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Note

High-performance liquid chromatographic method for the determination of aprotinin in body fluids

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Aprotinin, a basic proteinase inhibitor, is extensively used as a therapeutic agent in various diseases [1]. Various immunological or enzymic assays for the determination of its concentration in plasma or urine have been proposed [2–6]. However, it is likely that the unavailability of a routine direct assay system, which would allow a reliable estimation of aprotinin concentrations in biological fluids, makes it difficult to confirm the clinical effectiveness of inhibitor therapy [1].

The immunological assays [2–4] are specific and highly sensitive, but they require relatively complex and time-consuming sample preparation. Furthermore, it is known that immunological methods show a non-linear calibration function, which does not go through the origin: calibration based on non-linear standard curves is tedious, and reproducibility varies over the measuring range.

The enzymic methods [1,5] are based on the aprotinin inhibition of bovine trypsin-catalysed hydrolysis of an appropriate chromogenic substrate, under defined conditions, followed by spectrophotometric detection. These procedures lack specificity owing to interferences arising from endogenous trypsin inhibitors. They also have a rather low sensitivity, an indirect determination procedure and are limited by the competition between substrate and inhibitor for the active centre of the enzyme. The proposed use [6] of porcine pancreatic kallikrein, instead of bovine trypsin, avoids only the restriction due to endogenous trypsin inhibitors.

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In this note we propose a high-performance liquid chromatographic (HPLC) assay, with prior isolation of aprotinin by immobilized kallikrein, which allows a reliable routine estimation of effective inhibitor concentrations in biological fluids. This is a new method, which permits a specific and direct determination of aprotinin; the analysis of this inhibitor is accomplished without the use of a substrate and is effective in very dilute solutions.

EXPERIMENTAL

Reagents and materials

All chemicals were of analytical-reagent grade or the highest purity available and stored, when necessary, as recommended. Acetonitrile was of HPLC grade (Carlo Erba, Milan, Italy). Aprotinin and porcine pancreatic kallikrein were from Sigma (St. Louis, MO, U.S.A.). Water was distilled once and then deionized using a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Econo columns, 10 cm×1.0 cm I.D. (Bio-Rad Labs., Richmond, CA, U.S.A.), were used for affinity chromatography.

Solutions

Buffer A (pH 2.2) was 10.0 m*M* H_3PO_4 in 0.2 *M* $NaClO_4$. Buffer B (pH 8.3) was 0.1 *M* Tris–HCl solution in 0.5 *M* NaCl.

Aprotinin stock solutions (ca. 60.0 μg/ml) were prepared in buffer B and stored in a dark, cool place; the molarity of these solutions was determined by our previous method [7].

Buffer, plasma and urine standard solutions of aprotinin (2.0–40.0 μg/ml) were prepared by diluting appropriate aliquots of aprotinin stock solution with buffer A, plasma or urine, respectively.

Chromatography

A Model Twincle liquid chromatograph (Jasco, Tokyo, Japan), equipped with a Model VL-614 injection valve (loop capacity 100 or 1000 μl) and a Uvidec 100-III Model Jasco variable-wavelength UV detector, connected to a Chromatopac C-R3A data elaborator, was used. The column was a LiChrosorb RP-18 (250 mm×4.6 mm I.D., 7 μm particle size) from Merck (Darmstadt, F.R.G.) kept at 25±0.2°C in a Violet thermostat Clar 0.55. Isocratic elution was performed with buffer A–acetonitrile (70:30, v/v) at a flow-rate of 1.0 ml/min, and the effluent was monitored at 200 nm.

Affinity chromatography was performed on porcine pancreatic kallikrein bound to cyanogen bromide-activated Sepharose 4B according to the method of the manufacturer (Pharmacia, Uppsala, Sweden). Prolonged washing cycles of alternate high and low pH were necessary to obtain an acceptable HPLC background signal.

Procedure

The pH of a known volume of sample (plasma or preacidified urine, pH 2.0) containing 2.0–40.0 μg of aprotinin was adjusted to 8.3 (by addition of buffer B or 2 *M* NaOH, respectively). After centrifugation (4390 *g*) for 10 min, the supernatant liquid and washing buffer solutions collected were transferred by peristaltic pump (P1, Pharmacia, flow-rate 1×10) to the kallikrein-Sepharose 4B column, previously equilibrated with buffer B. The eluate was passed twice through the same column. The column was then washed with at least ten bed volumes of the same buffer solution to remove unbound substances prior to elution. The aprotinin tightly bound to immobilized kallikrein was fully recovered in a 5.0-ml volumetric flask by successive elution with 0.8 ml of 1.0 *M* H_3PO_4 and buffer A. A 1000-μl aliquot of the resulting solution was injected in the chromatograph.

Calibration graphs

The concentration of aprotinin in plasma or urine was determined from a calibration curve of peak area versus amount (μg) of inhibitor, constructed from aprotinin buffer standard solutions directly injected.

RESULTS AND DISCUSSION

The proposed procedure includes a specific isolation of aprotinin on immobilized kallikrein; this step is followed by analysis on a reversed-phase C_{18} column with UV detection.

In a previous paper [7], we reported well defined conditions for reversed-phase HPLC analysis of aprotinin. Fig. 1A shows a typical peak obtained by direct injection (1000 μl) of a buffer standard solution of inhibitor (4.0 μg/ml, peak a, retention time 9.3 min). The same HPLC conditions were used in this study for the analysis of aprotinin collected after passage through the column of immobilized kallikrein followed by acid elution. Preliminary assays to check the analytical recovery of aprotinin were performed by comparison of the peak areas obtained from four standard solutions containing the same amount of inhibitor (20.0 μg in 5.0 ml): three of these samples (treated buffers) were processed as indicated, the fourth sample (untreated buffer) was directly injected and provided the 100% value. In these experiments, good results in terms of recovery (greater than 98.9%) and reproducibility of peak areas of aprotinin were obtained.

We then analysed some samples of human plasma and urine to ascertain that no endogenous components interfered with aprotinin peak. Fig. 1B shows a chromatogram resulting from the injection (1000 μl) of the final acid solution, after the processing of 50.0 ml of fresh human plasma as described; thus, even high volumes of samples do not produce interfering peaks. Fig. 1C is a chromatogram obtained by starting from 5.0 ml of human plasma spiked with

4.0 μg of aprotinin and subjected to the extraction procedure described: the peak of the inhibitor is the same as that obtained by injection of untreated buffer (Fig. 1A). Analogous experiments performed with urine samples gave similar results.

Recoveries of aprotinin from spiked human plasma and urine samples were determined in duplicate at each of the following aprotinin amounts: 2.0, 5.0, 10.0 and 40.0 μg. An identical series of aprotinin amounts in untreated buffer standard solution provided the 100% values. Three injections for each sample, tests and reference, were performed. The mean (±S.D.) recovery from the eight determinations over the calibration range indicated was 98.5±1.15 and 99.2±1.24% for plasma and urine, respectively.

The precision of the proposed method was evaluated by replicate ($n=10$) analyses of human plasma and urine samples spiked with two different amounts of aprotinin (Table I). The intra-assay precision was assessed by analysing the samples on the same day and showed a mean coefficient of variation (C.V.) of 3.6% for plasma and 3.5% for urine. The inter-assay precision, evaluated over ten days, showed a mean C.V. of 6.2% for plasma and 5.8% for urine.

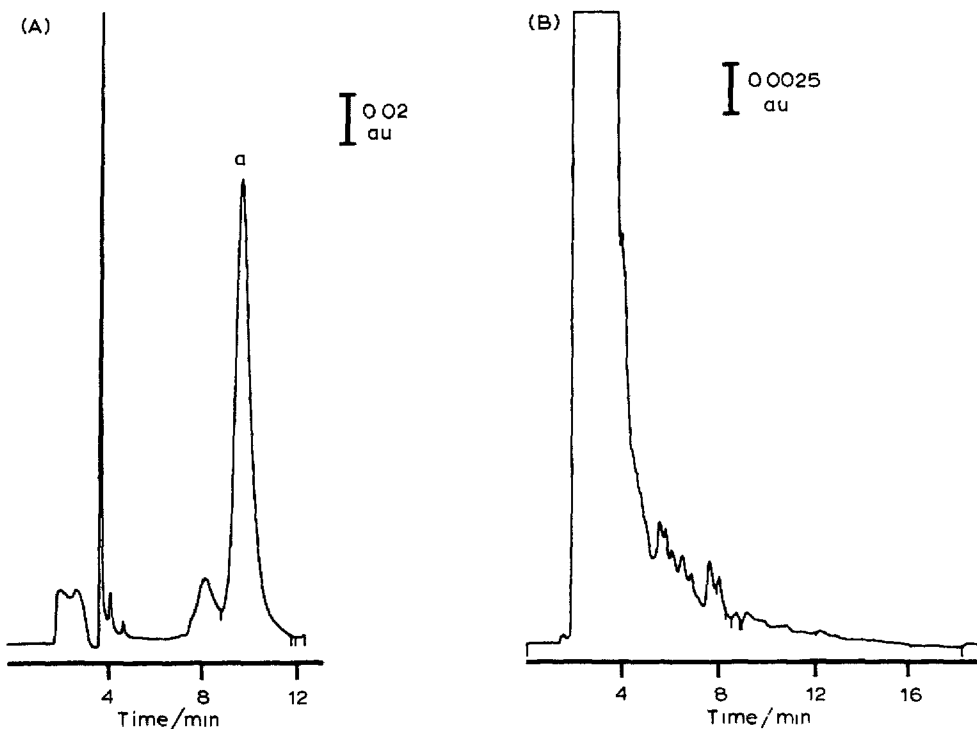

Fig. 1.

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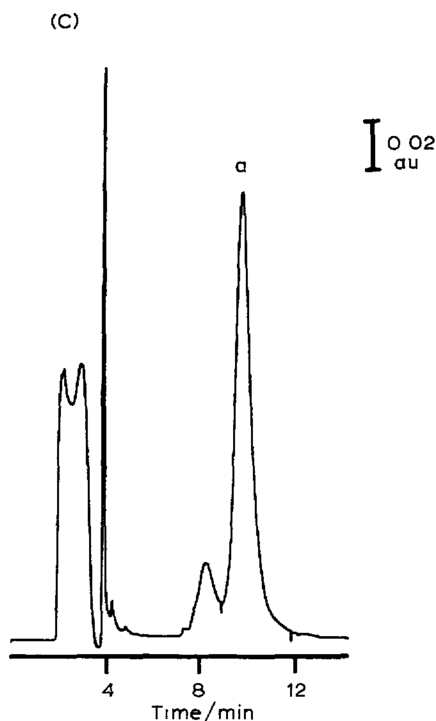

Fig. 1. Chromatograms of (A) a 1000-μl aliquot of standard buffer solution of aprotinin (4.0 μg/ml) directly injected, (B) a 1000-μl aliquot of the final acid solution, after processing of 50.0 ml of fresh human plasma as described, and (C) a 1000-μl aliquot of the final acid solution, after processing of 5.0 ml of fresh human plasma, spiked with 4.0 μg of aprotinin. Mobile phase, acetonitrile–0.1 *M* orthophosphoric acid in 0.5 *M* sodium perchlorate (30 70) (pH 2.2); flow-rate, 1.0 ml/min; detection, 200 nm.

The calibration data, when plotted as the area of peak a versus the amount (μg) of inhibitor from treated plasma, urine and buffer standard solution, gave in each case a straight line passing through the origin. The equations of the curves were calculated by least-squares linear regression, and a good linear relationship was obtained in all cases. The mean calibration curves in plasma, urine and treated and untreated buffer were significantly ($p \leq 0.05$) similar, and therefore the mean calibration curve in untreated buffer might be used for the calibration of aprotinin. This was advantageous since it reduced the number of treatments of plasma or urine standards for control of the quality of the kallikrein-Sepharose 4B columns.

The amounts of aprotinin present in 1 ml of the treated sample volume was calculated from the chromatograms from the following formula:

$$\text{aprotinin in the sample } (\mu\text{g/ml}) = \frac{a \times b}{c \times d} \quad (1)$$

TABLE I

INTRA- AND INTER-ASSAY PRECISION OF APROTININ DETERMINATION IN HUMAN PLASMA AND URINE ($n=10$)

Amount added (μg)	Intra-assay		Inter-assay	
	Amount found (mean ± S.D.) (μg)	C.V. (%)	Amount found (mean ± S.D.) (μg)	C.V. (%)
Plasma				
2 0	1.95 ± 0.08	4.1	2.20 ± 0.15	6.8
10.0	9.48 ± 0.29	3.1	9.80 ± 0.54	5.5
Urine				
2.0	2.09 ± 0.10	4.8	2.03 ± 0.13	6.4
10.0	9.85 ± 0.21	2.1	9.80 ± 0.51	5.2

where a = amount (μg) of aprotinin from calibration graph, b = volume (ml) collected from immobilized kallikrein elution, c = volume (ml) injected and d = volume (ml) of treated sample. In our procedure a = 2.0–40.0, b = 5.0, c = 1.00.

The limit of determination, defined here as the lowest injected amount of aprotinin that gave a 5:1 signal-to-noise ratio, was 0.2 μg. The sensitivity of the method depends on the initial volume of the sample. For example, by applying eqn. 1 in our procedure, for d = 100.0 ml and a = 0.2 μg, aprotinin in the sample results in 0.05 μg/ml. The lower limit of detection of the immunological assay [4] was ca. 0.02 μg/ml.

It can be concluded that the sensitivity, accuracy and precision make the described procedure a routine assay suitable for monitoring therapeutic levels of aprotinin in humans. The freedom from interferences allows the method to be applied to aprotinin analysis in other body fluids. Further, the proposed method allows preconcentration of the inhibitor, thus enhancing the sensitivity. Although the reversed-phase separation of aprotinin from endogenous trypsin inhibitors is feasible [8], the routine assay of the aprotinin by such a method is unsuitable owing to the need to wash the column after any isocratic elution: this increases the analysis time for column reequilibration.

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