Hydroxyproline determination in serum and gingival crevicular fluid

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A high-performance liquid chromatographic assay for total hydroxyproline in serum and gingival crevicular fluid has been developed. The C1q component of the first complement factor was precipitated out of the samples with a 0.02 M sodium acetate solution. Aliquots of the supernatants were dried, vapor hydrolyzed with 6 M hydrochloric acid at 105°C for 24 hours and derivatized with phenylisothiocyanate. The derivatives were chromatographed using a multilinear solvent gradient with detection at 254 nm. The regression of peak areas on the concentrations of hydroxyproline was linear with a correlation coefficient of 0.99 within the range of assayed quantities (2.5 to 25 ng). Analytical recovery was $90.5 \pm 2.54\%$ ($\bar{X} \pm SD$) and the determination level was 2.5 ng (19.1 pmol). The precision of the assay as determined by the coefficient of variation for five consecutive runs of six concentrations of hydroxyproline in serum hydrolysate ("within-run" precision) and five series run on different days ("between-run" precision) was $1.8 \pm 1.28\%$ and $4.2 \pm 2.59\%$ ($\bar{X} \pm SD$), respectively. Hydroxyproline concentrations in GCF from single sites during developing experimental gingivitis in the beagle dog showed an irregular pattern of low and high concentrations ranging from 5.2 to 17.4 ng/μl.

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Introduction

Inflammation and destruction of gingival connective tissue are early and continuing events in the periodontal disease process. Gingival crevicular fluid (GCF) is an inflammatory exudate of the dento-gingival vascular plexus (1, 2, 3). Due to the low compliance of the gingival tissues and the high hydraulic conductance of the crevicular epithelium, the inflammatory exudate carries most of the breakdown products resulting from the periodontal disease process out of the tissues and into the gingival crevice where they appear in the GCF (4). One logical bioassay for periodontal disease activity would be the detection and quantification of these products in GCF.

Collagens account for about 60% of the total proteins in normal healthy gingiva (5) and are also major constituents of the periodontal ligament and alveolar bone. In the healthy state, a high collagen turnover occurs in both gingiva (6) and periodontal ligament (7, 8) and is carried out by fibroblasts via phagocytosis and lysosomal degradation (9, 10). Enhanced production of enzymes capable of degrading collagen is associated with the onset and progression of periodontal disease (11–14) resulting in about a 70% loss of the collagen in the advancing front of the periodontal lesion (15).

It is generally accepted that degradation of collagen can be monitored by the release of hydroxyproline (Hyp) which makes up about 10% of this protein. Consequently, in active periodontal disease with increased degradation and a net loss of collagen in the periodontal connective tissue, the GCF would be expected to have increased concentrations of Hyp.

C1q (MW 409 600), a subunit of the first complement factor, contains 4.3% Hyp by weight (16). Systemic as well as local factors can alter its concentration in the tissues. It is seen coating bacteria collected from gingival crevices in patients with chronic periodontitis (17). Consequently, in order to accurately monitor increased degradation and loss of gingival collagen by determination of Hyp in GCF, it is imperative to remove any C1q from the samples. This can be done by precipitation of C1q with a 0.02 M sodium acetate buffer (18).

The first reasonably sensitive and reproducible procedure for determination of Hyp was described by Neuman & Logan (19). Because of the complexity of the biological mixtures in which Hyp is being determined, countless modifications or separate procedures have been developed to increase the sensitivity or to improve the specificity of the assay. A few attempts have been made

Table 1. Flow program

Time	Flow (ml/min)	%A[1]	% B[2]	Curve[3]
Initial conditions:	1.0	100	0	6
4.0	1.0	100	0	6
10.0	1.0	49	51	5
10.5	1.0	0	100	6
11.5	1.0	0	100	6
12.0	1.5	0	100	6
12.5	1.5	100	0	6
20.0	1.5	100	0	6
20.5	1.0	100	0	6
30.0	1.0	100	0	6
31.0	0.2	50	50	6

[1]A solvent: Sodium acetate (19 g), water (1000 ml) and triethylamine (0.5 ml) are mixed and titrated to pH 6.40 with glacial acetic acid. Mix 967 ml of this solution with 19.8 ml acetonitrile and 13.2 ml water.
[2]B Solvent: Acetonitrile:water (6:4 by vol.).
[3]Curve: As defined by Waters Associates: #6 (linear), #5 (convex). The final 11 min of the gradient bring the system into stand-by mode after unattended, overnight chromatography.

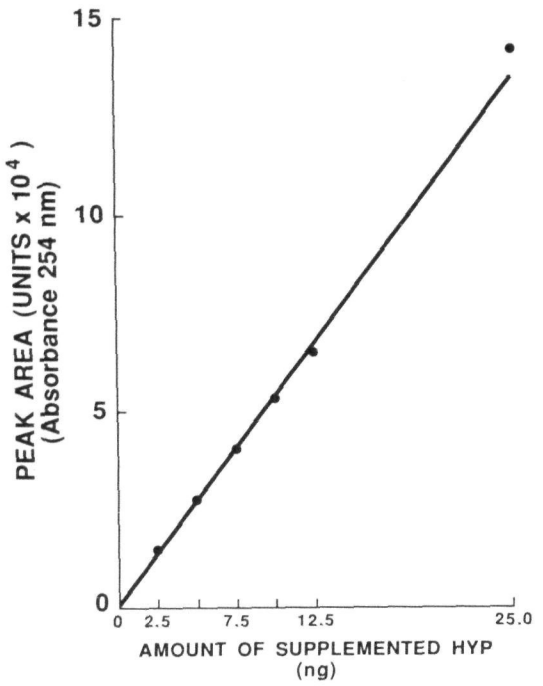

Fig. 1. Standard addition calibration curve ($y = -1914 + 5707x$; $r = 0.99$) for authentic *trans*-4-hydroxy-L-proline. Each point represents the mean of five consecutive determinations.

to determine Hyp in GCF. However, the procedures are not sufficiently sensitive and therefore require pooled samples rather than specimens taken from discrete sites (20), and do not take into account the effects of C1q (21, 22, 23).

This study presents a high-performance liquid chromatography assay for low nanogram quantities of collagen-derived Hyp in 1 to 2 μl volumes of GCF and serum.

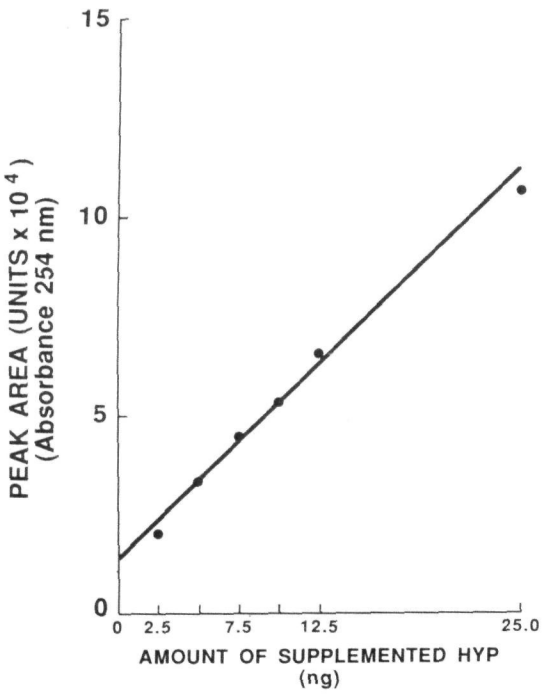

Fig. 2. Standard addition calibration curve ($y = 14438 + 3835x$; $r = 0.99$) for serum supplemented with *trans*-4-hydroxy-L-proline before hydrolysis. Each point represents the mean of five consecutive determinations.

Material and Methods

Apparatus

The high-performance liquid chromatography system (Waters Associates, Milford, MA) consisted of the Model 840 Data and Chromatography Control Station, two Model 510 solvent pumps, a Model 441 absorbance detector (fixed wavelength 254 nm; 10 mm flow cell) and a Model 710B Automatic Sample Processor. A Pico-Tag column was kept at a temperature of 38 ± 0.1°C by means of a Temperature Control System and the eluents were kept under a blanket of helium with an Eluent Stabilization System. A Savant Model SVC100H Speed Vac Concentrator (Savant, Farmingdale, NY) was used for desiccation to avoid splashing of the fluid samples and a Pico-Tag Work Station (Waters Assoc.) was used for vapor hydrolysis of the dried specimens.

Reagents

HPLC Grade acetonitrile, sodium acetate and methanol were from Fisher Scientific, (Livonia, MI) and glacial acetic acid was from Mallinckrodt (St. Louis, MO). Triethylamine (TEA), phenylisothiocyanate (PITC) and constant boiling hydrochloric acid were Sequanal Grade from Pierce (Rockford, IL). Pico-Tag Sample Diluent (0.005 M dibasic sodium phosphate titrated to pH 7.4 with 1 M phosphoric acid:acetonitrile; 19:1 by vol.) was obtained from Waters Associates. Stock solutions of *trans*-4-hydroxy-L-proline and *cis*-4-hydroxy-D-proline (Sigma Chemical Co., St. Louis, MO) were made up to a concentration of 1.25 mg/l, and 2 ml aliquots blanketed with argon were stored at −20°C until used. A 0.02 M sodium acetate buffer (Trihydrate, Grade 1, Sigma Chemical Co.), pH 5.5, was stored at 4°C until used.

Standard curves

Serum was obtained prior to each experiment by collecting blood from a beagle dog foreleg vein with serum separators (Microtainer, Becton Dickinson, Rutherford, NJ) that were left at 4°C for 60 min, centrifuged at $12700 \times g$ for 10 min and the serum retrieved.

Standard curves were generated for authentic Hyp (Matrix 1) and authentic Hyp in serum hydrolysate (Matrix 2a). Six concentrations of supplemented Hyp were made up by adding 17.5, 35.0, 52.5, 70.0, 87.5 and 175 ng of *trans*-4-hydroxy-L-proline to the samples tubes. In Matrix 1, the Hyp was pipetted into 6×50 mm borosilicate glass culture tubes (Corning Glass Works, Corning,

Table 2. Precision of assay for *trans*-4-hydroxy-L-proline expressed as the coefficient of variation (CV) of peak areas for quintuplicate samples of six concentrations of hydroxyproline

Matrix	Hyp conc.[1] (ng/μl)	Mean peak area (units $\times 10^4$)	SD	CV (%)
1[2]	2.5	13994	252	1.8
	5.0	28639	764	2.7
	7.5	40413	381	0.9
	10.0	53977	1121	2.1
	12.5	65301	236	0.4
	25.0	142850	813	0.6
2a[3]	2.5	20689	893	4.3
	5.0	33252	491	1.5
	7.5	44799	916	2.0
	10.0	53457	550	1.0
	12.5	65918	549	0.8
	25.0	108202	1567	1.4
2b[4]	2.5	6216	363	5.8
	5.0	11778	547	4.6
	7.5	14566	158	1.1
	10.0	20166	1631	8.1
	12.5	22752	767	3.4
	25.0	39029	761	1.9

[1] Concentration of supplemented *trans*-4-hydroxy-L-proline.
[2] Authentic *trans*-4-hydroxy-L-proline samples that were analyzed in five consecutive runs.
[3] Serum samples that were supplemented with *trans*-4-hydroxy-L-proline before hydrolysis, and assayed in five consecutive runs.
[4] Serum samples that were supplemented with *trans*-4-hydroxy-L-proline before hydrolysis, and assayed on different days.

NY) and the samples were brought to dryness. In Matrix 2a, the Hyp was added to 7 μl serum samples in micro sedimentation polypropylene tubes (Sarstedt, St. Louis, MO) and the samples were brought to dryness. Then 100 μl of 0.02 M sodium acetate were added to the tubes that were left overnight at 4°C for precipitation of C1q. After centrifugation at $12700 \times g$ for 15 s, 90 μl of the supernatants were brought to dryness in 6×50 mm glass culture tubes. Five consecutive series of these samples were processed and chromatographed on the same day for determination of "within-run" precision.

A second series of six concentrations of authentic Hyp in serum (Matrix 2b) was made up by adding 25, 50, 75, 100, 125 and 250 ng of *trans*-4-hydroxy-L-proline to 10 μl serum in micro sedimentation tubes and the samples were brought to dryness. Precipitation of C1q was done in 100 μl of 0.02 M sodium acetate, as described above, and 10 μl aliquots of the supernatants were brought to dryness in 6×50 mm glass culture tubes. Five series of these samples were processed and chromatographed on different days for determination of "between-run" precision.

Samples

Three beagle dogs were given weekly prophylaxes and had their teeth brushed every weekday until clinically healthy periodontal tissues were established, as determined by the color and consistency of the gingivae and the absence of GCF following gentle mechanical stimulation of the gingiva. Then dental plaque was allowed to form by refraining from all oral hygiene measures. Starting 1 wk later, samples were collected once per wk over a 5-wk period of developing gingivitis. The animals were fed no later than 24 h before sampling. GCF from mandibular premolars was collected with tared 2 μl capillary glass tubes (Drummond Scientific, Broomall, PA) after gentle mechanical stimulation of the gingiva. Again, the tubes were weighed on an analytical balance, after which the GCF samples were retrieved by flushing 60 μl of 0.02 M sodium acetate through the microcapillary tubes. After precipitation of C1q, as described above, 50 μl of the supernatants were brought to dryness in 6×50 mm glass culture tubes.

Hydrolysis

Up to 12 sample tubes were placed in a glass reaction vial with a resealable teflon closure (Waters Assoc.) and 200 μl of HCl were dispensed into the reaction vial, not the sample tubes. Oxygen was removed from the reaction vials by three cycles of alternate evacuation (20 s) and flushing with nitrogen (5 s) using the Pico-Tag Work Station. The reaction vials were sealed under vacuum at the end of the third cycle. The samples were hydrolyzed at 105°C for 24 h and then brought to dryness.

Derivatization

Twenty-five microliters of fresh redrying solution (methanol:water:TEA, 2:2:1 by vol.) were added to each sample which was vortexed and brought to dryness. Then 25 μl of fresh derivatization solution (methanol:water:TEA:PITC; 7:1:1:1 by vol.) were added to the samples that were vortexed, centrifuged for 20 s in the Speed Vac Concentrator, left at room temperature for 20 min and brought to dryness. Seventy microliters of Sample Diluent:methanol (4:1 by vol.) were added to the samples (35 μl to each of the "between-run" samples) that were vortexed for 10 s and then passed through Millex-HV4 filters into low volume inserts (Waters Assoc.).

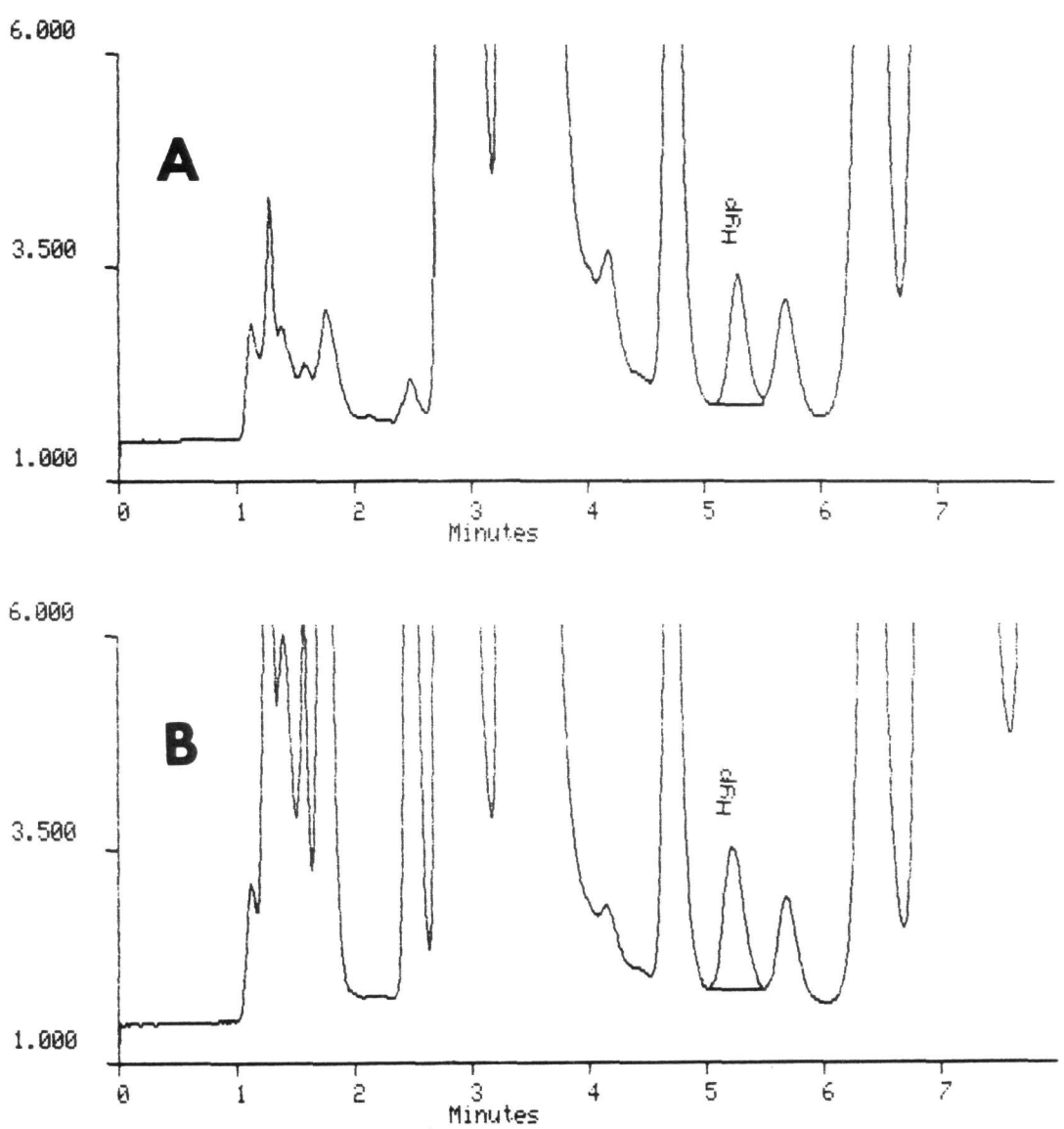

Fig. 3. Chromatograms showing hydroxyproline peak in *A*. Serum (3.2 ng) and *B*. Gingival crevicular fluid (4.1 ng). Retention time is 5.29 min. Attenuation: 0.005 absorbance units full scale.

Chromatographic conditions

Two eluents were used: A) Sodium acetate (19 g), water (1000 ml) and TEA (0.5 ml) were mixed and titrated to pH 6.40 with glacial acetic acid. Then, 19.8 ml acetonitrile and 13.2 ml water were added to 967 ml of this solution. B) Acetonitrile:water (6:4 by vol.). The flow program (Table 1) started isocratic with 1 ml/min of 100% A solvent for 4 min followed by a gradient washing step for 8.5 min and a 7.5 min re-equilibration phase. At 20 min the system was ready for the injection of 10 μl of the next sample. The final 11 min of the flow program brought the system into a stand-by mode after unattended, overnight chromatography. Before chrom-

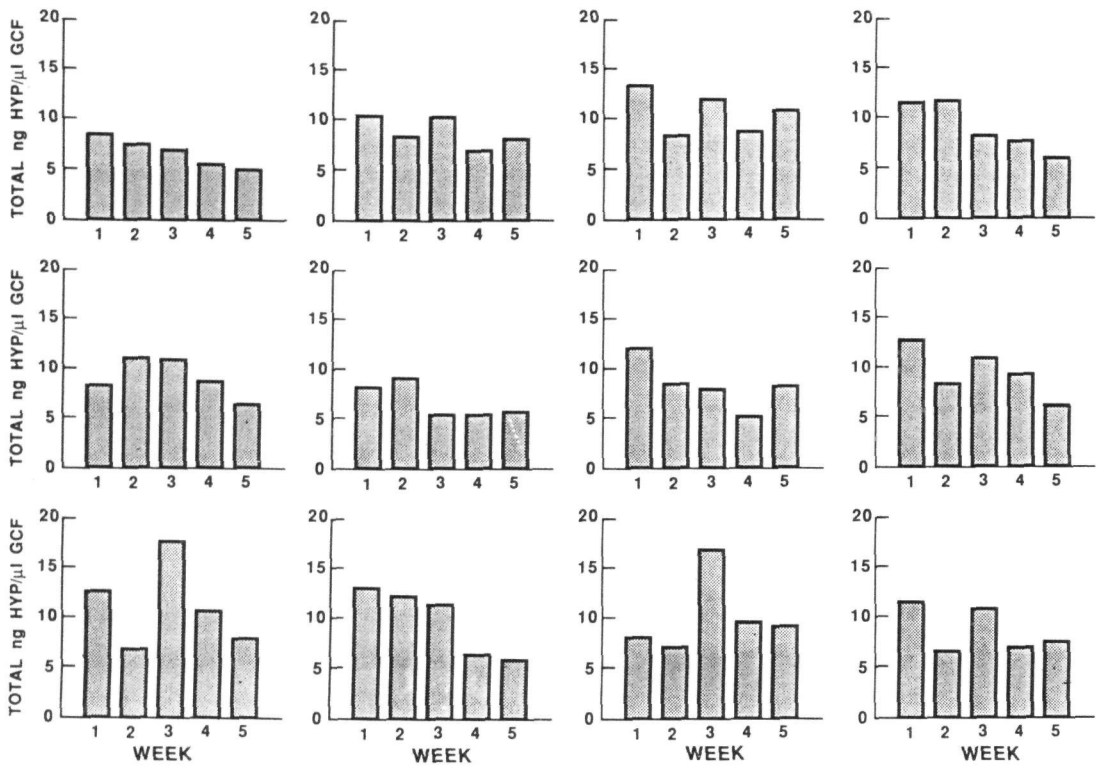

Fig. 4. Variations in total *trans*-4-hydroxy-L-proline in gingival crevicular fluid from 12 gingival crevices that were sampled 1 to 5 wk after initiation of experimental gingivitis.

atography of the first actual sample, the system was equilibrated by doing at least three blank runs. Absorbance was determined (254 nm) at a sampling rate of 4 points/s. To save hard disk storage space, only the first 8 min of each chromatogram were recorded for subsequent integration of the peak areas.

Analytical variables

Calibration curves were generated for quintuplicate samples using the standard-addition method. Peak areas were plotted against concentrations of supplemented Hyp (range 2.5 to 25.0 ng) for regression analysis. The curves for authentic Hyp and authentic Hyp in serum hydrolysate were compared by analysis of covariance. In each matrix, precision was determined for five samples of six different concentrations of supplemented Hyp by calculating the coefficient of variation (CV). Analytical recovery was determined by comparing the peak areas of six hydrolyzed aliquots of authentic *trans*-4-hydroxy-L-proline with the mean peak area of six non-hydrolyzed aliquots.

Results

Standard curves

The standard curves showed a linear correlation between peak areas (absorbance) and concentration of supplemented *trans*-4-hydroxy-L-proline (Figs. 1 and 2) with a correlation coefficient of 0.99. The regression equations (Matrix 1: $y = -1914 + 5707x$, Matrix 2a: $y = 14438 + 3835x$) showed that the calibration curve for Hyp standards in serum hydrolysate had a higher value for the y-intercept, accounted for by the Hyp content in serum, and a significantly ($p < 0.0001$) lower slope factor than the calibration curve for authentic Hyp.

Precision and analytical recovery

The precision of the assay was determined for all three matrices and, as shown in Table 2, the coefficient of variation for the six concentrations of Hyp ranged from 0.6 to 2.7% (Matrix 1), 0.8 to 4.3% (Matrix 2a) and 1.1 to 8.1% (Matrix 2b). The coefficient of variation for the slope factors of the five standard curves in Matrix 2b was 2.5%. The analytical recovery of the hydrolyzed *trans*-4-hydroxy-L-proline samples was $90.5 \pm 2.54\%$ ($\bar{X} \pm SD$) as compared with the non-hydrolyzed samples.

Clinical samples

The retention time for Hyp in the GCF samples was the same as for Hyp in Matrix 1, 2a and 2b (5.29 min) with good resolution of the Hyp peak (Fig. 3). One week after plaque was allowed to form (Fig. 4), GCF from 12 sites had a concentration of Hyp that ranged from 8.4 to 13.6 ng/μl (11.3 ± 2.15 ng/μl; $\bar{X} \pm SD$). Over the following 4-wk period, each site showed an irregular pattern of increases and decreases in Hyp concentration in the GCF that ranged from 5.2 to 17.4 ng/μl (9.0 ± 2.60 ng/μl; $\bar{X} \pm SD$).

Discussion

A sensitive and reproducible method for the determination of Hyp in GCF and serum has been established. It requires only 1 to 2 μl volumes of these specimens and has a determination level of about 2 ng of Hyp.

The slope factor of the calibration curve for serum hydrolysates supplemented with *trans*-4-hydroxy-L-proline prior to hydrolysis was found to be significantly lower than the slope factor of the calibration curve for authentic *trans*-4-hydroxy-L-proline ($p < 0.0001$). This might be explained by the demonstrated 9.5% loss of authentic *trans*-4-hydroxy-L-proline during hydrolysis which has a greater effect on serum samples with higher concentrations of supplemented authentic Hyp and consequently lowers the value of the slope factor. The loss is explained by the epimerization of approximately 10% of the *trans*-4-hydroxy-L-proline to *cis*-4-hydroxy-D-proline (24) that does not elute with the *trans* isomer.

In the five experiments performed on different days (Matrix 2b) the coefficient of variation for the slope factors of the standard curves was as low as 2.5% and the coefficient of variation for determination for the six concentrations of Hyp was only $4.2 \pm 2.59\%$ ($\bar{X} \pm SD$). Therefore, external standards were considered appropriate and manageable for the low level analyses of the small volume clinical samples, assuming that they would add the least measurement error to the assay.

There was no need for any sample clean-up other than precipitation of C1q despite the fact that the protein concentration in GCF can be as high as 93 μg/μl (25). However, to maintain established chromatographic conditions, 20 ml of methanol was pumped through the column (flow rate 0.2 ml/min; column temperature 38°C) after every 40–50 clinical samples which consistently eluted substantial amounts of contaminants that had been retained on the column.

When changing to a new Pico-Tag column, a slight modification of the A solvent was required to achieve the optimal retention time and resolution of the Hyp peak. This was accomplished by

an increase or decrease (± 2 ml) in the amount of acetonitrile that was added to the acetate buffer.

The clinical samples showed variations in Hyp concentration with differences between sites and within sites over time. High and low values appeared in an irregular pattern during the first 5 wk of experimental gingivitis which suggests that, during this period of collagen breakdown in the gingival connective tissue (26), the metabolism of this protein is an irregular rather than a linearly continuous event.

Leukocytes capable of releasing substantial amounts of hydrolytic enzymes accumulate in the gingival tissues during the onset and progression of periodontal disease (15). Collagenase is found at the advancing front of the periodontal lesion (27) and the collagenolytic activity increases with increasing severity and activity of the disease (28, 29). In addition to extracellular degradation of mature collagen, intracellular degradation of newly synthesized collagen occurs. The basal level of intracellular degradation of nascent collagen in healthy connective tissue is estimated to be $15 \pm 5\%$ and has minimal effect on collagen production (30). However, enhanced intracellular degradation above the baseline level occurs when underhydroxylated, non-helical collagen is synthesized (31) and when the fibroblasts are exposed to agents such as prostaglandin E_2 that increase intracellular levels of cAMP (32). In fact, the concentrations of prostaglandin E_2 and cAMP increase in the gingival tissues with increasing severity of the disease (33, 34, 35). In addition, collagen-poor, cell-rich gingival connective tissue incorporates more [G-^{3}H]-proline *in vitro* than does healthy tissue and a larger portion of the protein-bound activity is found in the culture medium (36) suggesting an increased synthesis of collagen in the diseased tissue. Since there is no accumulation of collagen, there is reason to believe that the reported increase in synthesis is counterbalanced by an enhanced intracellular degradation of nascent collagen.

Because of the high collagen turnover rate in gingiva, zero production of collagen due to enhanced intracellular degradation may contribute to loss of collagen in periodontal disease (6). Consequently, the amount of collagen-derived Hyp in GCF from this process, as well as extracellular degradation of mature collagen, may reflect collagen loss and disease activity in the periodontal disease process. Therefore, this sensitive and reproducible method for determination of Hyp in complex biological samples may serve as a useful, non-invasive assay for loss of collagen during progressive periodontal disease and may have the potential for assessment of periodontal disease activity.

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