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Short Communication

High-performance liquid chromatographic separation of aprotinin-like inhibitors and their determination in very small amounts

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method for the determination and quantitative recovery of fully active aprotinin (the basic pancreatic trypsin inhibitor or Kunitz inhibitor) and aprotinin-like inhibitors in amounts down to 0.5 μg is reported. The method, which allows separation of aprotinin isoinhibitors characterized by small differences in the primary structure with respect to aprotinin itself, appears to be suitable for the quantitation and identification of aprotinin-like inhibitors in human biological fluids, in which they appear to be present at very low levels.

INTRODUCTION

Aprotinin, also known as the basic pancreatic trypsin inhibitor (BPTI) or Kunitz inhibitor, is a polypeptide of 58 amino acid residues which shows a broad specificity towards many serine proteases [1]. This miniprotein, as well as three isoforms that are characterized by small differences in the primary structure with respect to aprotinin itself [2–4], is present in virtually all bovine organs. Although the function of these molecules is still obscure, a therapeutic application has been proposed, for aprotinin, in diseases connected with hyperactivity of proteolytic enzymes (acute pancreatitis, hemorrhagic shock, multiple trauma, etc.) [1]. In order to estimate aprotinin in biological fluids, many assays, prevalently of the immunological type, have been proposed [5–9]. Recently, Raspi *et al.* [10], reported a reversed-phase high-performance liquid chromatographic

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(RP-HPLC) method suitable for the direct determination of aprotinin in body fluids. Our interest is the search for aprotinin-like inhibitors in humans, where they appear to be present in small amounts [11,12], and this paper describes a method that allows the separation of aprotinin isoinhibitors and their quantitative recovery (down to 0.5 μg) in body fluids.

EXPERIMENTAL

Chemicals and reagents

Aprotinin was a gift from Bayer (Wuppertal, Germany). Aprotinin isoinhibitors were purified from bovine spleen according to a procedure previously reported [2]. Bovine trypsin, porcine pancreas kallikrein, and the synthetic substrate BAPNA (N-α-benzoyl-DL-arginine-*p*-nitroanilide) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and trifluoroacetic acid were obtained from Baker (Deventer, Netherlands). All solutions were prepared using deionized water further distilled in an Aquatron distiller (Bibby Sterilin, Stone, UK).

Concentration of the inhibitors

Stock solutions of the inhibitors were prepared from lyophilized material; their concentrations were determined by titration with an aprotinin standardized trypsin solution, as previously reported [11].

High-performance liquid chromatography

The HPLC analyses were performed with a Perkin-Elmer Series 10 apparatus, equipped with an injecton valve (loop capacity 20 μl) and a diode-array UV–VIS detector (LC 135). The data were analysed with Omega 2 software. The analyses were performed with a reversed-phase column Supelcosil LC 18-DB (25 × 4.6 cm I.D., 5 μm particle size) equipped with a Supel-Guard LC 18-DB (2.5 × 4.6 cm I.D.) guard column, both obtained from Supelco (Bellefonte, PA, USA). The column was equilibrated in 0.1% trifluoroacetic acid (TFA) in water (solvent A) and eluted with a gradient obtained by mixing solvent A with a solution of acetonitrile–2-propanol (4:1, v/v) in 0.1% TFA (solvent B) at a flow-rate of 1 ml/min. The commercial aprotinin, or the samples of bovine inhibitors, to be injected were prepared from stock solutions by dilution with solvent A just before the use. The absorbance of the eluted fractions was recorded at 280 nm.

Antiproteolytic activity

The presence and the concentration of aprotinin, and aprotinin-like inhibitors, in the HPLC eluates was determined by measuring their antitryptic activity as previously reported [2]. The presence of organic solvents, which affects the trypsin activity, was taken into account by adding, in the reference test, an eluate volume from a run performed without the inhibitor.

RESULTS AND DISCUSSION

Under the elution conditions described above, aprotinin has a retention time of 38 min, as can be seen in Fig. 1, which is a typical chromatogram of a commercial sample of aprotinin. Fig. 2 shows a calibration curve obtained by injection of scaling amounts of commercial aprotinin, and of a sample of spleen inhibitor IV (which is identical with aprotinin) purified by fast protein liquid chromatography (FPLC). The eluted inhibitor maintains, in both cases, the antitryptic activity, determined as previously reported [2]. HPLC analyses were performed in triplicate and over a wide range of analyte concentration (0–15 μg). The mean value for the recovery of the inhibitor, as derived from the slope of the calibration curve in Fig. 2, was 95 ± 3%. The intra-assay precision and the inter-assay precision, calculated as a mean coefficient of variation, were 3.3% and 4%, respectively. The standard error of a point has a value of 0.15 μg, and is in agreement with the lower limit for inhibitory detectability allowed by the enzymic assay we used (see Experimental). Calibration curves were also established with the other three bovine spleen aprotinin isoinhibitors (inhibitors I, II and III), prepurified by FPLC–ion-exchange chromatography, and the results obtained for their recovery were superimposable on those obtained with BPTI (see also Table I).

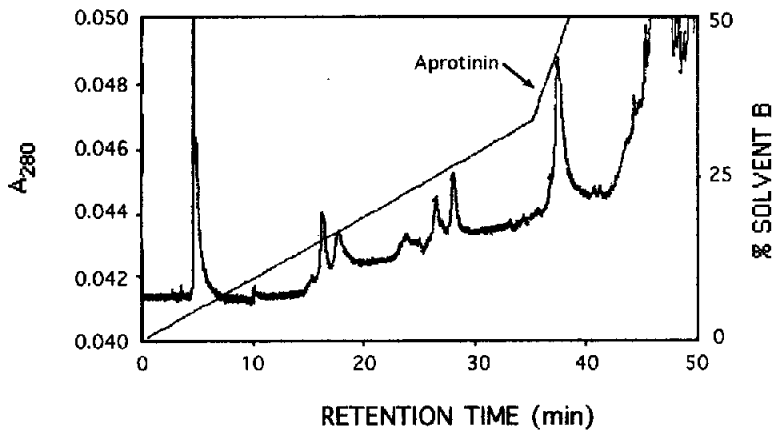

Fig. 1. Elution profile of a sample of commercial aprotinin (4.5 μg) on the reversed-phase C_{18} column. Flow-rate, 1.0 ml/min; detection wavelength, 280 nm. For other conditions see the Experimental.

The gradient shown in Fig. 1 allows the HPLC separation of the aprotinin isoforms expressed in bovine organs, and characterized by a primary structure very similar to that of aprotinin [2–4]. The retention times of the bovine spleen isoinhibitors (Table I) are consistent with the hydrophobicity of the molecules, which increases from inhibitor IV (aprotinin) to inhibitor I.

The validation of the proposed method for use with body fluids was achieved through analyses of human urine samples spiked with aprotinin, following different experimental protocols. The first procedure involved treatment of human urine (0.5 l) with trichloroacetic acid (2.5% final concentration). The supernatant, obtained after centrifugation, was brought to pH 8.6 and applied to a kallikrein–Sepharose 4B column (10 × 2 cm I.D.) equilibrated in 50 m*M* Tris–HCl buff-

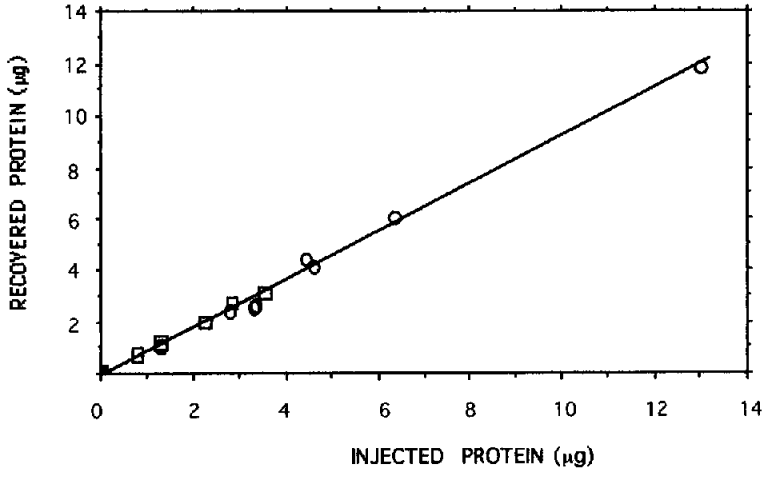

Fig. 2. Calibration curve obtained by injection into the reversed-phase C_{18} column of a sample of commercial aprotinin (○) or FPLC-purified bovine spleen inhibitor IV (□). The standard deviation (S.D. = 0.15 μg) is shown as one half of the dimension of the symbols used in the plot. Chromatographic conditions as in Fig. 1.

TABLE I

RETENTION TIMES AND RECOVERIES OF BOVINE SPLEEN APROTININ-LIKE INHIBITORS

Conditions as in Fig. 1.

	Retention time (min)	Amount injected (μg)	Amount recovered (μg)
Aprotinin (inhibitor IV)	38.0	6.36	6.04
Inhibitor I	44.7	n.d.	30.74
Inhibitor II	41.0	18	18
Inhibitor III	43.3	6	5.7

er (pH 8.6). The column was washed with the equilibrium buffer and then eluted with a solution of HCl at pH 1.5. The active fractions were pooled, brought to pH 8.6 with 1 *M* NaOH, and loaded on a Sep-Pak C_{18} cartridge (Millipore, Bedford, MA, USA). The cartridge was eluted with solvent A–solvent B (60:40) (see Experimental) in a final volume of 1500 μl, and then concentrated down to 50 μl using a Speed Vac centrifuge (Savant, NJ, USA). The use of volatile solvents simplifies this concentration procedure. The final solution was then loaded on the reversed-phase C_{18} column. Using this latter procedure for protein concentration, we were able to recover aprotinin (>99%) completely. The stepwise recoveries were also separately checked and found to be in good agreement with the overall values.

The intra- and the inter-assay precision values were very similar to those reported above when aprotinin solutions were directly loaded on the reversed-phase column. The standard error of a point agreed with the calculated value (0.15 μg) for aprotinin samples, and the statistical lower limit for the analyte detectability can be considered to be 0.45 μg, which is three times the standard deviation [13]. Alternatively, the fractions eluted from the kallikrein–Sepharose 4B column were injected into the C_{18} column after dialysis against doubly distilled water and concentration by lyophilization.

The results obtained clearly indicate that these latter two steps are responsible for a significant decrease (more than 20%) in the recovery of aprotinin used as internal standard. Thus, careful control of these steps is of great importance when working with large volumes, containing very small amounts of aprotinin-like inhibitors. On the basis of this evidence, we decided to use the first procedure for the identification of aprotinin-like inhibitors in human urine. In all the fifteen samples analysed, which were collected from the same donor, we found the presence of a proteic peak showing antitryptic activity with a retention time of 40.5 min, very similar to that exhibited by the aprotinin isoform spleen inhibitor II (see Table I). The concentration of the urinary inhibitor, calculated on the basis of the calibration curves reported in Fig. 2, appears to be 9.4 ± 0.5 μg/l of urine.

CONCLUSION

The method presented here allows the detection and the quantitative recovery of very small amounts of aprotinin-like inhibitors. It can be used for their quantitation and unambiguous identification in biological fluids. Owing to the low levels of aprotinin-like inhibitors in humans, this method could be of crucial importance for the purification and the further characterization of these molecules.

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