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QUANTITATIVE ANALYSIS OF HYALURONIC ACID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF STREPTOMYCES HYALURONIDASE DIGESTS

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SUMMARY

The separation and quantitative analysis of streptomyces hyaluronidase (HAase) degradation products of hyaluronic acid (HA) by high-performance liquid chromatography are described. The substituted 4,5-unsaturated tetrasaccharide and hexasaccharide which result from the digestion of HA are quickly separated on a silica gel (Zorbax SIL) column. The assay of HA is linear between 5 and 250 μg of HA. This procedure is suitable for some weakly acidic glycosaminoglycan contaminants having similar properties to those of HA.

INTRODUCTION

Hyaluronic acid (HA) is widely distributed in the connective tissues of various organs, generally together with other glycosaminoglycans (GAGs). Therefore, conventional quantitative analysis of HA consists in determining the constituent uronic acid as a marker after chromatographic separation of HA from other GAGs. However, this method has the disadvantages of being unable to avoid the contamination from other GAGs and that it cannot give an accurate result when HA is a minor component or when some weakly acidic GAGs having similar properties to those of HA are part of the contaminants. In a previous paper[1], we reported on the conditions for the separation and quantitative analysis of ΔDi-OSH* derived from HA and ΔDi-OSs derived from chondroitin, by high-performance liquid chromatography (HPLC) after degradation of HA-containing GAGs with chondroitinase, according to the methods of Lee and co-workers[2–4], Knudsen *et al.*[5], Adams and Muir[6] and Kahle and Tesarik[7]. The three peaks of ΔDi-4S derived from chondroitin 4-sulphate, ΔDi-OSH and ΔDi-OSs were situated very close together, so that the separation and accurate quantification were difficult when these three peaks coexisted and their proportions were greatly different.

* Abbreviations used: ΔDi-OSH = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose; ΔDi-OSs = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose.

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The hyaluronoglucosidase from *Streptomyces hyalurolyticus* (E.C. 4.2.2.1) found by Ohya and Kaneko[8] is highly substrate-specific, acting only on HA and degrading into tetrasaccharide and hexasaccharide which have 4,5-unsaturated uronic acids as the non-reducing terminals. Therefore, the separation of HA from other GAGs, which was impossible with testicular hyaluronidase or with chondroitinase, is possible with this streptomyces hyaluronidase.

We have investigated the rapid quantitative analysis by HPLC of unsaturated tetrasaccharide and hexasaccharide derived from HA after the degradation of GAGs with streptomyces hyaluronidase. This procedure does not require the separation and purification of samples, making it suitable for the quantitative analysis of HA present in a minute amount of sample.

MATERIALS AND METHODS

Materials

Chondroitin 4-sulphate (C-4S) and chondroitin 6-sulphate (C-6S) were prepared from bovine nasal and shark cartilage. Hyaluronic acid (potassium salt obtained from human umbilical cord; Grade I) was purchased from Sigma (St. Louis, MO, U.S.A.). Chemical analyses of standard HA are given in Table I. Standard unsaturated disaccharides (ΔDi-OSs, ΔDi-4S and ΔDi-6S) and streptomyces hyaluronidase (HAase, E.C. 4.2.2.1) were purchased from Seikagaku Kogyo (Tokyo, Japan).

HPLC

The apparatus comprised a Shimadzu liquid chromatograph LC-3 with a SPD-2A ultraviolet detector (Shimadzu Seisakusho, Kyoto, Japan). A prepacked Zorbax SIL column (150 × 4.6 mm) was used.

Enzymatic digestion

The digestion mixture contained 100 μg of standard HA, 30 μl of HAase solution (100 turbidity reducing units of HAase in 100 μl of 0.02 *M* acetate buffer, pH 6.0). After incubation at 55°C for 7 h, four volumes of absolute ethanol were added and the mixture left overnight at 4°C. The clear supernatant obtained by centrifugation was dried under a stream of nitrogen, the residue dissolved in 50 μl of mobile phase buffer and 30 μl were employed for HPLC.

TABLE I

ANALYSIS OF HYALURONIC ACID OBTAINED FROM HUMAN UMBILICAL CORD (SIGMA, GRADE I)

	HA (mg/g)	*Method of determination*
Uronic acid	383.98	Dische[9]
Protein	32.21	Lowry *et al.*[11]
Neutral sugars	65.90	Winzler[12]

HPLC separation of enzymatic digests

The mobile phase comprised acetonitrile–methanol–0.5 *M* ammonium formate (10:6:3, v/v/v), pH 6.0. HPLC separation was carried out at a flow-rate of 0.3 ml/min, at room temperature. Ultraviolet (UV) (at 232 nm) absorption spectra were recorded; details are given with each figure. The UV positive fractions were pooled for uronic acid determination by using the method of Dische[9], with glucuronic acid as standard. The relationships between UV absorption (area = peak height × a.u.f.s. × 1/2 peak width) and amount of uronic acid (μg) were determined.

RESULTS AND DISCUSSION

Degradation of chondroitin and HA with streptomyces HAase

To confirm the resistance of chondroitin to the digestion with HAase, chondroitin, prepared from C-4S and C-6S by the method of Kantor and Schubert[10], and HA were digested with HAase and then subjected to HPLC. As shown in Fig. 1C, HA digested with HAase gave peaks A, I and II. The peaks A were negative to uronic

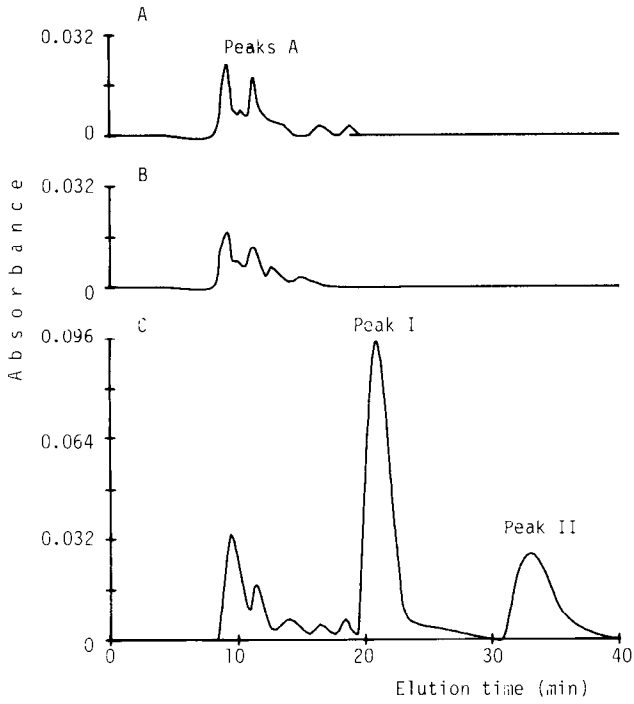

Fig. 1. HPLC of the degradation products of chondroitin and hyaluronic acid with streptomyces hyaluronidase. Fifty micrograms of substrate were dissolved in 50 μl of 0.02 *M* acetate buffer (pH 6.0). (A) Chondroitin derived from chondroitin 6-sulphate, digested with 60 TRU of enzyme in 50 μg of chondroitin at 55°C for 2 h. (B) Chondroitin derived from chondroitin 4-sulphate, digested with 60 TRU of enzyme in 50 μg of chondroitin at 55°C for 2 h. (C) Hyaluronic acid obtained from human umbilical cord, digested with 20 TRU of enzyme in 50 μg of HA at 55°C for 2 h. Peaks: I = 4,5-unsaturated tetrasaccharide; II = 4,5-unsaturated hexasaccharide. Solvent system: acetonitrile–methanol–0.5 *M* ammonium formate (pH 6.0) (10:6:3, v/v/v); Column: Zorbax SIL, 150 × 4.6 mm I.D. Flow-rate: 0.3 ml/min. Injected amount: 30 μg of the degradation products. UV detection at 232 nm, 0.32 a.u.f.s.

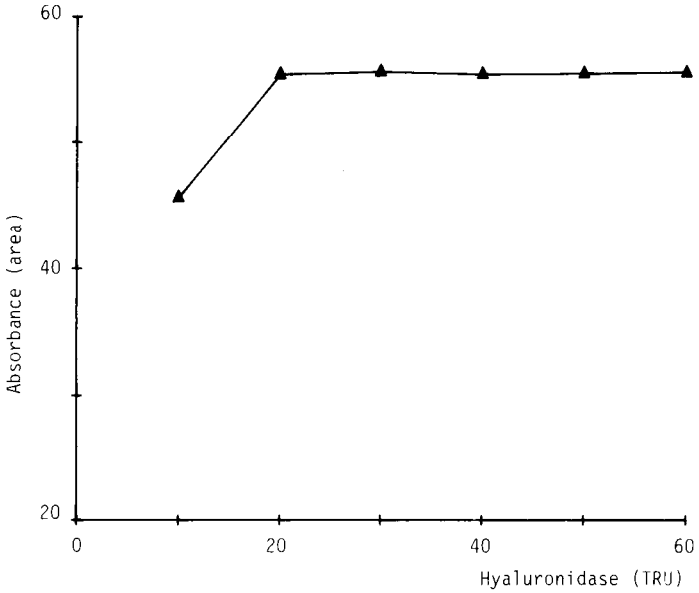

Fig. 2. Degradation of hyaluronic acid (100 μg) with various amounts of streptomyces hyaluronidase. Incubation was carried out by adding from 10 to 60 TRU of the enzyme to 100 μl of 0.02 *M* acetate buffer (pH 6.0) at 55°C for 2 h. Degradation products were assayed using the HPLC system in Fig. 1. Absorbance is expressed as the sum of the areas of peaks I and II. Peak area: a.u.f.s. × peak height × 1/2 peak width.

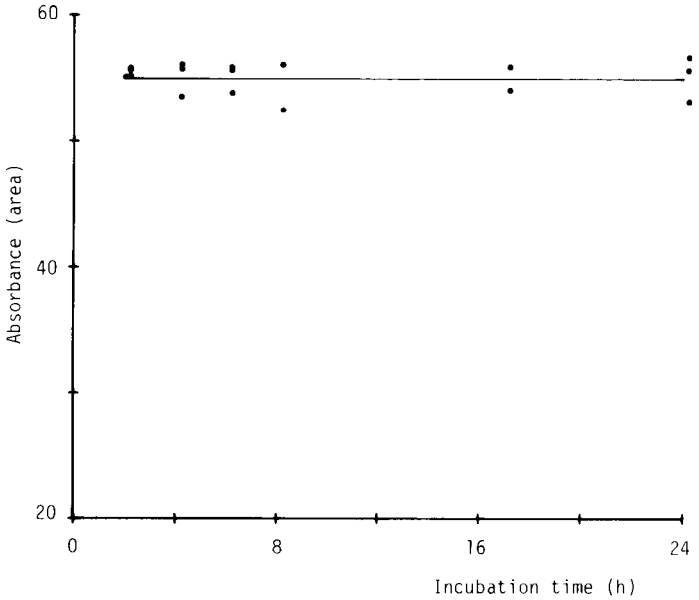

Fig. 3. The rate of enzymatic degradation of hyaluronic acid (100 μg). Incubation was carried out by adding 40 TRU of streptomyces hyaluronidase to 100 μl of 0.02 *M* acetate buffer (pH 6.0) at 55°C, for 2, 4, 6, 8, 17 and 24 h. Degradation products were assayed using the HPLC system in Fig. 1. Absorbance expressed as in Fig. 2.

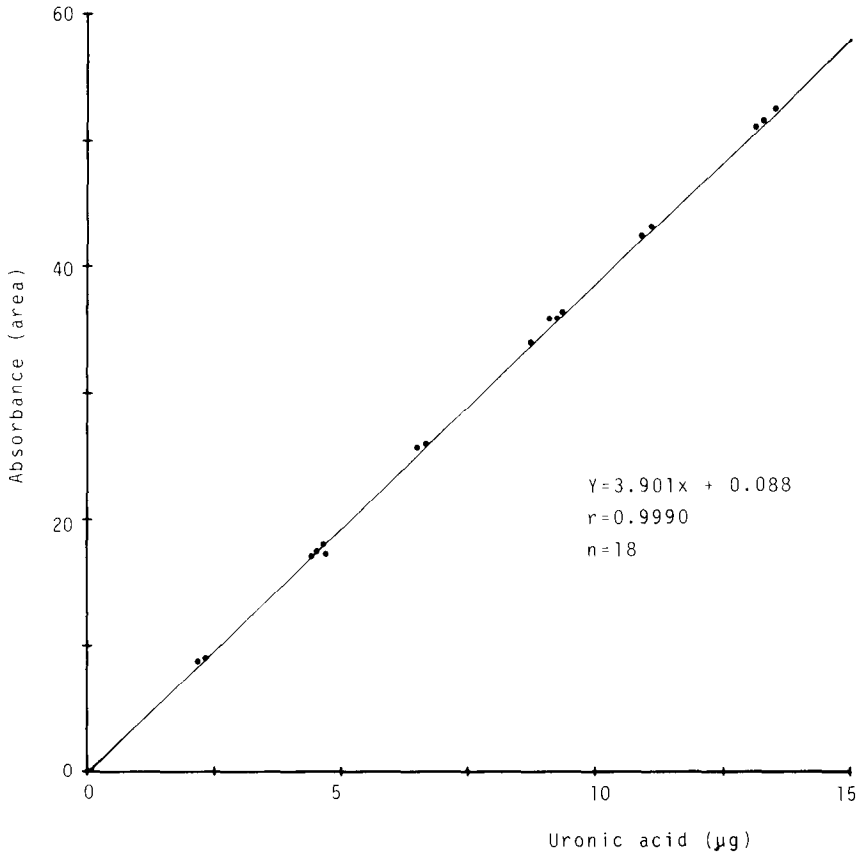

Fig. 4. Linear relationship between the UV absorbance obtained by HPLC and the chemically determined uronic acid content of standard ΔDi-OSs. Methods as in the text. Fractions showing UV absorption were pooled and analyzed for uronic acid content by the method of Dische. UV absorption expressed as in Fig. 2.

acid reaction whereas peaks I and II were positive; therefore determination was carried out only on the latter peaks. No peak was detected after digestion of chondroitin (Fig. 1A and B), which confirmed the resistance of chondroitin to digestion with HAase.

Optimal amount of enzyme for HA digestion

HPLC was performed with samples obtained by digestion of 100 μg of standard HA by HAase in amounts increasing by 10 turbidity reducing units (TRU) in the range from 10 to 60 TRU. The results shown in Fig. 2 confirmed that the recovery of the product was constant in the range 20–60 TRU and that $\geqslant$20 TRU of the enzyme were sufficient for degradation of 100 μg of HA.

Optimal degradation time with HAase

HPLC was performed with samples obtained by degradation of 100 μg of standard HA with 40 TRU of HAase at 55°C for 2, 4, 6, 8, 17 and 24 h. The results

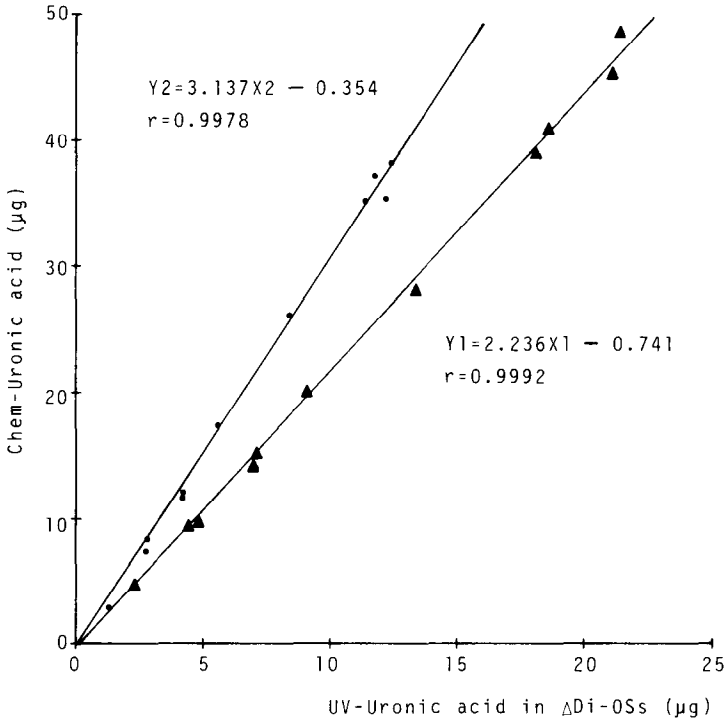

Fig. 5. Correlation between the uronic acid contents in the HA-degradation products determined from the UV absorbance by HPLC and by the chemical method of Dische. Methods are described in the text and Fig. 4. UV absorption values (area) of HA-degration products were converted into corresponding uronic acid contents in the standard ⊿Di-OSs by calculation. Compounds (see Fig. 1C): I (▲) = 4,5-unsaturated tetrasaccharide derived from HA with streptomyces hyaluronidase. II (●) = 4,5-unsaturated hexasaccharide derived from HA with streptomyces hyaluronidase.

shown in Fig. 3 suggested that the amount of the degradation products was constant from 2 to 24 h.

Correlation between UV absorbance and chemically determined ⊿Di-OSs

Samples of standard ⊿Di-OSs at various concentrations were subjected to HPLC to give the UV absorbances (areas), and the fractions showing UV absorption were pooled and analyzed for uronic acid content by the method of Dische. Eighteen samples of ⊿Di-OSs with different lot numbers were analyzed in this way and the results are summarized in Fig. 4. A high correlation (coefficient: r = 0.9990) was found between the UV absorbance and the chemically determined uronic acid content. Therefore, we conclude that the measurement of UV absorbance with HPLC enables the determination of the uronic acid content without any chemical analysis.

Correlation between UV absorbance and chemically determined HA-degraded products

The HA-degraded products were subjected to HPLC as described in Materials and Methods to give UV absorbances (areas), and the fractions showing UV absorption were pooled and analyzed for uronic acid content. The correlation between

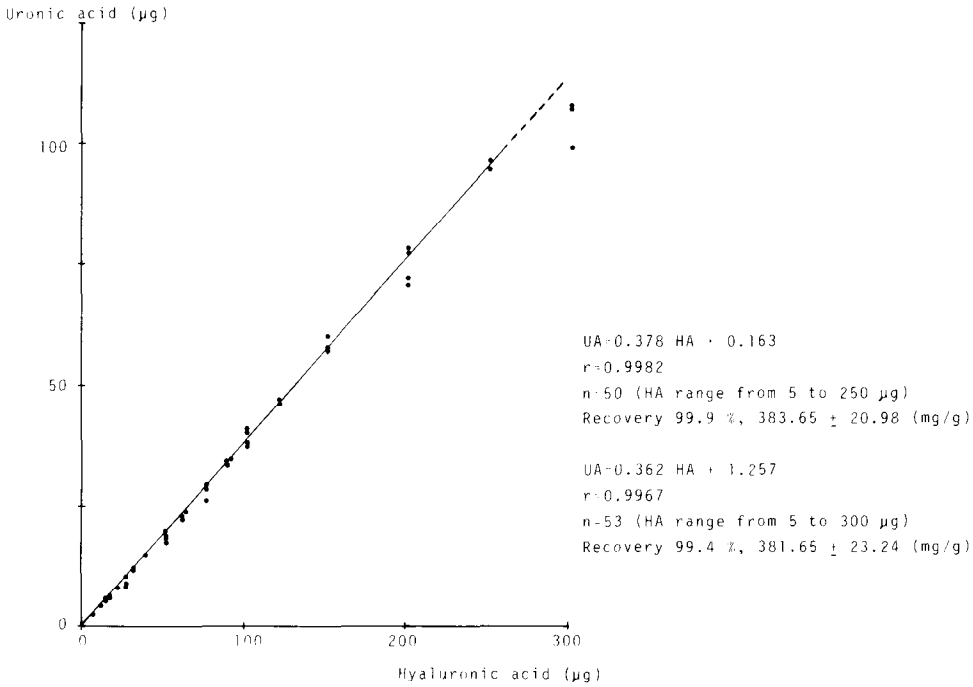

Fig. 6. Correlation between the content of uronic acid determined from the UV absorbance with HPLC and the amount of hyaluronic acid. HA in the range from 5 to 300 μg was digested with streptomyces hyaluronidase (0.3 TRU HAase per μg HA). Degradation products were assayed by HPLC. Uronic acid contents were calculated from UV absorbance using the equation in Fig. 5.

the amounts of ΔDi-OSs obtained from UV absorbance, X_1 and X_2, and the uronic acid contents in the recovered fractions determined by the method of Dische, Y_1 and Y_2, was examined. As shown in Fig. 5, for peak I, $Y_1 = 2.236\,X_1 - 0.741$, $r = 0.9992$, *i.e.*, the peak contained uronic acid about 2 times the amount of ΔDi-OSs per unit UV absorbance, and for peak II, $Y_2 = 3.137\,X_2 - 0.354$, $r = 0.9978$, *i.e.*, the peak contained uronic acid of about 3 times the amount of ΔDi-OSs per unit UV absorbance. These results confirmed that peak I comprised unsaturated tetrasaccharide and peak II of unsaturated hexasaccharide. The amount of uronic acid was then calculated by substituting the UV absorbance of peak I into equation for Y_1 and that of peak II into the equation for Y_2.

Recovery and accuracy of HPLC analysis of HA

The correlation between the content of uronic acid determined from the UV absorbance with HPLC and the amount of HA subjected to the analysis was examined for 53 samples containing 5–300 μg of standard HA (Fig. 6). Uronic acid contents determined with this method were transformed into uronic acid amounts per gram of HA, and then compared with the uronic acid amounts in the standard HA shown in Table I give the recovery. The average, with standard deviation (S.D.), of the uronic acid contents determined for the 53 samples was 381.65 ± 23.24 mg/g, with a recovery of 99.4%; the relationship between the uronic acid content (UA) and

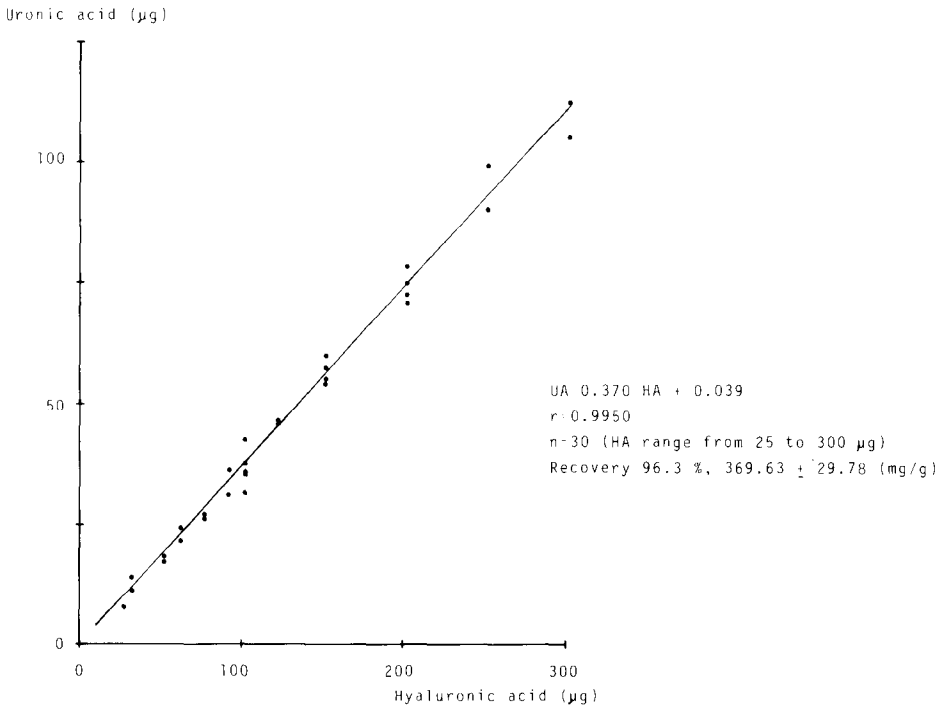

Fig. 7. Correlation between the content of uronic acid determined by chemical analysis and the amount of hyaluronic acid. HA in the range from 25 to 300 μg was digested with streptomyces hyaluronidase (0.3 TRU HAase per μg HA). Degradation products were assayed by HPLC. Uronic acid in the pooled fractions showing UV absorption was determined by the method of Dische (CUA).

the HA amount (HA) was UA = 0.362 HA + 1.257 with r = 0.9967. However, because at 300 μg of HA the measured value, *i.e.* the UA:HA ratio, tended to decrease, only the 50 samples containing 5–250 μg of HA were taken into account, showing that the average with S.D. was 383.65 ± 20.98 mg/g, the recovery 99.9% and UA = 0.378 HA + 0.163 with r = 0.9982. Thus, the recovery was higher with a lower S.D., and the regression curve was linear passing essentially through the origin, with a higher correlation coefficient.

The correlation between the content of uronic acid determined by the chemical method of Dische and the amount of HA subjected to the analysis is shown in Fig. 7. Because samples containing less than 25 μg of HA did not give accurate results with this method, only the values for samples containing $\geqslant$25 μg of HA are shown. The average with S.D. of the measured values for samples containing 25–300 μg of HA was 369.63 ± 29.78 mg/g, the recovery 96.3% and UA = 0.370 HA + 0.039 with r = 0.9950; the regression line passed essentially through the origin, but the recovery was slightly lower than that obtained from the UV absorption.

For the 30 samples in Figs. 6 and 7, the correlation between the uronic acid content determined from the UV absorbance (UV) and that determined chemically by the carbazole technique of Dische on the fractions showing UV absorption (CUA) was CUA = 1.008 UV −1.081 with r = 0.9974, suggesting essential equivalence of

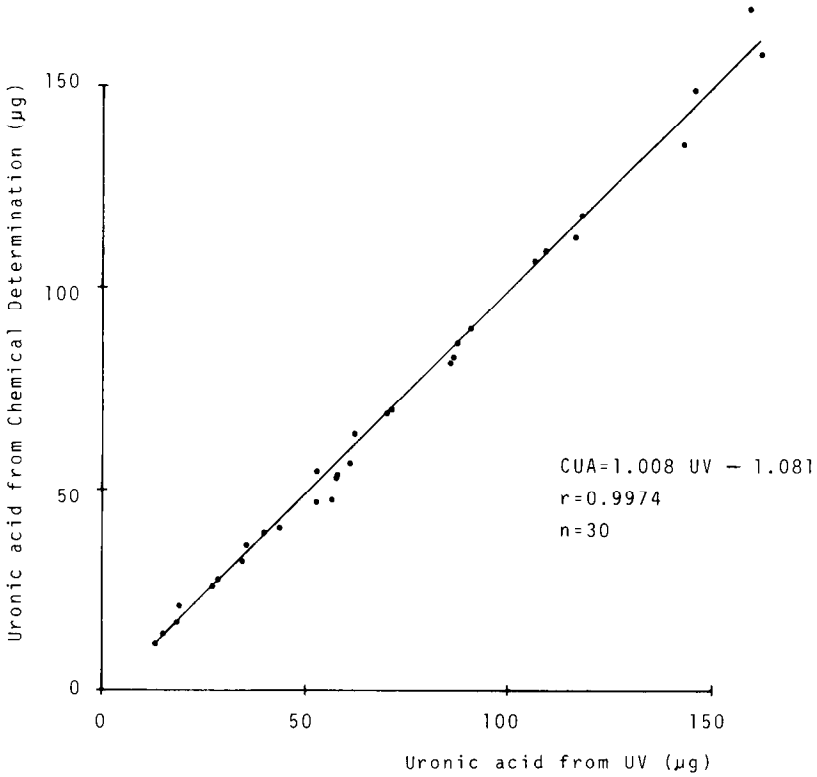

Fig. 8. Relationship for uronic acid between the UV absorption (UV) and the chemical analysis (CUA).

these methods (Fig. 8). We conclude that the measurement of UV absorbance can be utilized to determine uronic acid content and hence the HA content as well. The results in Fig. 6 indicate that when the uronic acid content was determined by measurement of UV absorbance with HPLC after digestion of HA with HAase, even as little as 5–25 μg of HA gave meaningful values but as much as 300 μg of HA resulted in a lower recovery. In contrast, when the uronic acid content was determined chemically after pooling the fractions showing UV absorption (Fig. 7), less than 25 μg of HA did not give accurate values, whereas amounts as much as 300 μg of HA resulted in good recovery.

These results indicate that even minute amounts of HA can be analyzed by the measurement of the UV absorbances of the unsaturated tetrasaccharide and hexasaccharide fractionated by HPLC after degradation of HA with streptomyces HAase, and if the fractions showing UV absorption are pooled and analyzed for the content of uronic acid, the range of analysis may be expanded to higher concentrations. It may be concluded that this method provides an easy, rapid, highly accurate and useful analysis of HA.

REFERENCES

1 I. Takazono and Y. Tanaka, *Kurume Univ. J.*, 30 (1981) 165.
2 G. J.-L. Lee, J. E. Evans and H. Tieckelmann, *J. Chromatogr.*, 146 (1978) 439.
3 G. J.-L. Lee and H. Tieckelmann, *Anal. Biochem.*, 94 (1979) 321.
4 G. J.-L. Lee and H. Tieckelmann, *J. Chromatogr.*, 222 (1981) 23.
5 P. J. Knudsen, P. B. Eriksen, M. Fenger and K. Florentz, *J. Chromatogr.*, 187 (1980) 373.
6 M. Adams and H. Muir, *Anal. Biochem.*, 109 (1980) 426.
7 V. Kahle and K. Tesařík, *J. Chromatogr.*, 191 (1980) 121.
8 T. Ohya and Y. Kaneko, *Biochim. Biophys. Acta*, 198 (1970) 607.
9 Z. Dische, *J. Biol. Chem.*, 167 (1947) 189.
10 T. G. Kantor and M. Schubert, *J. Amer. Chem. Soc.*, 79 (1957) 152.
11 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
12 R. J. Winzler, *Methods Biochem. Anal.*, 2 (1955) 279.