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Determination of an endothelin receptor antagonist in rat plasma by radioimmunoassay

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Abstract

A quantitative method based on radioimmunoassay for the determination of an endothelin receptor antagonist ($C_{31}H_{33}NO_7$, **I**) has been developed and validated. The immunogen was prepared by coupling **I** to the bovine serum albumin via the *N*-hydroxysuccinimide ester of **I** from which the radioligand was also prepared by the reaction with [^{125}I]-iodotyrosine. The method was specific and no immunoactive material other than the parent drug was detectable in mammalian plasma. This direct assay, using 50 μl of rat plasma is sensitive (0.4 ng/ml), without matrix interference, and has sufficient sensitivity, specificity, accuracy and precision for the analysis of dosed rat plasma samples.

Keywords: Endothelin receptor antagonist; Radioimmunoassay; [^{125}I]-iodotyrosine

1. Introduction

Endothelin is well documented to be one of the most potent, naturally occurring vasoconstrictive peptides whose synthesis and receptors are most abundant in vascular endothelium and smooth muscle, respectively. The autocrine pathway influences both blood flow and intravascular pressure. Endothelin elevations have been documented in chronic and acute renal disease, congestive heart failure and hypertension [1–5].

Compound **I** ($C_{31}H_{33}NO_7$, Fig. 1) is a specific endothelin receptor antagonist. A sensitive method for quantification of **I** in biological fluid was required to support the preclinical studies necessary to develop this drug candidate. This report describes the development and performance of a competitive binding radioimmunoassay (RIA) of **I** and its application to rat plasma.

2. Experimental

2.1. Materials and instruments

All of these animal studies were approved by the Merck Research Laboratories. Compound **I** was obtained from Merck Research Laboratories (Rahway, NJ).

Mono- and di-basic sodium phosphate, sodium azide, disodium ethylenediamine tetraacetic acid (EDTA), L-tyrosine, hydrochloric acid, acetonitrile,

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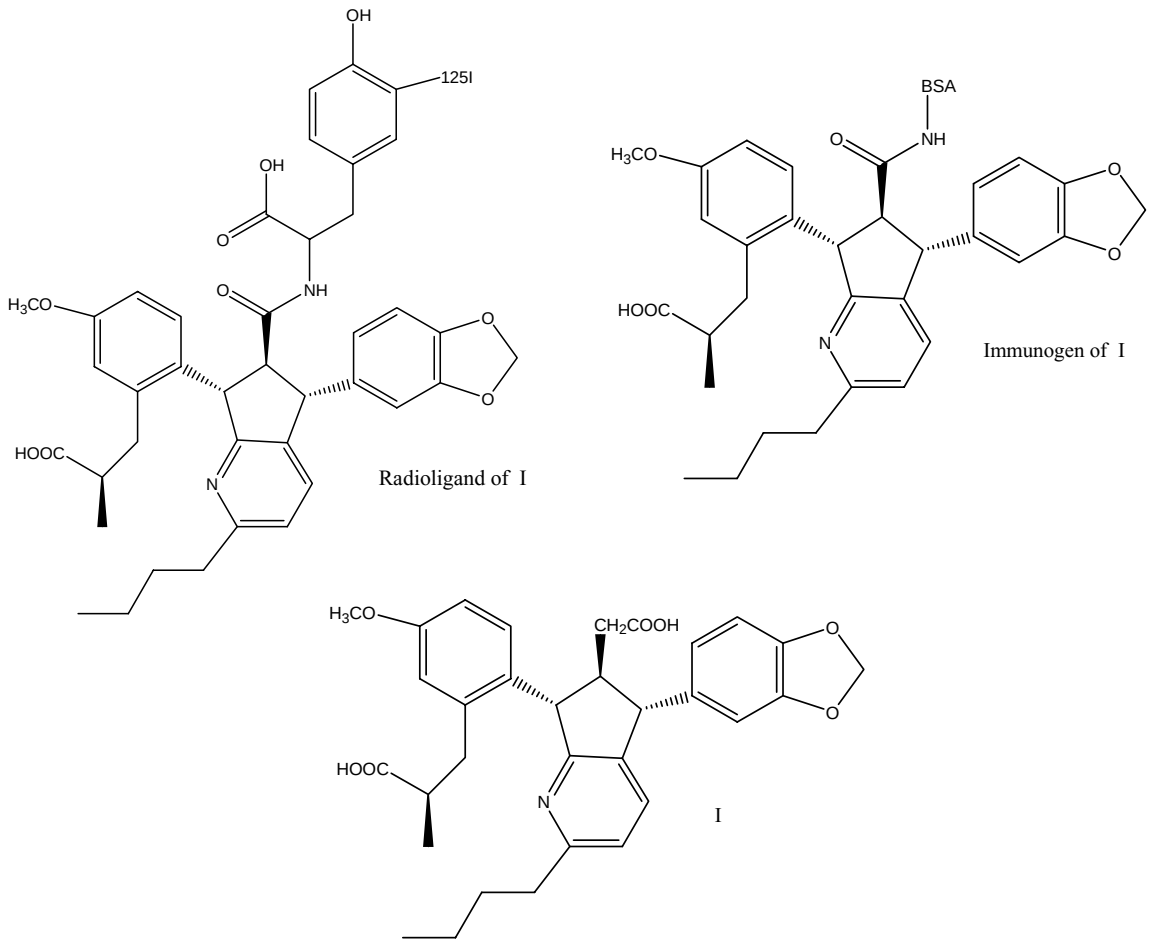

Fig. 1. Chemical structure of **I**, one of the three conjugated immunogens and radio ligands (conjugated with either one or both carboxylic acid groups) used in development of the immunoassay. "BSA" is bovine serum albumin moiety.

N,*N*-dimethylformamide (DMF), phosphoric acid, triethylamine and sodium tetraborate were obtained from Fisher (Fair Lawn, NJ). Bovine serum albumin (BSA), crotein, and sodium heparin were purchased from Sigma (St Louis, MO); sodium hydroxide and acetic acid were from Mallinkrodt (Paris, KY); sheep anti-rabbit-γ-globulin serum was obtained from Arnel Products (New York, NY); rabbit-γ-globulin and the BSA used to prepare the immunogen were supplied by Calbiochem (LaJolla, CA); ^{125}I-labelled sodium iodide was purchased from Amersham (Arlington Heights, IL); chloramine-T, sodium metabisulfite, *N*-hydroxysuccinimide and dicyclohexylcarbodiimide were obtained from Aldrich (Milwaukee, WI). Radioisotope detector model 170 was obtained from Beckman Instruments (Irvine, CA), fraction collector model FC203 was obtained from Gilson (Middletown, WI), multidetector gamma counter model Apex10/600 and automatic pipetting station (APS) were purchased from Micromedic Systems (Horsham, PA).

2.2. Preparation of the immunogen

The *N*-hydroxysuccinimide ester (NHS active ester) was prepared by reacting 0.038 mmol of **I** with 0.043 mmol *N*-hydroxysuccinimide, and 0.097 mmol of dicyclohexylcarbodiimide in 0.2 ml dry *N*,*N*-dimethylformamide (DMF) at room temperature

for 48 h. Without isolation of the active ester, this solution was added directly to bovine serum albumin (BSA, 20 mg, 0.3 μmol) in 0.8 ml 0.125 M potassium phosphate, pH 8.9. DMF (0.2 ml) was added to a control solution of BSA. Additions were made dropwise with constant stirring at 0–4 °C over a 2 h period. The stirring of this solution was continued overnight at 0–4 °C. Exhaustive dialysis against water for 72 h was carried out at 0–4 °C and the immunogen solution was diluted to 20 ml with distilled water. The extent of the incorporation of **I** into immunogen was approximately 8 mol/mol of BSA (Fig. 1) determinated by MALDI-TOF.

2.3. Immunization

The immunogen was emulsified with an equal volume of Freund's Complete Adjuvant (Sigma) and four female New Zealand white rabbits were immunized. Each rabbit received approximately 1 mg protein through multiple intradermal injections plus subcutaneous (sc) and intramuscular (im) injections. Rabbits were boosted at 1, 3 and 6 months with 0.5 mg protein in Freund's Incomplete Adjuvant (Sigma). Antisera were collected just prior to the 3 and 6 month boost and were stored at −20 °C.

2.4. Preparation of the radioligand

Nal^{125} (20 μl, 2mCi) and aqueous chloramine-T (25 μg in 5 μl) were added to L-tyrosine (1.1 nmol in 5 μl of 0.15 M borate buffer, pH 8.5) and 10 μl of 0.5 M potassium phosphate, pH 7.5. The reaction, conducted at room temperature, was stopped after 60 s by the addition of aqueous sodium metabisulfite (25 μg, 10 μl). Two hundred nanomoles of NHS ester of **I** in 5 μl DMF, 10 μl of 0.5 M monopotassium phosphate and 35 μl of DMF were added and the reaction proceeded overnight (20–24 h). Purification of the radioligand was carried out by high performance liquid chromatography on an μ-Boundpak ODS C18 column, 3.9 mm × 300 mm, 10 μm particle size (Water, Milford, MA), eluting with a 40-min linear gradient of 30–70% acetonitrile in 0.1% trifluoroacetic acid (w/v). Radioactivity was detected with a radioisotope detector and 1 ml fractions were collected using a fraction collector. A representative immuno-radiochromatogram is shown in Fig. 2. The fractions were tested for immunoreactivity and stored at −20 °C, under which conditions the radioligand (Fig. 1) had a useful life of approximately 3 months.

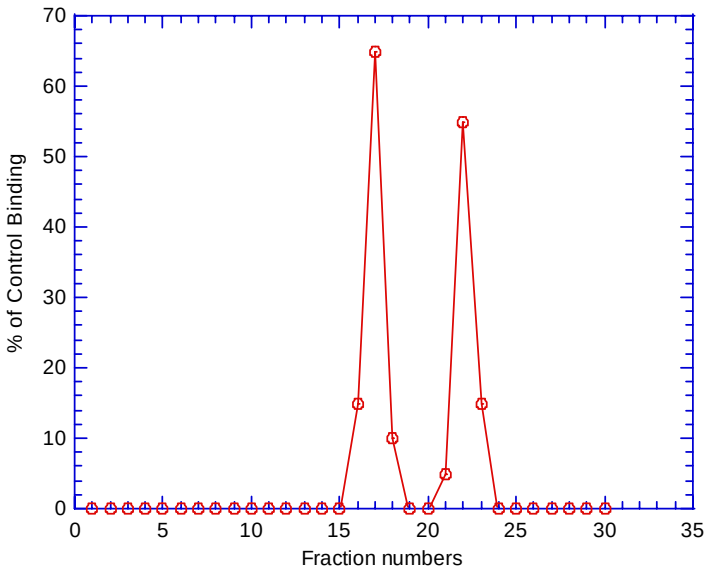

Fig. 2. Immuno-radiochromatogram for the radio-immunological activity (percent of control binding) corresponding to the [^{125}I]-iodotyrosine labeled at carboxylic acids of the **I**.

2.5. Immunoassay

The assay buffer used was 0.05 M phosphate containing 0.05 M EDTA, 0.05% sodium azide, and 2% (w/v) crotein, pH 7.5. The stock standard consisted of 0.25 ml aliquots of **I** in assay buffer at a concentration of 1 μg/ml. The standard curve, diluted from this stock, covered the range of 0.4–100 ng/ml. Quality control samples consisted of normal rat plasma containing the reference at the concentration of 1, 4 and 20 ng/ml. The rabbit antiserum was diluted according to titer in assay buffer containing rabbit-γ-globulin (0.05 ng/ml) for the double antibody precipitation. For convenience in pipetting, the radioligand was mixed with the anti-rabbit globulin. The matrix of standard were matched with 50 μl of rat control plasma, plasma and quality control samples were matched with 50 μl of assay buffer. Reagents were added to 12 mm × 75 mm glass culture tubes using an automatic pipetting station as follows: 50 μl of standard solution, quality control or test samples, 100 μl of specific antibody/γ-globulin reagent, 100 μl of second antibody/radioligand (∼20,000 dpm/tube), and 500 μl of assay buffer. Non-specific binding (nsb) was determined in tube lacking first (specific) antibody but containing the carrier γ-globulin. After overnight incubation at room temperature (18 h), the tubes were centrifuged for 45 min at 800 × *g*, the supernatants were decanted and the tubes were inverted to permit draining. Radioactivity in the pellets was determined by counting for 3 min in a gamma counter. All samples were assayed in triplicate. Using third-degree polynomial as a variant of the conventional log—logit transformation, a calibration curve of net control binding $[100(B - \text{nsb})/(B_0 - \text{nsb})]$ versus concentration was constructed, where B and B_0 are tracer bindings of sample and control plasma respectively (Fig. 3). The concentration of **I** in test samples was calculated by interpolation from the calibration curve.

3. Results

3.1. Antisera

Titration of rabbit antiserum collected 3 months after immunization demonstrated 50% binding (B_0/total counts) at a dilution of 1:3000. A stock solution was stored in aliquots with a dilution of 1:30 in assay buffer at −70 °C and further diluted prior to each analysis.

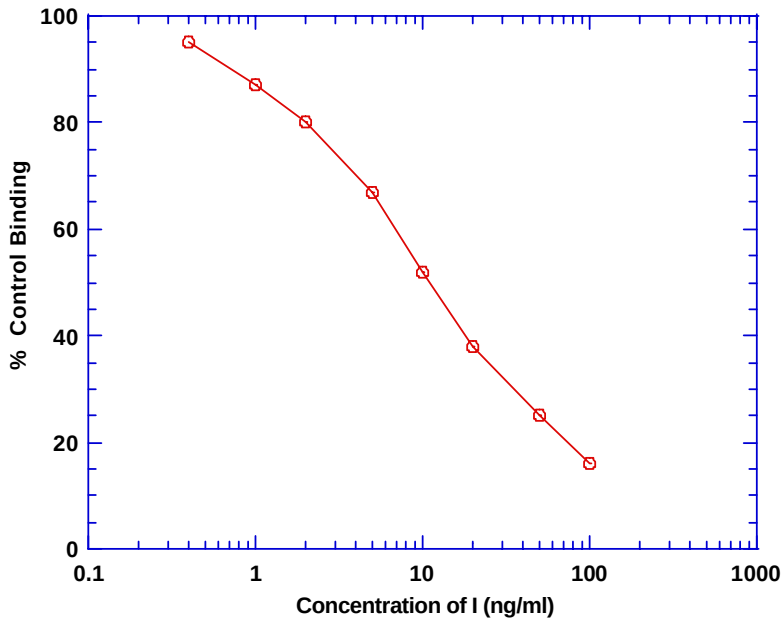

Fig. 3. A typical calibration curve for the determination of **I** in rat plasma.

Table 1
Intra-assay and inter-assay precision and accuracy data for the determination of **I** in rat plasma

Actual conc. (ng/ml)	Found conc. (ng/ml)					Mean	Accuracy[a]	CV (%)[b]
	Set 1	Set 2	Set 3	Set 4	Set 5			
Intra-assay ($n = 5$)								
0.4	0.45	0.39	0.51	0.42	0.49	0.45	113.00	10.90
1	1.05	0.90	0.95	0.92	1.11	0.99	99.00	9.10
2	2.05	2.34	2.11	1.90	2.17	2.11	105.70	7.60
5	5.21	4.89	4.45	4.77	5.11	4.89	97.72	6.10
10	10.25	10.33	9.60	9.78	9.85	9.96	99.62	3.20
20	19.40	18.85	22.20	18.23	18.65	19.47	97.33	8.10
50	46.32	46.78	48.33	47.44	50.36	47.85	95.69	3.30
100	97.10	102.33	93.28	94.88	104.56	98.43	98.43	4.90
Inter-assay ($n = 5$)								
1	0.93	1.14	0.97	1.11	0.91	1.01	101.20	10.50
4	3.53	3.89	3.70	4.10	3.74	3.79	94.80	5.70
20	18.95	18.26	20.18	19.41	20.68	19.50	97.48	5.20

[a] (Found/actual) × 100.
[b] Coefficient of variation.

Binding was independent of pH in the range of 6–8 and the assay was run routinely at pH 7.5.

3.2. Assay sensitivity and precision

A representative calibration curve is shown in Fig. 3. The assay was optimized such that 35% minimum of the total radioactivity was bound to drug free control plasma. By adopting 15% maximum for intra-assay relative standard deviation (R.S.D.) as a reliability criterion, the lowest quantifiable concentration was 0.4 ng/ml using 50 μl of plasma per assay tube. The upper limit was 100 ng/ml. Plasma containing higher than limit concentration was diluted prior to the follow-up analysis. The non-specific binding was approximately 1–2% of total counts. Inter-assay accuracy and precision were determined from the analysis of quality control samples obtained from separate assays (Table 1).

3.3. Parallelism and selectivity

Parallelism experiment was conducted on plasma from rats that were dosed with **I**. Samples were assayed following sequential dilution in drug free rat plasma. The measured concentrations of drug were multiplied by the appropriate dilution factor, yielding the results shown in Table 2. No significant dilution

Table 2
Parallelism experiments for **I** in rat control and post-dosed plasma samples

Sample no.	Initial	Concentrations (ng/ml)				
		1:2 Dilution	1:4 Dilution	1:8 Dilution	1:16 Dilution	1:32 Dilution
Rat plasma						
19	56.97	62.22	67.92	65.68	66.88	63.04
86	40.79	45.36	47.44	46.08	52.64	51.84
405	10.63	10.40	10.04	12.80	[a]	[a]
455	26.71	27.72	28.40	28.64	31.68	[a]
Control 40 ng/ml	37.27	40.56	44.16	46.08	47.20	50.88
Control 10 ng/ml	10.41	9.68	11.64	11.76	[a]	[a]

[a] Less than the lowest limit of quantification.

Table 3
Stability of **I** in rat plasma stored at −20 °C and assayed on two occasion, 6 months apart

Sample no.	Initial assay (ng/ml)	Re-assay (ng/ml)	Ratio
1	12.02	12.09	1.01
2	10.00	11.59	1.16
3	53.96	54.28	1.01
4	77.02	83.16	1.08
5	14.10	15.24	1.08

trend was observed of post-dose plasma samples compared to blank plasma spiked with known quantity of drug (control). This indicated that the selectivity of the assay was suitable for the analysis of **I** in preclinical studies.

3.4. Stability

Reanalysis of a set of post dosed rat plasma samples after the initial assay indicated that **I** was stable in samples stored at −20 °C over a period of 6 months (Table 3).

3.5. Analysis of post-dose rat samples

The RIA has been applied to the analysis of samples from preclinical rat studies. Plasma concentration-time curves obtained after rats received single dose of **I** by intravenous infusion are shown in Fig. 4.

4. Discussion

The immunogen was prepared via the active ester of carboxyl function groups of **I** to specifically avoid the formation of a bridge between the hapten and the carrier protein which could conceivably result in undesirable differences in the binding affinities of the antigen and radioligand. Two carboxyl groups on the molecule of **I** may accommodate this coupling, therefore, antibodies raised from this immunogen may recognize either one or both bridges associated with immunogen. Similarly, the radioligand prepared by reacting [^{125}I]-iodotyrosine with **I** may also yield multiple products as the result of conjugation at one or both carboxyl groups on the molecule. Fractions corresponding to the individual radioligand products were collected and screened for immunoreactivity in the RIA and subsequently pooled for assay use.

In the absence of major metabolite of this compound, the selectivity and accuracy of assay was determined both by analysis of spiked control plasma and the parallelism experiments. Assessment of recovery of **I** in spiked samples is an important test designed to detect abnormalities in binding caused

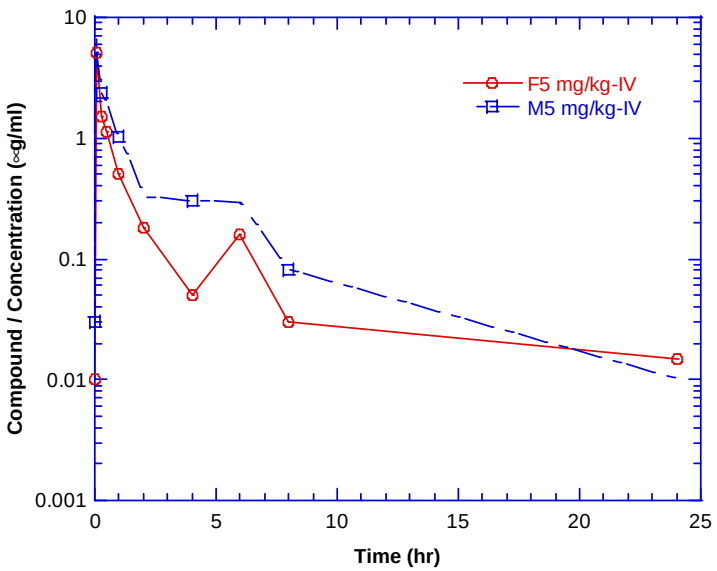

Fig. 4. Concentration–time profile of **I** in plasma of male (M) and female (F) rats receiving 5 mg/kg drug by intravenous infusion.

by differences in the endogenous plasma matrix. Parallelism experiment was performed to detect both matrix effects and any cross-reacting metabolite. A non-parallel displacement curve generally indicates the apparent increases or decreases in plasma concentration with dilution [6,7]. The developed assay satisfactorily passed both of these tests.

The assay-acceptance criteria require that the intra-assay and inter-assay relative standard deviation does not exceed 15%. The assay was satisfactory over the range of 0.4–100 ng/ml. Drug was stable in rat plasma up to 6 months when stored at −20 °C.

The advantage of using the RIA over other methods is that RIA requires very small sample volume and provides a convenient direct assay without the tedious and time consuming sample preparation steps. This is especially important in preclinical studies using animal species.

In conclusion, a simple, sensitive and specific direct radioimmunoassay has been developed and validated for the determination of the pharmacokinetics of **I** in rats. The assay required 50 μl of net plasma and had a limit of quantification of 0.4 ng/ml.

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