Journal of Chromatography, 206 (1981) 621–624
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,455

Note

High-performance liquid chromatographic determination of dityrosine in biological samples

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(Recieved October 23rd, 1980)

One of the protein-bound fluorescent compounds, 3,3′-bityrosine (dityrosine), was first found in resilin, a rubber-like protein in insects[1]. Dityrosine has been found in the hydrolysates of elastin from aortas of 12-day-old chick embryos[2], tussah silk fibroin and keratin[3], insoluble collagen from skin of an 18-year-old cow[4] and elastoidin[5]. Recently, it was observed that dityrosine was present in the hydrolysate of insoluble protein from human cataractous lenses[6]. In these studies, dityrosine was preparatively separated by means of ion-exchange or gel chromatography, including high-performance liquid chromatography (HPLC). This paper describes a sensitive and precise determination of dityrosine in the hydrolysate of biological samples by HPLC with fluorescence detection. Cow Achilles tendon was employed as a model biological sample to establish the assay procedure.

EXPERIMENTAL

Materials and reagents

All solvents and chemicals were of analytical-reagent grade. Dityrosine was a gift from Dr. Mineo Masuda. Cow (2-year-old) hooves were obtained from the slaughter house at Fukuoka, Japan.

Dowex 1-X8 (CH_3COO^-) column. Dowex 1-X8 (Cl^-) (200–400 mesh; 50 ml) was converted into the OH^- form in the usual manner, and then converted into the CH_3COO^- form by use of 1.0 *M* acetic acid (pH 3.2, 150 ml), and stored in a refrigerator (4°C). When required for use, the resin was suspended in 0.1 *M* acetate buffer (pH 3.2) and poured on to a glass tube (350 × 6 mm I.D.) to give a height of 18 cm after settling.

Column for HPLC. A stainless-steel tube (500 × 2.1 mm I.D.) was packed by the slurry technique; to 10 ml of methanol, 1.6 g of Hitachi Gel 3011-N (12 μm; anion exchanger, $-N^+R_3$ type; Hitachi, Tokyo, Japan) and 10 ml of water were added. The suspension was allowed to stand for *ca.* 30 min, than the supernatant was discarded. After the slurry had been poured into the packing reservoir, aqueous methanol (50%, v/v) was made to flow through the column at 150 kg/cm^2 for 30 min, by using a Hitachi 635A high-performance liquid chromatograph. Then the mobile phase (0.1 *M* acetate buffer, pH 3.2) was made to flow at 0.22 ml/min for 30 min. The column thus packed can be used for more than 50 injections with only a small decrease in the theoretical plate number.

0021-9673/81/0000–0000/$02.50

Apparatus

A Mitsumi liquid chromatograph equipped with a 7120 syringe-loading sample injector and a Hitachi 203 spectrofluorimeter fitted with a Hitachi flow-cell unit (cell volume, 18 μl) operated at an emission wavelength of 404 nm with an excitation wavelength of 287 nm was used. The column temperature was ambient (20–23°C) and the flow-rate of the mobile phase was 0.22 ml/min. Fluorescence spectra of the column effluents were measured with a Hitachi MPF-4 spectrofluorimeter in 10 × 10 mm cells. The slit widths in the exciter and the analyser in terms of wavelengths were set at 2 and 10 nm, respectively. The specta were uncorrected.

Procedure

Cow Achilles tendon (wet weight, 35 g) was dissected free from surrounding tissues, cut into small pieces and minced in a mortar with a pestle. The tissue was washed with 500 ml of 0.01 M Tris–hydrochloric acid buffer (pH 7.4) and then with 200 ml of *n*-hexane (three times in 18 h) with vigorous shaking. The residual tissue was dried *in vacuo*, homogenized in 200 ml of the Tris–hydrochloric acid buffer containing 0.15 *M* sodium chloride and centrifuged at *ca.* 15,000 *g* for 20 min to remove sodium chloride-soluble collagen. These homogenization and centrifugation steps were repeated four times. The precipitate was washed with 1000 ml of 0.5 *M* acetic acid to remove acid-soluble collagen and then lyophilized. Insoluble collagen (*ca.* 4 g) was obtained.

The collagen (1.0 g) was suspended in 25 ml of 6 *M* hydrochloric acid and heated in a screw-capped culture tube under a nitrogen atmosphere at 110°C in an oil-bath for 20 h, then the hydrochloric acid was removed *in vacuo* at 40°C. To the resulting viscous brown syrup water was added to give a total volume of 1.3 ml. The solution (0.2 ml) was poured on to the Dowex 1-X8 column and eluted with 0.1 *M* acetate buffer (pH 3.2). The initial eluate (4.5 ml) was discarded, then dityrosine was eluted with 4.0 ml of the buffer. The eluate was concentrated *in vacuo* at 40°C, followed by dilution of the concentrate with water to 0.6 ml. An aliquot (10 μl) of the resulting solution was injected on to the chromatograph. The amount of dityrosine was calibrated by means of the standard additions method: 0.1 ml of dityrosine standard solutions (10–40 nmol/ml) were added to 25 ml of the insoluble collagen suspension in 6 *M* hydrochloric acid and the same procedure was carried out.

RESULTS AND DISCUSSION

When the insoluble collagen hydrolysate was subjected directly to HPLC,the peak of dityrosine was overlapped by a peak of an unknown fluorescent compound. This interfering compound could be removed by chromatography of the hydrolysate on the Dowex 1-X8 column with 0.1 *M* acetate buffer (pH 3.2) as eluting solvent.

Fig. 1 shows a chromatogram of the hydrolysate free from the interfering compound. The retention time of dityrosine was 7.5 min. The fluorescence excitation and emission spectra of the component of peak 3 in the chromatogram were identical with those of authentic dityrosine at pH 3.2 and 10.0, and were different from those of the components of peaks 1, 2 and 4. These excitation and emission maxima are listed in Table I.

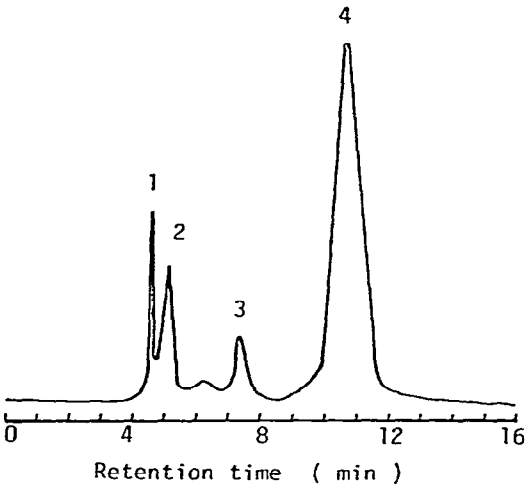

Fig. 1. Chromatogram of the hydrolysate of insoluble collagen from cow Achilles tendon. Peaks: 3 = dityrosine; 1, 2 and 4 = unidentified fluorescent compounds.

TABLE I

FLUORESCENCE EXCITATION AND EMISSION MAXIMA OF THE COMPONENTS OF PEAKS 1–4 IN THE CHROMATOGRAM IN FIG. 1 DISSOLVED IN BUFFERS OF pH 3.2 AND 10.0

Peak No.	*Buffer**	*pH*	*Excitation maximum (nm)*	*Emission maximum (nm)*
1	A	3.2	307	370
	B	10.0	290	365
2	A	3.2	270	395
	B	10.0	313	370
3	A	3.2	287	404
	B	10.0	320	404
4	A	3.2	375	430
	B	10.0	322	410

* A, 0.1 *M* acetate buffer; B, 0.1 *M* borate buffer.

The dityrosine content determined in insoluble collagen from an Achilles tendon was 9.1 nmol per gram of dry collagen (*ca.* 1 nmol per gram of wet tendon). The recovery of dityrosine was checked by adding known amounts of dityrosine (10, 20 and 40 nmol) to 1.0 g of dry collagen. The recoveries were 98 $\pm$ 3% (mean $\pm$ standard deviation). The lower limit of determination for dityrosine was 70 pmol/ml in the sample solution for HPLC.

This study has provided the first HPLC method for the sensitive determination of dityrosine. The presence of dityrosine in insoluble collagen from cow Achilles tendon was first observed by this method. This method has been applied to skins and tendons from cow and rat, and crayfish shell, and should be useful for the determination of dityrosine in various tissues.

ACKNOWLEDGEMENTS

We thank Dr. Mineo Masuda of the Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan) for his generous gift of dityrosine and Mrs. Chieko Nakano of the Nakano Pharmacy (Sue, Fukuoka, Japan) for financial support.

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