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Short Communication

Simple high-performance liquid chromatographic method for the determination of PGT/1A, a new immunostimulating drug, in biological fluids

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ABSTRACT

This paper describes a simple high-performance liquid chromatographic method for the determination of PGT/1A (3-L-pyroglutamyl-L-thiazolidine-4-carboxylic acid), a new immunostimulating drug, in plasma and urine. The column was packed with LiChrospher-NH_2 (5 μm), the mobile phase was 0.02 *M* monobasic potassium phosphate (pH 3.2 with concentrated phosphoric acid)–acetonitrile (25:75, v/v), the flow-rate was 1.2 ml/min, the detection wavelength was 210 nm and the apparatus was a Varian Model 5000. Plasma (1 ml) was added to 1.2 ml of acetonitrile and the supernatant injected; the urine was diluted 1:5. The retention time of PGT/1A was 9.4 min in plasma and 9.9 min in urine. The method was validated for recovery, accuracy and reproducibilty. The results after intravenous injection in twelve volunteers are also given.

INTRODUCTION

Significant activity in the immune system has been found due to peptides produced by thymus gland, but immunoenhancing activity also results from the presence of small peptides, such as TP-5 (Arg-Lys-Asp-Val-Tyr) and tuftsin (Thr-Lys-Pro-Arg).

Following our research on immunostimulating drugs, we found a new interesting peptide-like compound, PGT/1A (3-L-pyroglutamyl-L-thiazolidine-4-carboxylic acid) [1]. PGT/1A stimulates T lymphocytes in prednisolone-immunodepressed mice, enhancing the response to IV° delayed-type hypersensitivity and increasing the cytotoxic reaction in the rosette-forming cells test [2]. PGT/1A protects the mice against experimental bacterial infections [3,4], and increases the superoxide anion production and the migration peritoneal macrophages of in prednisolone-immunodepressed mice [5].

This paper describes a simple high-performance liquid chromatographic

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(HPLC) method for the determination of PGT/1A in plasma and urine, and some results after intravenous administration of the drug to volunteers.

EXPERIMENTAL

Reagents and materials

The solvents used were all of HPLC grade (LiChrosolv, Merck, Darmstadt, Germany, or Carlo Erba, Milan, Italy). The water was previously bidistilled using a glass distiller and filtered on a 0.45-μm membrane (Type HAWP, Millipore) 3-L-Pyroglutamyl-L-thiazolidine-4-carboxylic acid standard was prepared in our laboratories; the other reagents were all of analytical grade.

High-performance liquid chromatography

The apparatus used was Varian Model 5000, equipped with a UV-100 detector, a Vista Model 401 printer–plotter computer integrator and an automatic injection Valco valve. The column (250 mm × 4 mm I.D.) was packed with LiChrospher-NH_2, 5 μm (Merck). The column was kept at room temperature. The mobile phase was 0.02 *M* monobasic potassium phosphate (pH 3.2) with concentrated phosphoric acid–acetonitrile (25:75, v/v). The flow-rate was 1.2 ml/min and the detection wavelength 210 nm.

Standard solutions

A standard solution of 3-L-pyroglutamyl-L-thiazolidine-4-carboxylic acid was prepared at a concentration of 1000 μg/ml in water and stored at 4°C. The solution was diluted with phosphate buffer to a final concentration of 0.5–20 μg/ml. The calibration curves were obtained by adding known amounts of PGT/1A to human plasma and to dilute (1:5) human urine.

Assay procedure for plasma levels

To 1 ml of plasma were added 1.2 ml of acetonitrile. The mixture was stirred on a vortex mixer for 1 min and centrifuged at 3000 *g* for 10 min. The supernatant was drawn off with a syringe and added to 5 ml of chloroform. This mixture was stirred on a vortex mixer for 1 min and centrifuged at 3000 *g* for 10 min. Then 0.5 ml of the supernatant was added to 0.5 ml of acetonitrile, and 200 μl of this solution were injected into a column (25 mm × 4 mm I.D.) packed with LiChrospher-NH_2 (5 μm) (Merck) and connected to the automatic injection valve. The column was eluted with 200 μl of mobile phase before connection to the main column.

Assay procedure for urinary levels

The urine samples were diluted 1:5 (v/v) with water and filtered on a 0.45-μm membrane (Type HAWP, Millipore), and 10-μl aliquots were injected into the main column.

Quantitative evaluations

The PGT/1A content in plasma and in urine was determined by comparison of the sample peak area with peak areas of the calibration curve. No internal standard was used owing to the very simple procedure.

Human study

Male volunteers (mean age 36.3 ± 7.3 years; mean weight 73.4 ± 7.1 kg; mean height 178.1 ± 6.6 cm) were treated intravenously with 200 mg of PGT/1A. Blood samples were drawn before and 5, 15, 30 and 45 min and 1, 2, 4, 8, 12, 24 and 48 h after administration. Urine was collected before, from 0 to 8 h and from 8 to 24 h.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of PGT/1A in human plasma under our

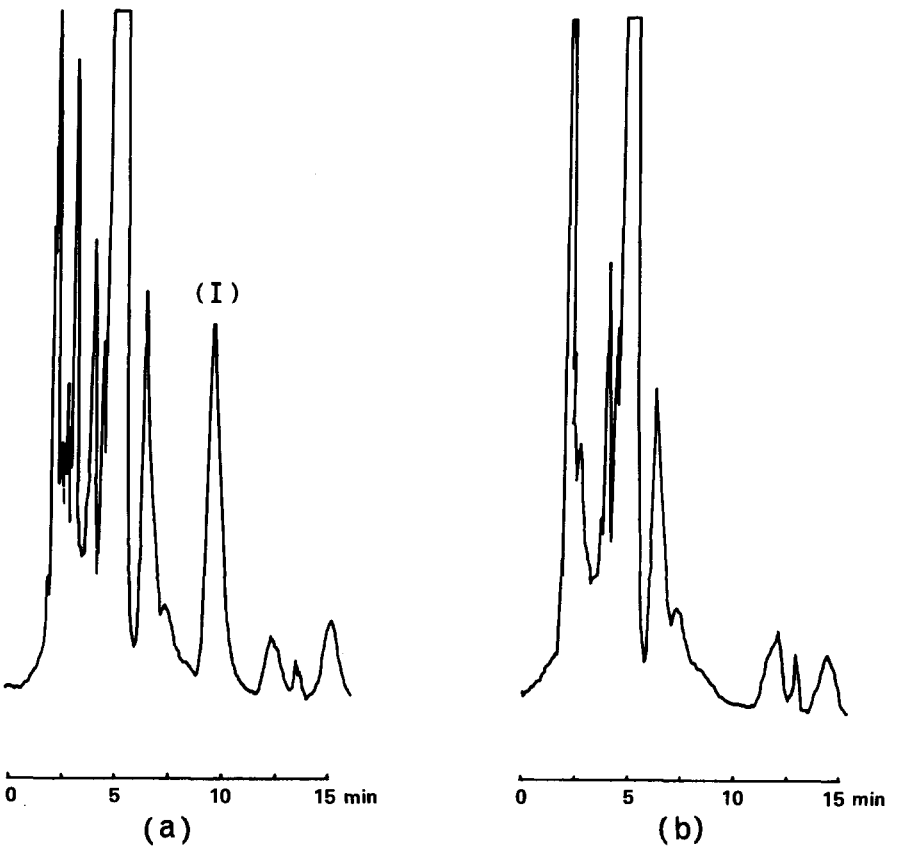

Fig. 1. Chromatograms obtained from (a) plasma spiked with PGT/1A (I) (20 μg/ml) and (b) drug-free plasma.

experimental conditions. The blank chromatogram shows that no interference arose from endogenous substances in the plasma.

Fig. 2 shows a typical chromatogram of PGT/1A in human urine under our experimental conditions. The blank chromatogram shows that no interference arose from endogenous substances in the urine.

The retention time of PGT/1A is 9.4 min in plasma and 9.9 min in urine. This difference is probably due to the different pH conditions of the samples obtained from the two biological fluids. The retention time of analyte is sensitive to changes of pH, and in both sample preparation procedures only the mobile phase is buffered.

In order to check the validity of the proposed method a known amount of PGT/1A was added to human plasma, and its recovery rate was determined by the HPLC method. As reported in Table I, the recovery of PGT/1A in plasma was 97.6–103.4%.

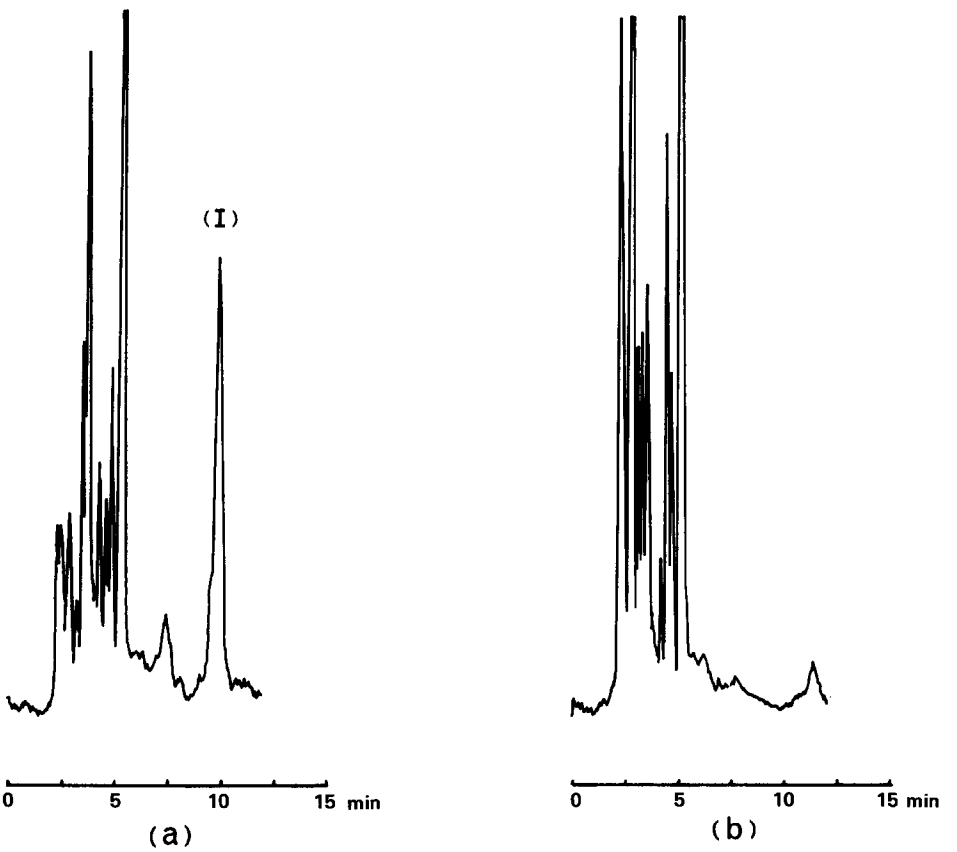

Fig. 2. Chromatograms obtained from (a) urine spiked with PGT/1A (I) (200 μg/ml) and (b) drug-free urine.

TABLE I

RECOVERY TEST FOR PGT/1A ADDED TO HUMAN PLASMA

Concentration (μg/ml)		Recovery (mean ± S.D., n = 5) (%)	Difference of mean from theoretical (%)
Added	Found		
20	19.97	99.85 ± 9.30	−0.15
10	9.94	99.40 ± 9.80	−0.60
5	5.17	103.40 ± 12.35	−3.40
2.5	2.44	97.60 ± 10.15	−2.40

The accuracy, defined as the difference between the found and true values, was within 3.4% (Table I), The plasma calibration curve was linear over the range 0.5–20 μg/ml. The relationship between PGT/1A plasma concentrations in this range and the peak areas was expressed as $y = 17\,697.2x + 10\,699.3$, where x is the amount of PGT/1A injected (expressed as μg/ml plasma) and y is the peak area. The correlation coefficient was $r = 0.9999$. The detection limit of PGT/1A was estimated as 0.5 μg/ml, at a signal-to-noise ratio of *ca.* 5:1.

The within-day and day-to-day reproducibilities of the method were checked at various plasma PGT/1A concentrations. Five determinations of each were done on the same day; the day-to-day reproducibility was assessed over thirty days. The results are given in Table II.

The urine calibration curve was linear over the range 4–400 μg/ml; the relationship between PGT/1A urinary concentrations in this range and the peak areas was expressed as $y = 1516.2x - 4468.4$, where x is the amount of PGT/1A injected (expressed as μg/ml) and y is the peak area. The correlation coefficient was $r = 0.997$. The detection limit of PGT/1A was estimated as 4 μg/ml, at a signal-to-noise ratio of *ca.* 6:1.

TABLE II

DAY-TO-DAY AND WITHIN-DAY REPRODUCIBILITY IN HUMAN PLASMA

Concentration (μg/ml)	Coefficient of variation (%)	
	Day-to-day (n = 3)	Within-day (n = 3)
20	9.31	4.91
10	9.86	5.57
5	11.99	7.69
2.5	10.25	8.58

TABLE III

DAY-TO-DAY AND WITHIN-DAY REPRODUCIBILITY IN HUMAN URINE

Concentration (μg/ml)	Coefficient of variation (%)	
	Day-to-day (n = 3)	Within-day (n = 3)
10	6.4	5.3
20	5.2	4.7
100	4.3	3.8
200	4.5	3.1
400	4.0	3.9

The within-day and the day-to-day reproducibilities of the method were checked at various urinary PGT/1A concentrations. Three determinations of each were done on the same day; the day-to-day reproducibility was assessed over thirty days. The results are given in Table III.

Plasma levels of PGT/1A after administration of 200 mg as intravenous bolus to male volunteers are shown in Fig. 3. The mean pharmacokinetic parameters can be obtained with a structural model of two exponentials after intravenous bolus, fitted to data using a peeling algorithm (Siphar program). The mean values

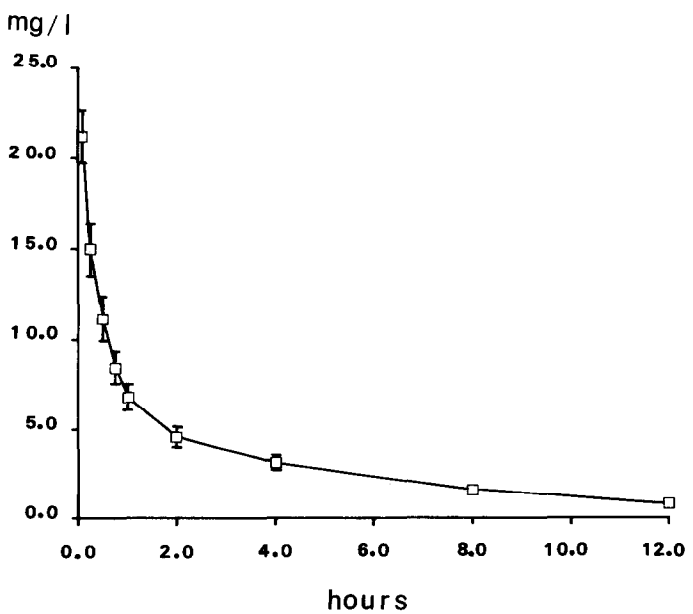

Fig. 3. Plasma levels of PGT/1A (mean ± S.E.) after bolus intravenous administration of 200 mg to twelve volunteers.

are as follows (mean ± S.E.): C_{max} = 21.2 ± 1.5 mg/l; $t_{1/2elim}$ = 3.8 ± 0.1 h; $AUC_{0-\infty}$ = 44.5 ± 5.2 mg l^{-1} h; total clearance = 5.1 ± 0.5 l h^{-1}; distribution volume = 27.2 ± 2.4 l.

The urinary recovery of unchanged PGT/1A was 95.2 ± 2.3%.

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