaction, after making good use of the dispersion. As this method can select the optimum conditions which can change the dispersion at will by setting some analytical conditions; once the conditions are set, the same dispersion is always obtained. Therefore good reproducible determination is possible even on a system which has not achieved equilibrium. And the method is a more rapid analytical technique than the conventional batch method; moreover, it involves the less consumption of the reagents and makes possible the assay of even a slight amount of sample. Further, there is no discrepancy among analysts for measurements using this method. Since then many conventional analyses have been modified to FIA, in the clinical analyses as well [30-32]. In our previous communication [33], an application of FIA to KISO method which is the microdetermination of Hyp in the connective tissue has been proposed; here we wish to report details of this work.

Experimental

Reagent

Borate buffer stock solution: Boric acid (38.65 g) and potassium chloride (140.75 g) were dissolved in water (500 ml) and then the pH was adjusted to 8.7 with potassium hydroxide.

Oxidant solution: sodium p-toluensulfonchloramide (Chloramine T, 0.1408 g) as oxidant was dissolved in ethylene glycol monomethyl ether (methyl cellosolve, 200 ml) and was added to the solution of stock buffer solution (160 ml) and water (640 ml).

20% (v/v) Triton X-100 solution: Triton X-100 (200 ml) as surfactant was dissolved in warm water (800 ml).

Ehrlich's reagent solution: p-dimethylaminobenzaldehyde (DBA, 102.6 g) was dissolved in methyl cellosolve (400 ml) and was added to sulfuric acid (conc. H_2SO_4: 100 ml, 20% (v/v) Triton X-100 solution: 160 ml, H_2O: 340 ml).

1 mg/ml L-Hydroxyproline (Hyp) standard stock solution: Hyp (0.100 g) was dissolved in water to 100 ml.

1 mg/ml L-prolin (Pro) and glycine (Gly) standard stock solutions: each Pro (0.100 g) and Gly (0.100 g) was dissolved in water to 100 ml.

Standard solution: A concentration series of Hyp was prepared; three standard stock solutions were mixed as shown in Table I.

All chemicals used were from Wako Pure Chemical Industries, LTD-make, and were of analytical-reagent grade except for surfactant. Water used was ultrapure.

Apparatus

Pump: A double-plunger micropump (Kyowa Seimitsu KHU-W-294) was used for the oxidant solution and the Ehrlich's reagent solution, fitting the air dampers (Kyowa Seimitsu KU-AIR 2) and the pressure gauge (Kyowa Seimitsu KPG-50L).

Sample injector [34]: A six-way injection valve (Ohyoh Bunko) was used for the sample injection; the volume of the

sample loop was 150 µl.

Spectrophotometer: The absorbance was measured with a double-beam spectrophotometer (Japan Spectroscopic Co. Ltd., UVDEC-340) with a microflow cell (Japan Spectroscopic Co,. Ltd., FIC-361, light pass: 10 mm, volume: 20 µl).

Recorder: A recorder (Riken Denshi Co. Ltd., SP-G12) with a precision attenuator with offset adjuster (Riken Denshi Co. Ltd., AT-74) was used for the record of the change of absorbance.

Procedure

A flow diagram of the FIA system is shown in Fig. 1. The flow lines were made from Teflon tubing (1 mm id, except back pressure coil, 0.5 mm id). Flow rates of (R1) and (R2) were fixed at 2.8 ml/min, respectively. The sample was injected with a sample injector into a current of the buffer and oxidant solution (R1) by a syringe without a needle (1 ml; e.g. Terumo, disposable syringe). Following the oxidation, the sample was decarboxylated in the mixing coil (C1) in a boiling water bath at 100°C. Then, the buffer and oxidant solution with pyrrole was mixed in a 1 : 1 ratio with Ehrlich's reagent (R2) at the point X, and a colored product was formed in a reaction coil (C2). Then, the product was passed through the flow cell fitting to the spectrophotometer by the continuous stream, and the absorbance change at 560 nm was recorded as a function of the concentration of the Hyp. To prevent bubble-forming, a back pressure coil (C3) was set after the spectrophotometer.

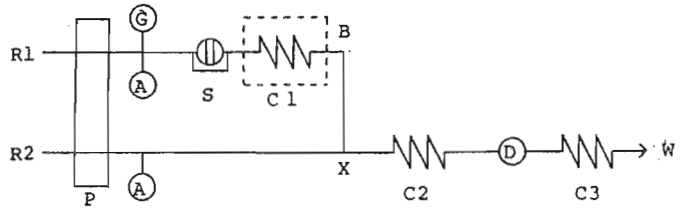

Fig. 1 A Flow Diagram for the Microdetermination of Hyp
R1: Buffer and oxidant solution; R2: Ehrlich's reagent solution; P: Plunger pump; G: Pressure gauge; A: Air damper; S: Sample injector; B: Boiling water bath at 100°C; C1: Mixing coil (1 mm id x 10 m); C2: Reaction coil (1 mm id x 15 m); C3: Back pressure coil (0.5 mm id x 4 m); D: Detector (1) Spectrophotometer, λ = 560 nm, (2) Flow cell, cell volume 20 µl, light pass 10 mm; W: Waste; Flow rate (both R1 and R2): 2.8 ml/min

Results and Discussion

The Application of KISO method to FIA

The reagent composition in the KISO method was applied to the double line FIA system. Both (R1) and (R2) solutions shown in Fig. 1 are prepared according to the KISO method. (R1) solution was the buffer and oxidant solution which contained methyl cellosolve, and (R2) solution was the Ehrlich's reagent solution which was alcoholic sulfuric acid solution. However, on the KISO method, the two solutions were not mixed, and on the FIA system at the original concentration, a precipitate was formed, which clotted in the coil (C2). While the precipitate was dissolved in water, it appeared because the ethanol in Ehrlich's reagent solution (R2) reduced the solubility of the salt. This unknown precipitate was then found to be potassium chloride. To prevent potassium chloride precipitating, the ethanol content was reduced, so DBA from Ehrlich's reagent solution, which was slightly soluble in water, was precipitated.

Study of the buffer solution

For these reasons, several buffer solution, were tested. For example, triethanolamine and triethanolamine-trifluoroacetic acid were found to decrease the stability of the base line. Ammonia buffer produced a chromogenic Schiff base by the reaction with DBA, which interferes with the absorbance at 560 nm. When the Schiff base was produced, chromogenic materials were observed to spray out of the pipe line. This phenomenon is due to the permeablilty of alcohol through the Teflon tubes under pressure (about 5 kg/cm^2). The above-mentioned results suggest that the requirements of the buffer solution are the following: (1) water soluble (2) ethanol soluble (3) not reactive with Ehrlich's reagent. In addition, the buffer should be resistant to the oxidation by Chloramine T. However, it is difficult to use one buffer solution satisfying all four requirements. To promote the

widespread use of this assay procedure, the optimum condition was found by changing the concentrations of borate buffer solution or of other reagents.

Study of the Ehrlich's reagent solution

Water tried as a solvent was not suitable because of the insolubility of DBA. Then, DBA was dissolved in water containing a definite amount of methyl cellosolve. To prevent a precipitating, the concentration of sulfric acid was increased and the concentrations of both buffer and DBA were decreased to a fifth part as much as are used in the KISO method. Then, when the concentration of sulfric acid was 1.5 times (10% (v/v)) that of the KISO method, (R1) and (R2) could flow without any precipitate forming. When the concentration of DBA increased beyond the designated percentage, it was deposited at the window of the flow cell and the stability of the base line was lost. This appeared as a drift of the base line.

Stabilizing the base line

Apart from this drift, a gradual ascent of the base line was observed. This rise happened because DBA's color was changed by the light and because chloramine T as oxidant was decomposed by the light. Therefore, when the reagent bottles were wrapped with aluminum foil, this rise stopped.

Study of the concentration of the oxidant

The influence of the concentration of the oxidant is shown in Fig. 2. When the concentration of the oxidant was decreased to a certain extent, the absorbance changes were increased. This was caused by decomposition of pyrrole with the excess oxidant. Besides, the slope became less steep with deficient oxidant. When both slope and linearity were considered, the concentration of the oxidant was chosen to be 1/80 (0.1408 g/l) for the KISO method.

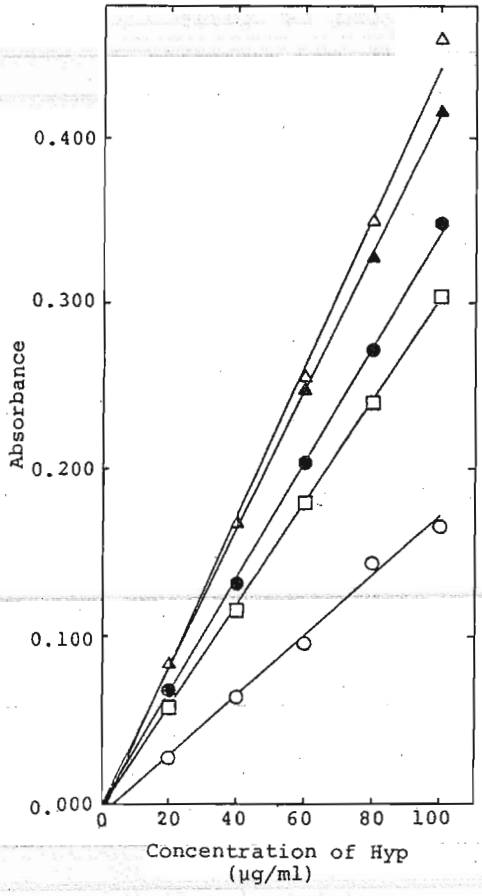

Fig. 2 Influence of the Concentration of Oxidant
○: 1/10, r = 0.9955 ●: 1/20, r = 0.9996
△: 1/40, r = 0.9985 ▲: 1/80, r = 0.9999
□: 1/160, r = 0.9999
where each ratio is to the KISO method

Study of the surfactant

When the samples were injected, the base line rose slightly. This is due to the chromogenic compound. As this rise was significant, Triton X-100 as non-ionic surfactant was added. Triron X-100 was added to (R1), (R1) and (R2), and (R2), respectively, and the influence of those solutions on the developed reaction was examined. (R1) and (R2) were prepared for the concentration of surfactant so as to give 0.04, 0.2, 0.4, 0.8 and 1.6% (v/v) ratios after mixing. Although at lower concentration it was impossible to prevent the base line from

rising, at 1.6% (v/v) which is about 110 times the critical miceller concentration, it was possible to prevent this rise completely. When the surfactant was added to (R2), the solubility of DBA increased significantly. Not only is a small amount of methyl cellosolve required, but also the preparation time of Ehrlich's reagent is shortened by 10 min.

A typical example of the calibration curve is shown in Fig. 3. A good linear relationship (r = 0.9997) was observed between the concentration of Hyp (from 0 to 80 μg/ml) and the absorbance. The gradient was large compared with that when no surfactant is used. The relative standard deviation was 1.05% (n = 24) with the 100 μg/ml standard sample, as shown in Table I. One sample per about 3.5 min could be injected.

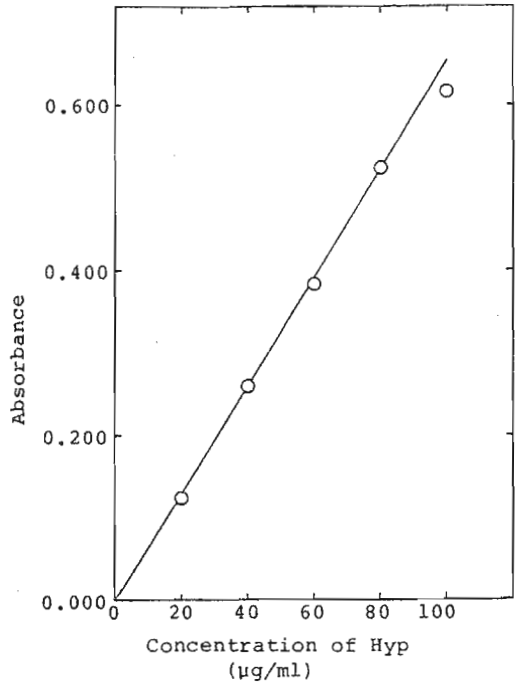

Fig. 3 Calibration Curve for Determination of Hyp

Table I Preparation of Standard Samples

	Concentration (μg/ml)		
No.	Hyp	Proline	Glycine
1	0	100	100
2	20	80	100
3	40	60	100
4	60	40	100
5	80	20	100
6	100	0	100

Conclusion

A more rapid and convenient microdetermination of Hyp was established by modification of the KISO method using FIA. In

this method, the processes of the extraction and dehydration with the column are not necessary, so the organic solvent used was methyl cellosolve only. The concentrations of the reagents were low compared with those in KISO method. More rapid analysis and more reduction of the reagents will become possible by the optimization of the conditions for FIA. Extensive studies of the medical applications of this method in microquantities of biological samples are now in progress and will be reported elsewhere.

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