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SPECIFIC ASSAY OF HYDROXYPROLINE BY GAS CHROMATOGRAPHY*

JOHN M. L. MEE
Department of Animal Sciences, University of Hawaii, Honolulu, Hawaii 96822 (U.S.A.)
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SUMMARY

A quantitative gas–liquid chromatographic (GLC) assay of hydroxyproline was developed. Sample analysis was readily achieved by adding blood serum or urine acid hydrolyzate into a micro-vial containing a known amount of an internal standard for the direct formation of N-trifluoroacetyl, *n*-butyl ester derivatives and GLC injection without laborious cleanup. Specificity (using the nitrogen detection system), sensitivity (detection in the nanogram range), and speed (less than 30 min) of the developed procedure can be used for the rapid determination of hydroxyproline in physiological fluids. An accelerated hydrolysis method (145° for 4 h) was evaluated for the determination of total urinary hydroxyproline. A low hydroxyproline yield resulting from incomplete hydrolysis of some segments of the collagen peptides present in the urine by the "overnight" hydrolysis process was demonstrated. Recovery of hydroxyproline added to various urine samples and analyzed after acid hydrolysis exceeded 90%.

INTRODUCTION

Hydroxyproline of animal origin is a characteristic amino acid of collagen. Collagen is a fibrous protein accounting for about 35 to 40% of the total body protein and is distributed in and around walls of all blood vessels and around cells. The excretion of hydroxyproline is closely associated with growth rate, malnutrition, collagen metabolism, specific disease, and carcinoid tumors[1–3].

Specific methods already described[4–9] for hydroxyproline assay involve the oxidation of the amino acid from acid hydrolysates of urine samples to pyrrole-2-carboxylic acid or pyrrole, followed by extraction, isolation, and pyrrole chromophore formation, which is then estimated colorimetrically. The analysis is lengthy and time-consuming. The difficulty lies in the control of oxidation and the subsequent color formation.

Liquid (ion-exchange) chromatographic techniques have been used to separate hydroxyproline from other components and then determine it by either pyrrole chromophore[10] or by ninhydrin reaction[11–16]. These methods, however, require sample pretreatment and cleanup and are generally time-consuming.

This paper describes a specific and convenient method, using a gas chromatograph equipped with a nitrogen detector, for assaying hydroxyproline in conjunction with amino acid analyses previously described[17–19].

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EXPERIMENTAL

The gas chromatograph used in this study was an MT-220 (Tracor, Austin, Texas, U.S.A.), four-column oven, equipped with a Coulson electrolytic conductivity detector. A 6 in. × 1/4 in. I.D. U-glass column packed with 0.325 w/w % EGA on 80–100 mesh AW HT Chromosorb G was used for the separation of hydroxyproline from other amino acids present in the sample mixture. N-Trifluoroacetyl amino acid *n*-butyl ester derivatives of the corresponding amino acids were prepared according to the conditions of Roach and Gehrke[20] and by means of the apparatus described by Mee and Brooks[21]. The GLC instrumental setting and parameters are shown in Table I.

The quantitative aspects of hydroxyproline analysis were checked by the procedure of relative yield or response factor. Ten μl of a 2.5 *mM* solution of hydroxyproline, amino acids, and internal standard (L-aminocaprylic acid) were introduced

TABLE I

GAS–LIQUID CHROMATOGRAPHIC CONDITIONS FOR THE SEPARATION OF AMINO ACIDS

Column	0.325 w/w% EGA on 80–100 mesh AW HT Chromosorb G *
Helium flow-rate, ml/min	
Carrier	60
Pyrolyzer	10
Hydrogen flow-rate in pyrolyzer, ml/min	50
Pyrolyzer temperature, °C	820
Initial temperature of column, °C	140
Programmed temperature, °C/min	5
Final temperature, °C	230
Inlet temperature, °C	210
Chart speed, in./min	0.5

*EGA = Ethylene glycol adipate; HT = heat-treated at 550° for 16 h.

TABLE II

DETERMINATION OF RELATIVE PEAK HEIGHT RATIO

Trial	*Peak height (chart unit)*		*Relative yield*,***
	Hydroxyproline	*Internal standard*	
1	13.6	14.0	0.97
2	6.0	6.0	1.00
3	13.2	13.0	1.02
4	14.0	14.5	0.97
5	16.4	16.5	0.99
Average			0.99 ± 0.01

* Equivalent to a 2.5 m*M* solution in 0.1 *N* HCl.

** Relative yield = (peak height hydroxyproline)/(peak height internal standard).

directly into a micro-vial for derivatization followed by GC injection. Five independent measurements were made and the relative peak height ratio was calculated as shown in Table II. The internal standard was chosen particularly because it is stable, foreign to the testing samples, and of close retention to hydroxyproline.

The serum samples (10–100 μl) obtained from chicken and swine blood were used for direct derivatization. One ml of 24-h urine volume and 1 ml of concentrated HCl were placed in a PTFE screw-capped tube (16×75 mm) sealed under nitrogen. The tubes were heated in an aluminum heating block at 100° for 22 h; then 0.1 ml of the acid hydrolysate was directly derivatized. A 3- to 5-μl aliquot of the final derivative mixture was used for injection into the gas chromatograph.

Initial studies, including the analyses of blanks and reagents, were tested prior to sample analysis. No extraneous peak nor interference was observed on the chromatogram. The region of relative retention time or temperature for internal standard was also carefully checked for interference, which might possibly be overlapping with the internal standard. The chromatograms indicated that no unknown peak would show at the retention of the internal standard.

An accelerated acid hydrolysis procedure using a higher temperature, 145°, for a shorter time, 2–4 h, was also investigated with urine samples to compare the results of an ordinary 100° overnight hydrolysis, *e.g.* 16 h.

Experiments on recovery of hydroxyproline from urine were designed by first selecting five urine samples of known levels of hydroxyproline determined by previous trials. Two levels of standard hydroxyproline, *e.g.* 1.64 and 3.28 μg per micro-vial basis, were chosen for addition. Relative yield ratio, weight (μg) per each vial, and mg% hydroxyproline were calculated. In turn, percent recovery of each urine hydrolysate fortified with hydroxyproline in the presence of other amino acids was also calculated.

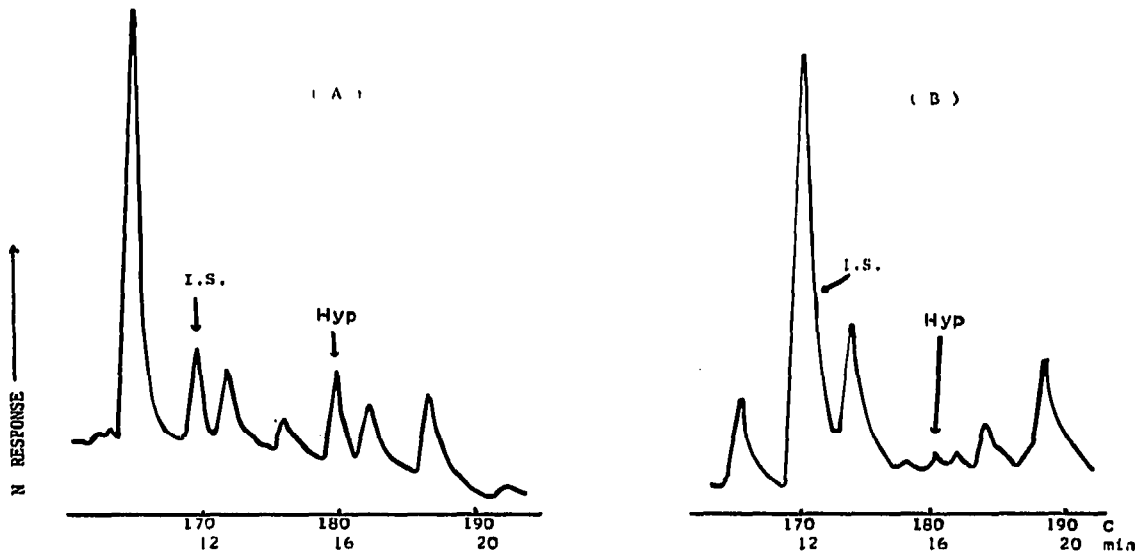

Fig. 1. Gas–liquid chromatograms showing the elution, the relative retention and the (hydroxyproline)/(internal standard) yield ratio in: (A) 1 μl of chicken serum and (B) 0.2 μl of swine serum. Hyp = hydroxyproline; I.S. = internal standard.

RESULTS AND DISCUSSION

Fig. 1 shows the gas–liquid chromatograms of separation of hydroxyproline from other protein amino acids in the serum on the EGA column. A complete run is less than 25 min. The amount of hydroxyproline and amino acids present in the serum was in the nanomole range. The quantitation of hydroxyproline can be readily achieved by measuring the response ratio (hydroxyproline)/(internal standard). This, in turn, can be calibrated against a mixture of hydroxyproline standard plus internal standard run according to Gehrke *et al.*[22]. An internal standard permits correction for volatization of solvent, dilution of final derivative mixture, and losses during sample manipulation. Quantitative analysis can be achieved with 25 ng of hydroxyproline.

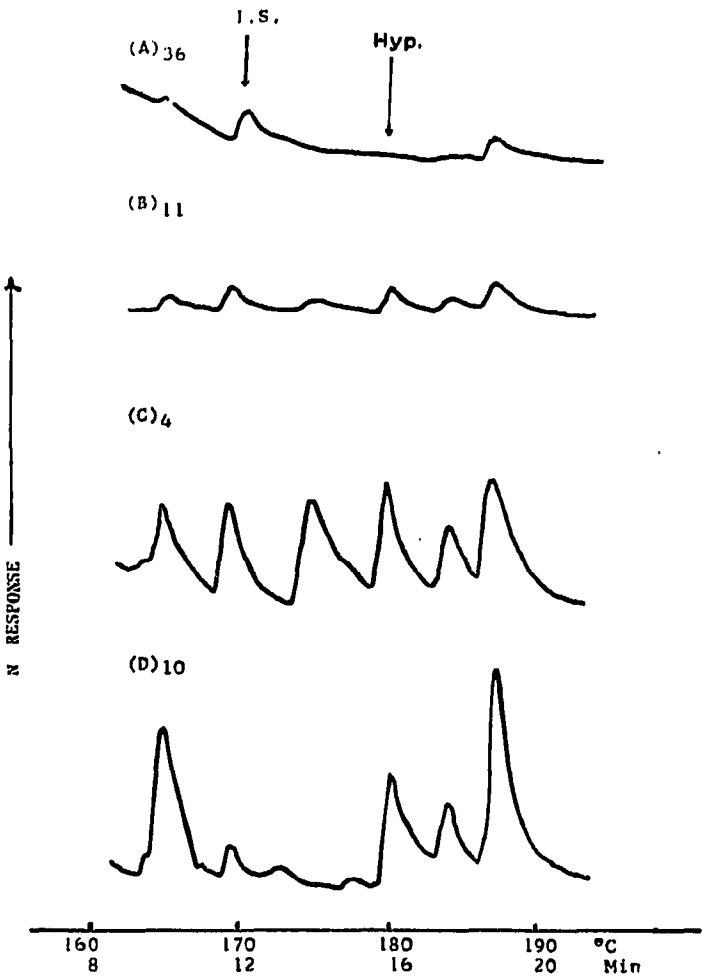

Fig. 2. Gas–liquid chromatograms showing the elution, the relative retention, and the (hydroxyproline)/(internal standard) yield ratio in acid-hydrolyzed urine of: (A) adult, (B) and (C) normal child, and (D) child suffering from bladder stone disease. The injected mixture contained *ca.* 2 μl of urine.

TABLE III

RELATIVE YIELD AND LEVELS OF HYDROXYPROLINE FOUND IN SOME HUMAN URINE

The urine was hydrolyzed in 6 *N* HCl at 100° for 22 h under nitrogen pressure.

Urine sample	*Relative yield**		*mg% of hydroxyproline***
AM (35)†	Trace		Trace
AS (40)	Trace		Trace
CH (11)	1.00	1.00	6.89
C (13)	0.72	0.75	4.85
CE (4)	1.12	1.10	7.80
C†† (10)	1.20	1.25	8.07
C†† (3)	1.79	1.75	11.61
C†† (9)	3.65	3.67	24.01

* Relative yield, Rp = (peak height hydroxyproline)/(peak height internal standard).

** mg% hydroxyproline $= \frac{Rp \times 3.28}{1 \times \text{sample}} \times 100$.

† Age in parentheses.

†† Child suffering from bladder stone disease.

TABLE IV

THE INFLUENCE OF ACID HYDROLYSIS ON THE AVERAGE HYDROXYPROLINE YIELD RATIO

The hydrolysis was carried out in 6 *N* HCl under nitrogen pressure.

Urine sample	*Average hydroxyproline yield ratio*			
	100°		*145°*	
	16 h (overnight)	*22 h*	*2 h*	*4 h*
U_M	0	0	0	0
U_M + hydroxyproline*	1.18	1.19	1.19	1.18
U_0	3.50	3.67	3.10	3.68
U_{13}	0.63	0.75	0.76	0.79

* 4.3 μg hydroxyproline per vial.

The relative yield by ratio (peak height hydroxyproline)/(peak height internal standard) is the basis for reproducibility and quantitation. Five independent measurements were made and the ratios were calculated (Table II). The average for five runs is 0.99 with a standard deviation of ± 0.01. These data reflect errors due to GLC analysis and storing of the N-trifluoroacetyl *n*-butyl esters. This precision is satisfactory for its applications. The high sensitivity and the speed of the GLC method provide a significant advantage over the ion-exchange and the wet chemistry methods.

Fig. 2 shows the gas–liquid chromatograms of urine analyses. These urine samples were from a normal adult, a normal child, and a child suffering from bladder

stone disease. The amount of sample injected represented 2 μl of urine. There was rarely any interference near 170°, where the internal standard was eluted. The urine analysis of one child having abnormally high levels of hydroxyproline was noted. The instrumental conditions and the amount injected were identical to Fig. 1.

The actual levels of hydroxyproline found in urine from adults, normal children and boys suffering from bladder stone disease are presented in Table III. The hydroxyproline content varies from a trace amount in urine from adults to as high as 24 mg per 100 ml of urine in children suffering from bladder stone disease. However, the severity of the bladder stone disease among these children was not known.

The process of acid hydrolysis is important to the actual amount of urinary hydroxyproline analyzed, as well as the kind of degraded collagen peptides present in the urine. The data in Table IV indicate that the classical overnight acid hydrolysis of 16 to 17 h gave an incomplete hydrolysis roughly equivalent to that of 145° for 2 h. Higher urinary hydroxyproline values were obtained at 100° for 22 h or 145° for 4 h. The authentic hydroxyproline added to the urine showed no effect upon hydrolysis. It is suggested that a longer time for 100° hydrolysis appears to be essential for higher hydroxyproline recovery from the collagen peptides in the urine.

The data in Table V show the recovery of hydroxyproline added to actual

TABLE V

RECOVERY OF HYDROXYPROLINE FROM URINE

The urine was hydrolyzed in 6 *N* HCl for 20 h under nitrogen pressure.

Urine hydrolysate	*Known level*		*Amount added per vial (μg)*†	*Amount found*		*% recovered*††
	*Rp**	*μg per vial***		*Rp**	*μg per vial***	
Tube No. 1						
M1	0	0	3.28	0.96	3.15	96
M2	0	0	1.64	0.49	1.61	98
Tube No. 2						
131	0.75	2.46	3.28	1.76	5.87	102
132	0.75	2.46	1.64	1.21	4.02	98
Tube No. 3						
101	1.25	4.10	3.28	2.20	7.22	98
102	1.25	4.10	1.64	1.69	5.53	96
Tube No. 4						
31	1.75	5.74	3.28	2.44	8.08	90
32	1.75	5.74	1.64	2.01	6.66	90
Tube No. 5						
91	3.67	12.04	3.28	4.44	14.58	95
92	3.67	12.04	1.64	3.76	12.34	90
Range						90–102

* *Rp* = (peak height hydroxyproline)/(peak height internal standard).

** $\frac{\text{Sample } Rp}{\text{Calibrated mixture } Rp} \times 3.28$.

† 10 ml of 2.5 m*M* calibrated mixture containing hydroxyproline and other common protein amino acids.

†† % recovered = (μg found per vial)/(μg known per vial + μg added per vial) × 100.

samples. Percent recovery was calculated on the μg per vial basis. Values of mg% hydroxyproline can be obtained by multiplication by 2. With the fortification of 3.3 to 5.6 mg% hydroxyproline (1.64 to 3.28 μg per vial) into a series of known urine samples ranging from 0 to 24 mg% hydroxyproline (0 to 12 μg per vial), the minimum recovery was above 90%. Percent recovery on all samples ranged from 90 to 102% This precision is considered satisfactory for many applications. The accuracy of the method should be comparable to this precision if the sample analyses are performed by other analysts.

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REFERENCES

1 N. B. Cabacungan, C. W. Miles, R. P. Abernathy and S. J. Ritchey, *Amer. J. Clin. Nutr.*, 26 (1973) 173.
2 D. J. Prockop and K. I. Kivirikko, in G. N. Rammachandran and B. S. Gould (Editors),Vol. II, Academic Press, New York, 1968, p. 215.
3 E. Wunsch, L. Stranch and G. Bries, *A Joint Meeting of the German, Swiss, and Austrian Societies for Clinical Chemistry in Vienna, November 1971.*
4 D. J. Prockop and S. Udenfriend, *Anal. Biochem.*, 1 (1960) 228.
5 K. I. Kivirikko, O. Laitinen and D. J. Prockop, *Anal. Biochem.*, 19 (1967) 249.
6 A. Dupont, *Clin. Chim. Acta*, 18 (1967) 59.
7 C. Dreux and J. Leymarie, *Ann. Biol. Clin.*, 26 (1968) 641.
8 A. L. Koevoet and J. D. Barrs, *Clin. Chim. Acta*, 25 (1969) 39.
9 G. Bondjers and S. Björkerud, *Anal. Biochem.*, 52 (1973) 496.
10 E. C. Le Roy, *Adv. Clin. Chem.*, 10 (1967) 213.
11 S. Moore and W. H. Stein, *J. Biol. Chem.*, 192 (1951) 663.
12 K. A. Piez, F. Irreverre and H. L. Wolff., *J. Biol. Chem.*, 223 (1956) 687.
13 S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, 30 (1958) 1185.
14 K. A. Piez and L. Morris, *Anal. Biochem.*, 1 (1960) 187.
15 P. B. Hamilton, *Anal. Chem.* 35 (1963) 2055.
16 P. X. Callahan, J. A. Shepard and S. Ellis, *Anal. Biochem.*, 49 (1972) 155.
17 J. M. L. Mee and C. C. Brooks, *J. Chromatogr.*, 62 (1971) 138.
18 J. M. L. Mee, *2nd Asian Congr. Nutr., Manila, Philippines, January 1973*, Abstr. No. IIIA, in press.
19 J. M. L. Mee and C. C. Brooks, *2nd Asian Congr. Nutr., Manila, Philippines, January 1973*, Abstr. No. II, in press.
20 D. Roach and C. W. Gehrke, *J. Chromatogr.*, 44 (1960) 269.
21 J. M. L. Mee and C. C. Brooks, *J. Chromatogr.*, 62 (1971) 141.
22 C. W. Gehrke, D. Roach, R. W. Zumwalt, D. L. Stalling and L. L. Wall, *Quantitative Gas–Liquid Chromatography of Amino Acids in Proteins and Biological Substances*, Anal. Biochem. Lab. Inc., Columbia, Mo., 1968.