Communications to the Editor

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A NEW CONVENIENT METHOD FOR MICRODETERMINATION OF HYDROXYPROLINE BY FLOW INJECTION ANALYSIS

Kazuhide Uchida,[a] Tetsuichi Shibata,[a] Masako Tomoda,[b] Shin-ichi Saito,[b] and Seiichi Inayama*,[a]

Pharmaceutical Institute, School of Medicine, Keio University,[a] 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan and General Science Laboratory, Faculty of Science and Engineering, Sophia University,[b] 7-1 Kioicho, Chiyoda-ku, Tokyo 102, Japan

A more rapid and convenient microdetermination of hydroxyproline (Hyp) was established by modification of the conventional KISO method using flow injection analysis. This new flow method is based on the color change reaction between pyrole, the oxidation and decarboxylation product of Hyp, and Ehrlich's reagent. A good linear relationship was obtained between the concentration of Hyp (from 0 to 80 μg/ml) and absorbance.

KEYWORDS —— microdetermination; hydroxyproline; collagen; connective tissue; KISO method; flow injection analysis

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 samples and are often hampered by several inconveniences, 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 superior batch method, the KISO method,[10,11] was established for the microdetermination of Hyp with good reproducibility. It has been used mainly in biochemical research on wound healing and 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 present communication concerns a new method based on flow injection analysis (FIA).[24,25] The term FIA was first used by Ruzicka and Hansen[26] to describe an analytical technique in which a discrete sample is injected into a continuously flowing carrier stream. FIA is now used as the prevailing batch method, since it is convenient and useful in the rapid and simple microdetermination of Hyp.

This FIA modification of the original KISO method is based on the color reaction of pyrrole, produced from Hyp by oxidation and subsequent decarboxylation, with Ehrlich's reagent. It has proved useful for the rapid and simple microdetermination

of the Hyp with spectrophotometric detection. A flow diagram is shown in Fig. 1. The hydrolysate solution (ca. 20 µg of Hyp in 0.5 - 0.75 ml) prepared from the tissue sample (ca. 0.5 mg)is injected with a sample injector[27] into a current of the buffer and oxidant solution. Following the oxidation, the sample is decarboxylated in a boiling water bath at 100°C. The buffer and oxidant solution with pyrrole from the sample passing through a mixing coil, is mixed in a 1 : 1 ratio with Ehrlich's reagent to develop the color. A colored product is formed in the reaction coil. Then the absorbance change at 560 nm is recorded as a function of the concentration of the Hyp. Both (R1) and (R2) solutions shown in Fig. 1 are prepared according to the KISO method.

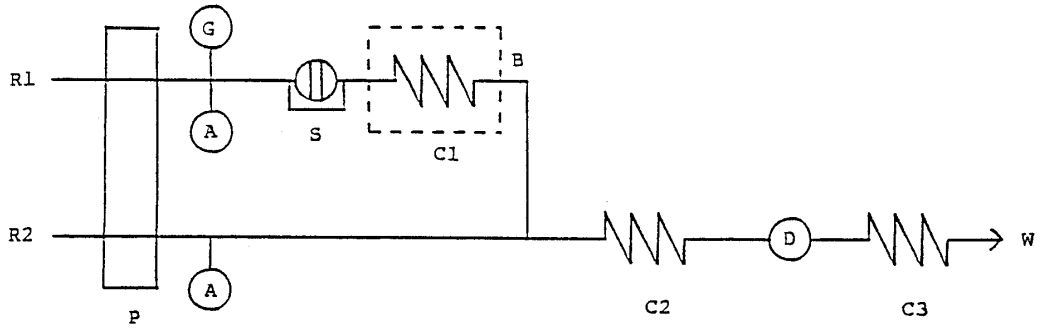

Fig. 1. A Flow Diagram for the Microdetermination of Hyp

R1: Buffer and oxidant solution; R2: Ehrlich's reagent solution; P: Plunger pump (Kyowaseimitsu KHU-W-294); G: Pressure gauge (Kyowaseimitsu KPH-50L); A: Air damper (Kyowaseimitsu KU-AIR 2); 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 (Nihonbunko UVIDEC-340), (2) Flow cell, cell volume 20 µl, light pass 10 mm (Nihonbunko FIC-361); W: Waste; Flow rate (both R1 and R2): 2.8 ml/min

Part of the potassium chloride in the buffer solution was precipitated and clotted in the coil (C2) due to reduction of its solubility by the addition of the ethanol in Ehrlich's reagent solution. But the ethanol content should not be reduced much because the p-dimethylaminobenzaldehyde (DBA) from Ehrlich's reagent solution is insoluble in water. For this reason, several buffer solutions were tested. For example, triethanolamine and triethylamine-trifluoroacetic acid were found to decrease the stability of the base line. Ammonia buffer produced a chromogenic Schiff base, which interferes with the absorbance at 560 nm.

When the Schiff base was produced, chromogenic material was observed as a spray out of the pipe line. This phenomenon is due to the permeablilty of alcohol in the Teflon tubes under pressure (about 5 Kg/cm^2). But we should not renounce use of Teflon tubes because Teflon is an easy-to-use semitranslucent material which allows acceptable processing and is acid-proof.

Next, water was tried as a solvent, but it was not good because of the insolubility of DBA. As a result of these experiments, the volume of concentrated sulfuric acid in Ehrlich's reagent was increased to 2.5 times (10% (v/v)) as much as is used in the KISO method, the concentration of DBA was set at 60% (w/w) (102.6 g/l),and buffer at 40% (w/w) (KCl 18.02 g/l, H_3BO_3 4.95 g/l, and KOH, pH 8.7). The organic solvent was ethylene glycol monomethyl ether (methyl cellosolve) only. 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.

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 sodium p-toluensulfonchloramide (chloramin T) as oxidant was decomposed by the light. Therefore, when the reagent bottles were wrapped with aluminum foil, this rise stopped.

The influence of the concentration of the oxidant is shown in Fig. 2; here, standard samples were prepared as shown in Table I. 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.

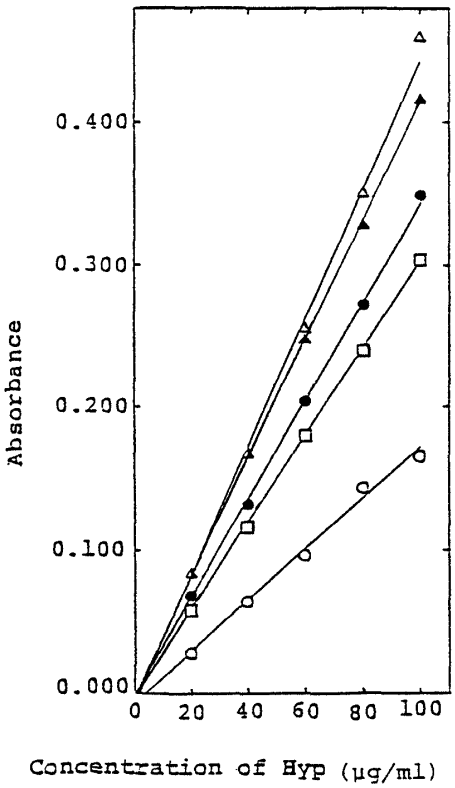

Fig. 2. Influence of the Concentration of Oxidant

○: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

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

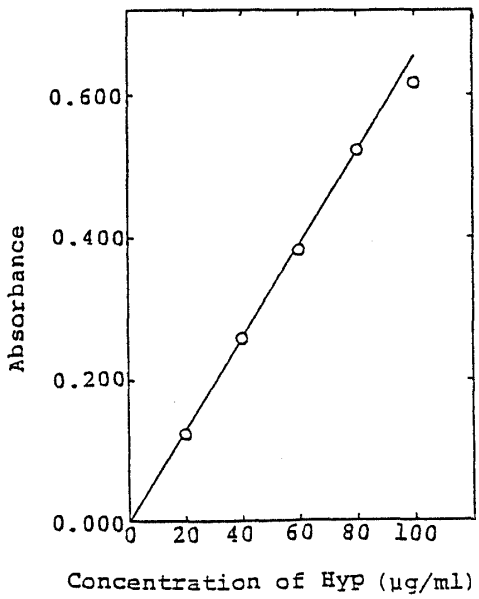

Fig. 3. Calibration Curve for Determination of Hyp

When samples were injected, the base line rose slightly. As this rise was significant, Triton X-100 as surfactant was added. The rise was suppressed with a concentration of Triron X-100 about 110 times the critical micellar concentration. When the surfactant is used, 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 (see Table I). The time required for one measurement was about 3.5 min.

Futher extensive studies of the medical applications of this new, rapid and convenient microdetermination of Hyp in microquantities of biological samples is now in progress and will be reported elsewhere soon.

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