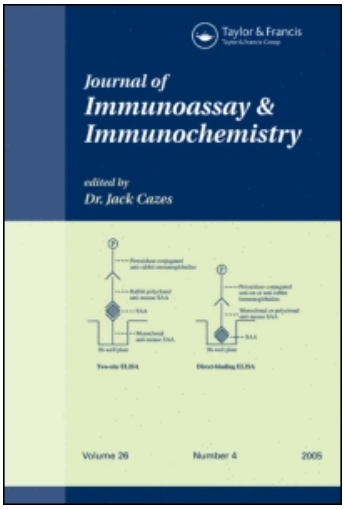

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Development and Validation of a Sensitive and Specific Radioimmunoassay for the Determination of Cicaprost in Biological Samples

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DEVELOPMENT AND VALIDATION OF A SENSITIVE AND SPECIFIC RADIOIMMUNOASSAY FOR THE DETERMINATION OF CICAPROST IN BIOLOGICAL SAMPLES

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ABSTRACT

Cicaprost is a potent, chemically and metabolically stable PGI_2-mimetic. Pharmacodynamic effects were observed after oral administration of ≈ 10 µg in man when plasma levels were in the low pg-range.
The present report describes the development of a selective antiserum and a tracer with high specific activity and their use for the RIA determination of Cicaprost in biological samples. Cicaprost-[^{3}H]-methylester with a specific activity of 819 GBq/mmol was used as a tracer. RIA-analyses were carried out with 0.05 - 0.5 ml plasma adjusted to pH 2 with 1 N HCl and extracted with 2.5 ml diethylether. Separation of antiserum bound and unbound Cicaprost was achieved by the charcoal method. Extraction recovery of Cicaprost was ≈ 90 % at pH ≈ 2. The detection limit of the assay was 10 - 20 pg/ml plasma. Coefficients of variations were 6, 3 and 9 % (within-day, n = 5) and 25, 12 and 10 % (day-to-day, n = 11) at 50, 100 and 200 pg/ml. HPLC-chromatograms of plasma extracts did not reveal any peak apart from Cicaprost, demonstrating the specificity of the method. The present RIA for Cicaprost exhibits high specificity and sensitivity and will be used for further bioanalyses in pharmacokinetic study.

[KEY WORDS:Cicaprost, radioimmunoassay, bioanalytics, pharmacokinetics, PGI_2]

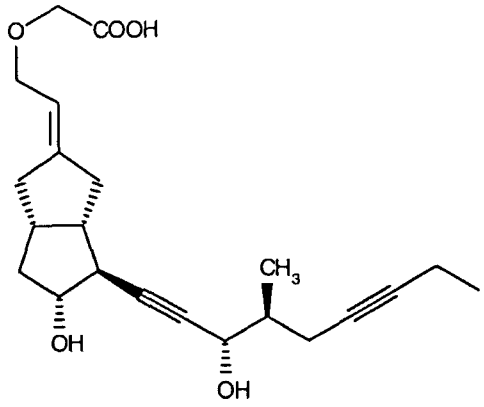

FIGURE 1

Structural formula of Cicaprost

INTRODUCTION

Cicaprost (INN 5-[(E)-(1S,5S,6S,7R)-7-hydroxy-6-(3S,4S)-3-hydroxy-4-methylnona-1,6-diynyl]-bicyclo [3.3.0]octan-3-yliden]-3-oxapentanoic acid; Fig. 1) is a novel, potent PGI_2-mimetic. The compound is synthesized as the pure 16-S-isomer [1].

From the endogenous pharmacophore prostacyclin, structural modifications led to cicaprost, a PGI_2-mimetic that is both chemically and metabolically stable and pharmacologically highly potent [2-6].

The compound exhibits pharmacotherapeutic effects after low oral doses (5 - 10 μg) due to its complete bioavailability in man [5]. Therapeutic plasma or serum levels were in the low pg-range [5,6], similar to another PGI_2-mimetic, Iloprost, which is mainly used as an i.v. infusion treatment principle [7].

In order to determine Cicaprost levels in biological samples a radioimmunoassay was developed and validated. As compared to other eicosanoid derivatives the prerequisites for the practical use of a RIA-method were promising in case of Cicaprost, because the drug was shown to be metabolically stable in man and several animal species. Thus, at least the problem of possibly cross-reacting metabolites should not occur. Because of the very low dose and plasma levels, a tracer with high specific activity had to be used.

METHODS AND MATERIALS

Synthesis of the Immunogen:

Cicaprost (50 mg = 138 μmol) was dissolved in 3 ml tetrahydrofurane (THF), mixed with 235 μl of a 10 %

(v/v) solution of triethylamine in THF (167 μmol) and cooled to -10°C. After the addition of 225 μl of a 10 % (v/v) solution of isobutylchloroformate in THF (167 μmol), the temperature of the reaction mixture was slowly raised to 4°C over a period of 20 min. Subsequently the reaction mixture was added to a solution of 200 mg bovine serum albumin in 8 ml aqueous THF (1:1 v/v) containing 200 μl 1 N NaOH. The pH of the reaction was adjusted to 8. After a reaction time of 2 h at 0°C, the solution was dialyzed against distilled water. The final product was lyophilized and dried in a desiccator. Colorimetric determination of the free amino groups according to Habeeb [8] showed that 37 moles of hapten per mol albumin were covalently coupled.

Immunization

Five rabbits were each immunized by subcutaneous injection of 1 mg immunogen in an emulsion of 1 ml aqueous sodium chloride (0.154 mol/l) and 1 ml Freund's complete adjuvant. The injections were applied at two sites on the lower back of the animals. Booster injection with 1 mg immunogen in complete adjuvant were applied in monthly intervals.

After the third booster injection, blood was drawn from the ear artery to determine the titre of the antiserum.

Determination of the Titre

Assay buffer: (BSA-buffer) Phosphate buffer (1/15 mol/l, pH 7.0) containing NaCl (0.15 mol/l), sodium azide (0.1 %, w/v) and bovine serum albumin (0.1 %, w/v).

Tracer: [^{3}H]-Cicaprost-methylester with a specific activity of 819 GBq/mmol was synthesized by the Dept. of Isotope Chemistry, Schering AG. The radiochemical purity was > 98 % as determined by HPLC. A solution containing 50,000 dpm/ml in assay buffer was used in the assay.

Charcoal: 1.25 % (w/v) charcoal (Aktivkohle p.A. Merck) in BSA-buffer.

To determine the titre of the antiserum, 0.8 ml assay buffer, 0.1 ml tracer (5,000 dpm) and 0.1 ml of various antiserum dilutions were incubated for

16 h at 4°C. Separation of free and bound ligand was performed by the addition of 0.2 ml charcoal suspension. The titre is defined as the final dilution of the antiserum in the assay at which 50 % of the added tracer is bound.

RIA

Plasma samples (0.05-0.2 ml) were diluted with physiological saline to a finale volume of 0.5 ml, adjusted to pH 2 with 1 N HCl and extracted with 2.5 ml diethylether on an Heidolph^{R} rotor for 30 min. A standard curve was set up by pipetting 0.1 ml BSA-buffer containing 1.9 to 1000 pg Cicaprost/0.1 ml into disposable glass tubes adding drug-free plasma (0.1-0.2 ml), filled with 0.3-0.4 ml physiological saline to a final volume of 0.5 ml with physiological saline adjusted to pH 2 with 1 N HCl and extracted just like the plasma samples. The tubes were centrifuged, placed in a mixture of ethanol/dry ice to solidify the aqueous phases and the organic phases were decanted into new disposable glass tubes. The solvent was evaporated under a gentle stream of nitrogen and subsequently 0.8 ml BSA-buffer was added to the dried extracts.

0.1 ml tracer solution and 0.1 ml antiserum dilution were pipetted into each tube. Tubes for measuring the total radioactivity (RA) and non-specific binding (NSB) contained 0.9 ml BSA-buffer and 0.1 ml tracer solution. Tubes for measuring the maximal binding (C0) contained 0.8 ml BSA-buffer, 0.1 ml tracer solution and 0.1 ml diluted antiserum.

After incubation for 16 h at 4°C, 0.2 ml charcoal suspension was added to all tubes at 0°C (0.2 ml buffer to the RA-tubes) and the tubes were left in an ice bath for 20 min. After centrifugation for 20 minutes at 2300 x g and 4°C the supernatants were decanted into vials and counted in a scintillation counter after the addition of 4 ml Atomlight TM (NEN).

All samples were analyzed in duplicated (standard curve in triplicate) and evaluated by computer, using the 4-parameter model described by Rodbard [9,10].

Cross-reactions of the Antiserum

Cross-reactions were not tested, since no metabolites of Cicaprost are known.

Assay scheme (volume in ml)

	RA	NSB	CO	Standard curve	Samples
Sample	-	-	-	-	0.05-0.2
Standard	-	-	-	0.1	-
phys. saline	ad 0.5	ad 0.5	ad 0.5	ad 0.5	ad 0.5
drug-free plasma	0.1-0.2	0.1-0.2	0.1-0.2	0.1-0.2	-
1 N HCl	0.1	0.1	0.1	0.1	0.1
Extraction	2.5	2.5	2.5	2.5	2.5 ml diethyl-ether
Buffer	0.9	0.9	0.8	0.8	0.8
Tracer	0.1	0.1	0.1	0.1	0.1
Antiserum	-	-	0.1	0.1	0.1
Incubation	16 h at 4°C				
Charcoal	-	0.2	0.2	0.2	0.2
Buffer	0.2	-	-	-	-
Incubation	20 minutes at 0°C				
Centrifuge 20 min. at 2300 g and 4°C and decant the supernatant in scintillation vials. Add cocktail and count.					

Extraction of [3H]-Cicaprost

The recovery of [^{3}H]-Cicaprost was tested by adding 0.1 ml tracer solution to 0.4 ml blank plasma. Spiked samples were adjusted to pH 2, 4 and 7.4 with 1 N HCl and/or buffer solutions and extracted with 2.5 ml diethylether for 30 min. The organic phase was decanted in LSC-vials, evaporated and redissolved in 0.5 ml distilled water. After the addition of 10 ml Atomlight™ the radioactivity was measured in a liquid scintillation counter. All analyses were carried out 5-fold.

Precision and Accuracy of the Method

Within-day and day-to-day variation of the assay were characterized by repeated measurements of a plasma sample in one assay (n = 5) and by analyses of control plasma samples containing 50, 100 and 200 pg/ml Cicaprost in 11 different assays. Accuracy was tested by comparing the amount of Cicaprost added to blank plasma samples with the concentration determined by RIA. Detection limit and the concentration dependent coefficient of variation of each assay were determined using the method of

Rodbard [10]. Mean response (i.e. cpm) and corresponding variances thereof were pooled over the successive assays and used for the calculations. From these data a correlation between variance and response could be calculated, which in turn resulted in a precision profile and detection limit for each assay (Fig. 2).

Specificity of the Method

The specificity of the radioimmunoassay was tested by subjecting diethylether extracts of plasma and native urine samples to HPLC-separation.

The biological samples from man originated from a phase I study, where Cicaprost was administered orally three times daily at a dosage of 10 μg as tablet preparation SHT 490 A in 6 male volunteers for 5 days. Plasma samples obtained at 4 to 6 h p.adm. were combined. 4.8 ml of the pool was extracted (condition described above). The residue was redissolved in 500 μl of 0.01 M aqueous $(NH_4)_2CO_3$-solution (eluent A of HPLC, see below) and 200 μl was injected onto an HPLC-column. Urine (200 μl) was injected directly. The plasma samples from dogs were

obtained in a pharmacokinetic and -dynamic study, where Cicaprost was administered orally at 15, 30 and 60 μg/kg to female dogs. Plasma samples (0-4 h p.adm.) were combined and extracted as described above. For the HPLC of monkey plasma a combined samples from a toxicological study were used.

HPLC-conditions:

column:	Spherisorb ODS II, 5 μm, 250 x 4.6 mm
eluent:	A: dist. water containing 0.01 M $(NH_4)_2\ CO_3$ B: methanol
gradient:	in 60 min from 0 % to 100 % of B
temperature:	ambient
flow rate:	2 ml/min
detection:	fractionating of the eluent (10 fractions/3 min) and assaying individual fractions after evaporation and redissolving in BSA buffer by RIA.
evaluation:	plotting concentrations of immunoreactive compounds of individual fractions vs. retention time.

To obtain a reference chromatogram, 200 pg of Cicaprost was analyzed as described above.

RESULTS

Extraction of 3H-Cicaprost from Plasma

Cicaprost could be extracted from plasma samples with a recovery of 59 % at pH 7.4, 76 % at pH 4 and 88 % at pH 2. Therefore, plasma samples were extracted with diethyl ether at a pH 2 prior to the radioimmunoassay.

RIA-analysis

Due to the higher specific activity of the Cicaprost-[^{3}H]methylester as compared to Cicaprost tritiated in position 4, the antiserum dilution in the present assay was 1:1,400,000 as compared to 1:400,000 in an earlier experiment where ^{3}H-Cicaprost was used as a tracer [6]. The total amount of tracer added could be reduced to 5,000 dpm corresponding to approx. 5 pg per test tube. In general, samples void of Cicaprost did not show any interfering compounds imitating a Cicaprost level. Al-

though in general no Cicaprost could be detected in drug-free samples, due to unknown reasons, an apparent level of Cicaprost (20-30 pg/ml) was detected in some few samples (3-5 %), obtained prior to Cicaprost administration. These levels were quite close to the limit of quantification, so that further dilution of samples was not possible. Furthermore HPLC-investigations on the nature of the cross-reacting compound could not be conducted due to the low total concentration.

Detection limit, Precision and Accuracy

The detection limit of the assay was approx. 2-4 pg/test tube and thus, depending on the plasma volume used (in general 0.1 to 0.2 ml) 10 to 20 pg/ml.

Data on within-day and day-to-day reproducibility of the method and its accuracy and pecision are given in Tab. 1 and 2.

A precision profile is shown in Fig. 2.

TABLE 1

Day to day variation of the RIA measurement of Ci-caprost (determined in spiked plasma samples)

concentration added (pg/ml)	**50**	**100**	**200**
concentration	62.0	119.7	216.9
measured (pg/ml)	73.2	118.4	220.8
	59.5	114.0	226.5
	70.7	120.2	221.6
	43.0	103.0	233.5
	48.5	124.9	282.2
	35.0	91.1	213.2
	59.0	117.5	251.0
	71.0	107.0	238.0
	34.5	83.0	200.5
	63.5	106.0	218.5
Mean	56.4	109.5	229.3
S.D.	14.1	13.1	22.0
% of variation	24.9	12.0	9.6
% of precision	112.8	109.5	114.7

TABLE 2

Within-day variation of the RIA measurement of Cicaprost (determined in spiked plasma samples)

concentration added (pg/ml)	**50**	**100**	**200**
concentration measured (pg/ml)	62	117	198
	65	122	197
	66	116	232
	59	125	234
	58	119	223
Mean	62	120	217
S.D.	4	4	18
% of variation	6	3	9
% of precision	124	120	109

Specificity

The specificity of the method was high, as tested by the chromatography of the extracts of plasma and urine samples of dog, cynomolgus monkey and man after Cicaprost administration and checking the immunoreactivity of eluent fractions.

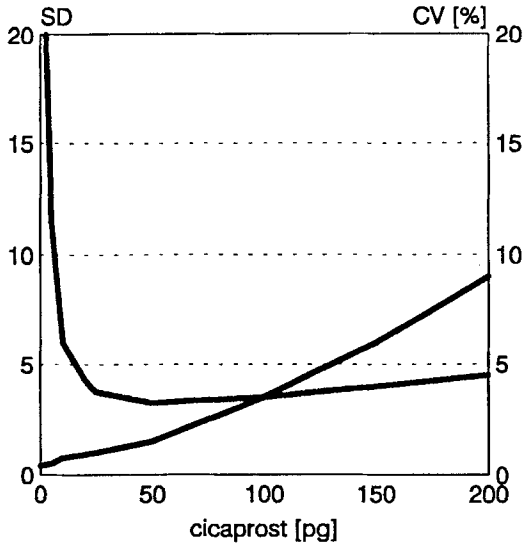

FIGURE 2

Precision profile of RIA-measurement of Cicaprost plasma levels

Fig. 3 shows the corresponding chromatograms. No cross-reacting drug-related or endogenous peaks, which exceeded the detection limit of the assay, were observed.

DISCUSSION

A new radioimmunoassay for Cicaprost has been developed, using a conjugate of Cicaprost and bovine serum albumin as immunogen. The antisera raised with

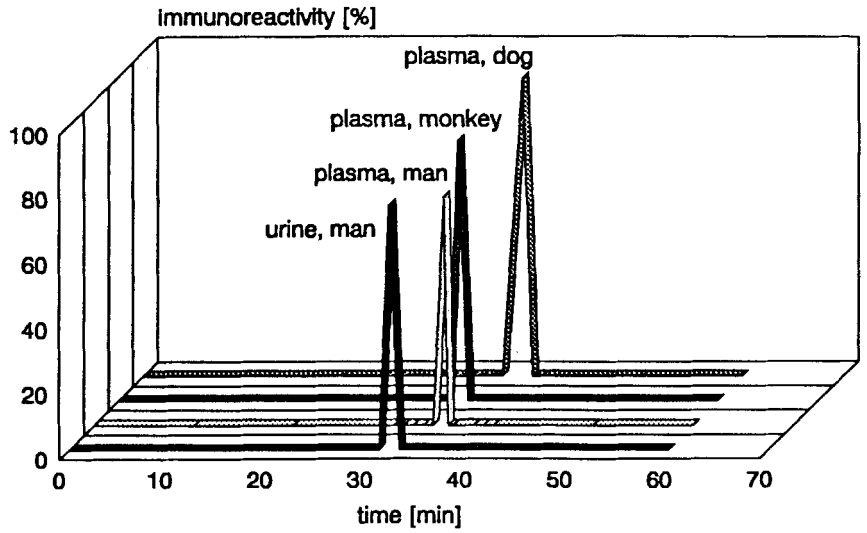

FIGURE 3

Chromatograms of diethylether extracts of plasma and urine samples of man (a: plasma, b: urine) and plasma of cynomolgus monkey (c) and dog (d).
Each eluent fraction was subjected to Cicaprost-RIA to search for possible cross-reacting endogenous compounds.

this immunogen showed a remarkable high cross-reaction with Cicaprost methylester and therefore Cicaprost-[^{3}H]-methylester was used as tracer in the assay, since this was available with a high specific radioactivity. The cross-reaction of the antiserum with biodegradation products of Cicaprost was not investigated because Cicaprost was metabolically stable in man and animals like monkeys. However in

other species (e.g. male rats) degradation products occur, but structures have not been elucidated up to now.

The specificity of the assay was assessed by subjecting plasma extracts of human subjects, dogs and monkeys treated with Cicaprost to reversed phase HPLC and testing each fraction with the radioimmunoassay. Only one immunologically active peak, which coincided with Cicaprost, was found in all extracts.

Although in dogs several degradation products of Cicaprost were observed after ^{3}H-label administration these metabolites did not interfere with the RIA applied.

In the present study, the standards for the calibration curve were extracted to increase the accuracy of the method. The sensitivity of the method was sufficient for measurement of biological samples obtained after pharmacodynamically effective doses [i.e. 5-10 μg p.o.]. The coefficients of day-to-day and within-day variation of the RIA were in an acceptable range. Up to now no independent alternative

method for the analytical determination of Cicaprost (e.g. GC/MS/NCl or HPLC with fluorescence derivatization) was not established to cross-validate the present RIA method. Therefore all attempts of the present series of investigations aimed at demonstrating the specificity, sensitivity and reproducibility of the method.

All data reported unequivocally demonstrated that the RIA is well suitable for bioanalytical measurements in pharmacokinetic studies.

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