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MEASUREMENT OF ANGIOTENSIN CONVERTING ENZYME INHIBITORS IN SERUM BY RADIOINHIBITOR BINDING DISPLACEMENT ASSAY

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Abstract—The principle of enzyme radioinhibitor binding displacement was developed to measure the concentration of angiotensin converting enzyme (ACE) inhibitors in rat serum. ^{125}I MK351A, a tyrosyl derivative of enalaprilic acid, and a potent ACE inhibitor, bound in a concentration and time dependent manner to ACE.

Binding of ^{125}I MK351A to rat serum ACE was reduced in a concentration dependent manner *in vitro* by the ACE inhibitors MK521 (lisinopril), S9780, and Ro 31-3113-000 (Cilazapril diacid). This relationship was used to measure MK521 and S9780 in rat serum four hours after oral gavage with MK521, S9490-3 the prodrug ester of S9780, at 1, 2 and 4 mg/kg, or ½ hour after intraperitoneal injection of Ro 31-3113-000 (0.0125–0.7 mg/kg).

Serum MK521 concentrations, estimated by radio inhibitor binding displacement, and radioimmunoassay, correlated well ($r = 0.94$, $N = 9$, $P < 0.001$).

Serum MK521, S9780 and Ro 31-3113-000 concentrations measured by radioinhibitor binding displacement assay were dose related, and inversely related to serum ACE enzymatic activity.

The radioinhibitor binding displacement assay method using ^{125}I MK351A as a ligand for ACE has application to the measurement of any competitive inhibitor of ACE.

Inhibitors of angiotensin converting enzyme (ACE) form a new class of compounds proving of great clinical interest in the treatment of hypertension and heart failure [1–3]. Measurement of ACE inhibitor drug levels has been difficult, requiring either sophisticated chromatography [4], specific radioimmunoassay [5], or extraction of the drug and measurement of ACE inhibition *in vitro* by enzyme kinetic assay [6]. Apart from the necessity of studying the pharmacokinetics of ACE inhibitors, determining the plasma concentration of ACE inhibitors and the relationship to inhibition of plasma and tissue ACE activity is important in understanding the mode of action of these new antihypertensive agents.

In this paper we describe a simple method of radioinhibitor binding displacement and its application to the estimation of serum levels of ACE inhibitors in the rat.

MATERIALS AND METHODS

(a) *Radioinhibitor binding and its displacement by ACE inhibitors.* ^{125}I MK351A was used as the index ligand for binding to ACE. MK351A is a tyrosyl derivative of enalaprilic acid, and a potent competitive inhibitor of ACE. MK351A was provided by Drs Hichen and Ulm of Merck, Sharp & Dohme (Rahway, NJ). MK351A was iodinated by the chloramine T method of Hunter and Greenwood [7]. MK351A (250 ng) was dissolved in 0.5 M potassium phosphate buffer (25 μl, pH 7.5) and reacted for 45 sec with 1 mCi Na ^{125}I and 10 μl chloramine T (1 mg/ml). The reaction was terminated with 10 μl sodium metabisulphite (5 mg/ml). ^{125}I MK351A was separated from free ^{125}I by passage over a 1 × 15 cm column of SP Sephadex C25 equilibrated in 0.01 M ammonium acetate buffer (pH 3.0). The radioinhibitor was eluted with 0.1 M ammonium acetate buffer (pH 3.5) [5]. ^{125}I MK351A was stable on storage at −20° for at least 3 months.

For ACE binding and radioinhibitor binding displacement experiments, pooled rat serum was used as a source of ACE. The rat serum was diluted 1:320 in 0.05 M Tris buffer (pH 7.0) containing 0.3% bovine serum albumin, 75 mM NaCl and 50 μM $ZnSO_4$. The diluted serum (250 μl) was mixed with 50 μl ^{125}I MK351A (40,000 cpm, 10 pg/tube) together with 10 μl of a serial dilution of the relevant ACE inhibitor as standard, or 10 μl of the experimental serum sample. Final concentrations of the ACE inhibitor standards (MK521, S9780 and Ro 31-3113-000) were 10^{-12} to 10^{-4} M. After overnight equilibration at 20° ACE bound ^{125}I MK351A was precipitated by addition of 1 ml of absolute alcohol. Following centrifugation the supernatant was discarded and the radioactivity of the pellet counted in a LKB 1260 Multigamma II counter. ACE inhibitor concentration in the serum sample was read directly from the radioinhibitor binding displacement curve constructed from the serial dilutions of the ACE inhibitor standard under study, after correction for non-specific binding (which was always less than 5% of total counts added). Ten microlitres pooled rat serum was added to the standard radioinhibitor binding curve to correct for endogenous ACE binding in the serum sample.

(b) *Animal studies.* ACE inhibitors studied were MK521 (lisinopril), the lysine derivative of enalaprilic acid (supplied by Dr D. Jeremy, Merck, Sharp & Dohme, Australia); S9780 and S9490-3 (supplied by Mdm. M. Devissaguet, Institut de Recherches Internationales Servier, France) and Ro 31-3113-000

the parent diacid of cilazapril (supplied by Dr E. Kaplan, Roche Products Pty. Ltd., Dee Why, N.S.W.). Adult female Sprague–Dawley rats (200–250 g) were gavage fed MK521 or S9490-3 (1,2 and 4 mg/kg, 3 rats for each dose level).and sacrificed 4 hr later, or received an intraperitoneal injection of 0.0125, 0.25, 0.05, 0.1, 0.3 or 0.7 mg/kg Ro 31-3113-000 and were sacrificed half an hour later. Trunk blood was collected for measurement of ACE inhibitor by the radioinhibitor binding displacement method (MK521, S9780, Ro 31-3113-000) and by radioimmunoassay (MK521). Serum ACE activity was measured in control rats (N = 6) and in all rats treated with ACE inhibitor by the enzyme kinetic method of Cushman and Cheung which utilizes hippuryl-L-histidyl-L-leucine as substrate for ACE [8].

MK521 was measured by specific radioimmunoassay using antibody provided by Drs Hichen and Ulm (Merck, Sharp & Dohme, Rahway, NJ). The radio ligand used was ^{125}I MK351A. An 8 μl aliquot of plasma from each sample was added to tubes containing 20,000 cpm ^{125}I MK351A, and a 1 : 19,000 dilution of specific antibody in 0.1 M potassium phosphate buffer (pH 7.5), containing 0.05 M disodium EDTA, 1 g/l bovine serum albumin, 0.005% w/v rabbit gamma globulin, and 0.3125 units of goat anti rabbit gamma globulin antibody. The total incubate volume was 798 μl. Bound radioactivity was separated from free by centrifugation after 48 hr incubation at 4°. A standard curve was constructed using pure MK521 (Batch L-154, 826-00T 35) provided by Merck, Sharp & Dohme [5].

(c) *Statistical methods*. Comparisons between groups were by Student *t* test, with $P < 0.05$ required for significance. Linear correlation coefficients were calculated by the method of least squares. Results are expressed as mean ± SEM.

RESULTS

^{125}I *MK351A binding displacement by ACE inhibitors*

ACE bound ^{125}I MK351A was 70 to 80% of added ^{125}I MK351A in the absence of unlabelled competitive ACE inhibitor.

MK521, S9780 and Ro 31-3113-000 displaced ^{125}I MK351A bound to rat serum ACE in a concentration dependent manner. ACE bound ^{125}I MK351A was displaced by 50% (DD_{50}) at a concentration of 2.3 ng/ml by MK521, 5.2 ng/ml by S9780, and 0.13 ng/ml by Ro 31-3113-000 (see Fig. 1).

Serum ACE inhibitor concentrations

Serum MK521 concentration (ng/ml) was estimated by radioinhibitor binding displacement and by specific radioimmunoassay in each serum sample. There was a good correlation between estimates by each method ($r = 0.94$, N = 9, $P < 0.001$). Serum lisinopril concentration increased in a dose related manner (Table 1).

Serum S9780 concentration (ng/ml) 4 hr after oral gavage of S9490-3 are shown in Table 1. Similar serum concentrations were achieved at 1 and 2 mg/kg, rising to higher levels with the 4 mg/kg dosage.

Serum Ro 31-3113-000 concentration (ng/ml) ½ hour after intraperitoneal injections are shown in

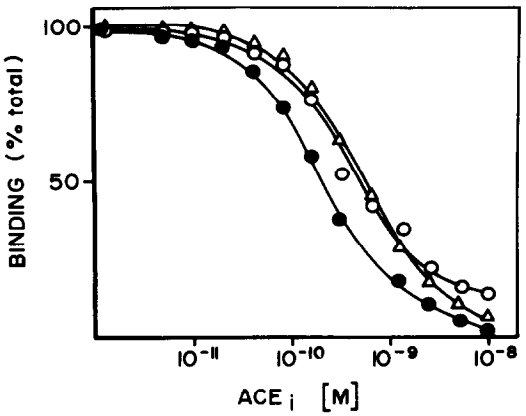

Fig. 1. Radioinhibitor binding displacement from rat serum ACE by MK521, S9780 and Ro 31-3113-000. ^{125}I MK351A was displaced from rat ACE in a concentration related manner by each ACE inhibitor (ACE_i[M]). MK521 = ○, S9780 = △, Ro 31-3113-000 = ●.

Table 1. Mean drug levels at each dose correlated closely with intraperitoneal dosage ($r = 0.989$; $P < 0.001$).

ACE inhibitor concentrations related to ACE enzymatic activity

There was a close correlation between serum drug concentration and inhibition of serum ACE enzymatic activity for all three compounds. In six normal rats the mean serum ACE activity, measured by the enzyme kinetic assay of Cushman and Cheung [8] was 48.3 ± 3.2 nmol histidyl-leucine/ml/min. Serum ACE activity of rats treated with ACE inhibitors was measured within the same assay, and the results expressed as percentage of the mean of the normal group (Table 1). After treatment with each ACE inhibitor, ACE activity (% of normal) fell in an ACE inhibitor dose related manner (Table 1). There was a concentration dependent relationship between serum ACE activity (% of normal) and serum ACE inhibitor concentration (ng/ml), as measured by the radioinhibitor binding displacement assay (Fig. 2). *In vivo* serum ACE inhibitor concentration at which 50% of normal ACE activity was inhibited (ID_{50}) was estimated at 5 ng/ml for MK521 (lisinopril), 12 ng/ml for S9780 and 0.12 ng/ml for Ro 31-3113-000 (Cilazapril diacid).

DISCUSSION

We have applied the technique of radio inhibitor binding displacement to develop an assay system applicable to all competitive ACE inhibitors. The assay technique utilizes the unique competitive ACE inhibitor MK351A, which is a tyrosyl derivative of enalaprilic acid. MK351A is easily iodinated, and has been used by us [9, 10] and others [11] to develop a direct binding assay for the quantitation of ACE in serum and tissues. We have now further utilized this radioligand to develop a simple radioinhibitor binding displacement assay for quantitation of competitive ACE inhibitors.

Table 1. Serum concentration of ACE inhibitors and serum ACE activity following treatment of rats with ACE inhibitors MK521, S9490-3 and Ro 31-3113-000

ACE inhibitor	Dose (mg/kg)	ACE inhibitor serum concentration (ng/ml) RIDA	RIA	ACE activity (% of normal serum)
MK521	0	0	0	100 ± 7
	1	5.7 ± 0.7	5.9 ± 0.4	47 ± 1
	2	17.3 ± 3.6	13.5 ± 2.2	26 ± 3
	4	41.6 ± 3.5	39.3 ± 6.4	12 ± 2
S9780	0	0	—	100 ± 7
	1	23.6 ± 1.8	—	22 ± 2
	2	20.4 ± 2.3	—	22 ± 6
	4	44.7 ± 8.2	—	14 ± 1
Ro31-3113-000	0	0	—	100 ± 7
	0.0125	0.062 ± 0.004	—	81 ± 10
	0.025	0.102 ± 0.002	—	61 ± 6
	0.05	0.45 ± 0.17	—	21 ± 6
	0.10	0.63 ± 0.17	—	14 ± 4
	0.30	2.76 ± 0.33	—	0 ± 0
	0.70	4.83 ± 0.09	—	1 ± 1

RIDA = radioinhibitor binding displacement assay; RIA = radioimmunoassay.

Radioligand techniques have been widely applied in studies localizing and characterizing enzymes, using specific antibodies labelled with radioactive compounds, but have been less widely applied with radioactive labelled inhibitors. ^{3}H captopril has been used to quantitate ACE in tissues [12]; however, this compound is relatively unstable, may form dimers and bind to endogenous reducing compounds [4].

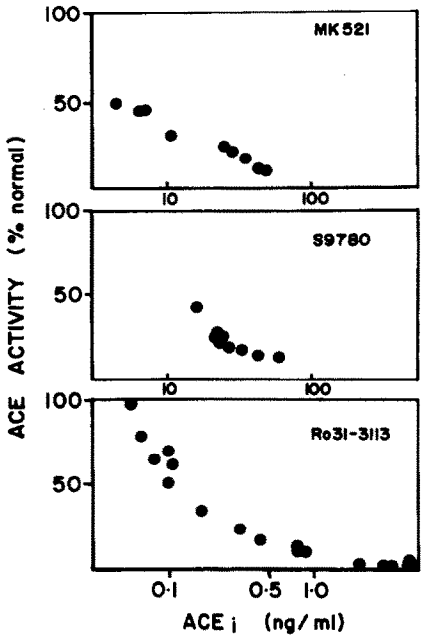

Fig. 2. Correlation between serum concentration of ACE inhibitor (ng/ml) measured by radioinhibitor binding displacement assay and serum ACE activity (% of normal) in rats following treatment with MK521 (upper panel), S9490-3 (the ester of S9780, middle panel), or Ro 31-3113-000 (lower panel).

Captopril may also bind to ACE in a noncompetitive manner [13]. ^{125}I MK351A does not contain a sulphydryl element, and has low nonspecific binding in rat serum [9, 10].

Previous work using an ^{125}I MK351A displacement assay by Fyhrquist *et al.* [14] has demonstrated a progressive increase in ^{125}I MK351A displacement following a single dose of captopril. As captopril is unstable comparisons of drug concentration were not reported by these workers. Fyhrquist *et al.* [14] also reported that other ACE inhibitors displaced ^{125}I MK351A from ACE, and suggested this could be utilized to estimate ACE inhibitor concentrations [14].

In this study ^{125}I MK351A bound to rat serum ACE was displaced by MK521, S9780 and Ro 31-3113-000 in a concentration related manner. We have utilized this to develop a simple assay system to measure MK521, S9780 and Ro 31-3113-000 in rat serum. MK521 was estimated by both radioinhibitor binding displacement assay and by direct radioimmunoassay to confirm the correlation of concentrations measured by both techniques. There was an excellent concordance between results. Furthermore, increasing doses of oral or intraperitoneal ACE inhibitor gave increases in serum drug levels, and serum ACE enzymatic activity was reduced. Log_{10} drug concentration and ACE inhibition for each ACE inhibitor were related (Fig. 2), supporting the contention that the radioinhibitor binding displacement assay measures circulating levels of the ACE inhibitor. *In vivo* ID_{50} was estimated from the curve relating serum drug concentration and % ACE inhibition. Values obtained for each inhibitor (MK521, 5 ng/ml; S9780, 12 ng/ml; Ro 31-3110-000, 0.12 ng/ml) corresponded to the ID_{50} measured *in vitro* using rat serum ACE in the assay of Cushman and Cheung (MK521, 2.3 ng/ml; S9780, 5.2 ng/ml; Ro 31-3110-000, 0.13 ng/ml) [15]. The relationship between serum ACE activity and drug level, once

determined for a particular ACE inhibitor and animal species, could potentially be used to estimate drug levels. This is, however, only applicable in acute experiments as during chronic ACE inhibitor treatment serum ACE activity is induced, with a resultant shift of the ACE inhibitor concentration versus ACE inhibition curve to the right [16, 17]. Several ACE inhibitors have been measured by specific radioimmunoassay methods, that are usually relatively simple [5]. A specific antibody must, however, be raised for each new drug. Antibodies also have the potential of cross-reaction with biologically inactive drug fragments produced during drug metabolism. In contrast, the radioinhibitor binding displacement method can be applied to any competitive ACE inhibitor, and is specific for the active component responsible for ACE inhibition.

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