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Determination of an echinocandin, MK-0991, in mammalian plasma by radioimmunoassay

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Abstract

A quantitative method based on radioimmunoassay for the determination of the antifungal agent, CANCIDAS™ (MK-0991) has been developed and validated. The immunogen was prepared by coupling MK-0991 to bovine serum albumin through a two-step reaction with difluorodinitrobenzene. An antiserum specific to MK-0991 was selected for RIA. The assay was based on the competitive immunoassay principle in which the drug competes with iodinated drug for a limited quantity of specific antibody. The bound tracer was separated via goat anti-rabbit globulin. The assay demonstrates good accuracy and reproducibility at plasma concentration down to 10 ng/ml. The specificity of the RIA method was confirmed by cross-validating against an established HPLC method.

Keywords: Radioimmunoassay; Cancidas; Echinocandia; Antifungal; Quantitation

1. Introduction

MK-0991 (Fig. 1) is a semi-synthetic acylated cyclic lipopetide compound in the echinocandin class isolated from the fermentation of *Glarea lozoyensis*. MK-0991 is an antifungal agent that acts by interfering with β-(1,3)-glucan synthesis in cell wall formation in fungi. MK-0991 is under development for the treatment of *Candida* and *Aspergillus* infections in humans [1–6]. A sensitive method for the quantitation of MK-0991 in biological fluids was required to support the preclinical studies necessary to develop this drug candidate. This paper describes the development and validation of a competitive binding radioimmunoassay (RIA) and its application to mammalian plasma. The selectivity of the RIA method is confirmed by the analysis of plasma samples after dosing rats with MK-0991, using both RIA and the HPLC method with fluorescence detection developed concurrently in human plasma for supporting clinical studies [7].

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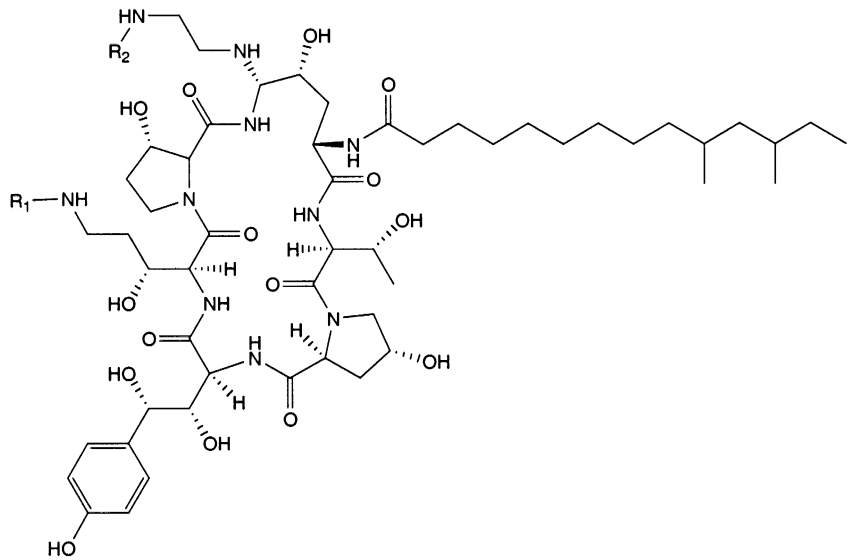
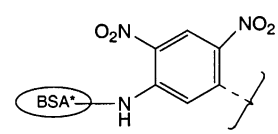
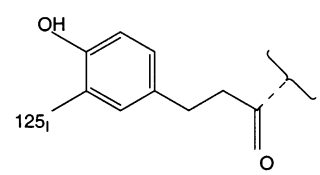
2. Experimental

2.1. Reagents

The following reagents were purchased from the indicated vendors: rabbit IgG and goat anti-rabbit gamma-globulin from Arnel Products Co. (New York, NY); bovine serum albumin (BSA) was obtained from Calbiochem (San Diego, CA); control plasma (in EDTA) was supplied by Bio-



IDENTITY	SUBSTITUENT ON R1 or R2 PARENT MOLECULE
MK-0991	H—
MK-0991 Immunogen	
MK-0991 Radioligand	

* BSA = Bovine Serum Albumin

Fig. 1. Chemical structures of MK-0991, MK-0991 immunogen and MK-0991 radioligand.

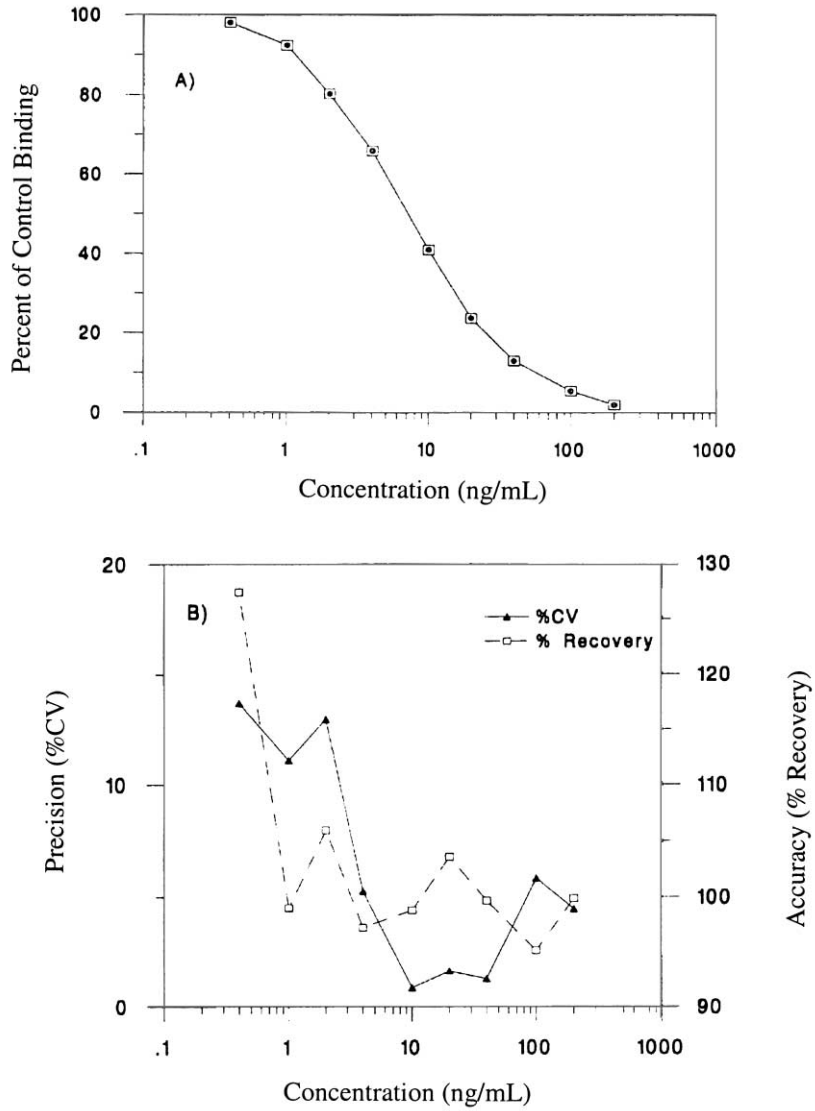


Fig. 2. Effect of plasma volume (μl per tube) on radioligand binding.

logical Specialty (Colmar, PA); potassium phosphate, ethylenediaminotetraacetic acid (EDTA) and sodium azide were from Sigma (St. Louis, MO); I-125-labeled sodium iodide and I-125 labeled Bolton–Hunter reagent were purchased from Amersham (Arlington Heights, IL); chloramine-T, sodium metabisulphite and 1,5-difluoro-2,4-dinitrobenzene were obtained from Aldrich (Milwaukee, WI). MK-0991 and its metabolites were obtained within Merck Research Laboratories (Rahway, NJ). The remaining agents were widely available and not dependent on the source.

2.2. Preparation of the immunogen

The immunogen was prepared by coupling MK-0991 to BSA via a dinitrophenyl bridge.

MK-0991 (0.03 mmol) in deionized H_2O was reacted with 0.06 mmol of 1,5-difluoro-2,4-dinitrobenzene in DMSO at room temperature for 2 h. Without isolation, the reaction mixture was added to bovine serum albumin (20 mg, 0.31 μmol) in 0.77 ml of 0.125 M potassium phosphate, (pH 8.5). Additions were made in small (0.1 ml) aliquots with the solution stirring at room temperature over a 2-h period. The solution was stirred overnight at room temperature. Exhaustive dialysis against water for 96 h was carried out at 0–4°C and the immunogen solution was diluted to 20 ml with distilled water. The extent of incorporation of MK-0991 into the immunogen was estimated by measurement of the difference in mass between the immunogen and the control BSA using MALD1-TOF/MS at ≈ 4 mol/mol of BSA.

2.3. Immunization

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

2.4. Preparation of the radioligand

Radioligand was prepared by adding 8 nmol of MK-0991 in 10 μl of 0.1 M borate buffer (pH 8.5) to 1 mCi of I-125 labeled Bolton–Hunter reagent. The reaction mixture was incubated overnight in the refrigerator. Purification of the radioligand was carried out by high performance liquid chromatography (HPLC) on a μBondapak C18 column (10 μm, 3.9×300 mm, i.d.) (Water, Milford, MA). A 35-min linear gradient of 25–90% acetonitrile in 0.1% TFA was programmed and executed with a flow rate of 1 ml/min. Radioactivity was detected with a Model 170 radioisotope detector (Beckman Instruments, Irvine, CA) and 1-ml fractions were collected using a Gilson Model FC203 Fraction Collector (Middleton, WI). The fractions containing the bulk of the radioactive products were tested for immunoreactivity and stored at -20°C, under which conditions the radioligand had a useful life of ≈ 3 months.

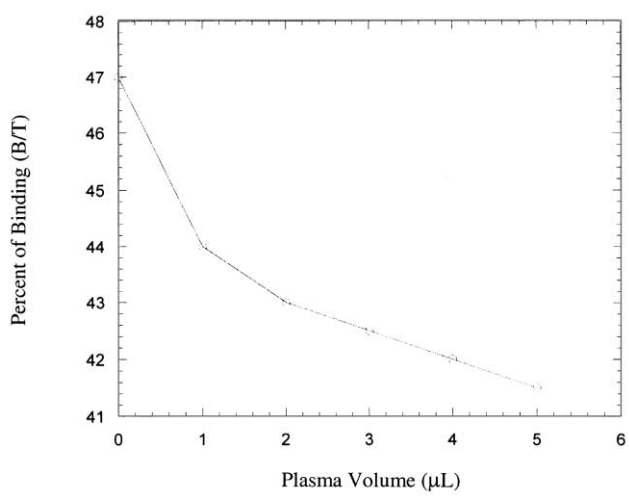


Fig. 3. Calibration curve (A) and precision and accuracy profiles; (B) for the determination of MK-0991 in plasma by RIA. Drug concentrations are expressed as ng per ml of plasma diluted 1:10 with buffer.

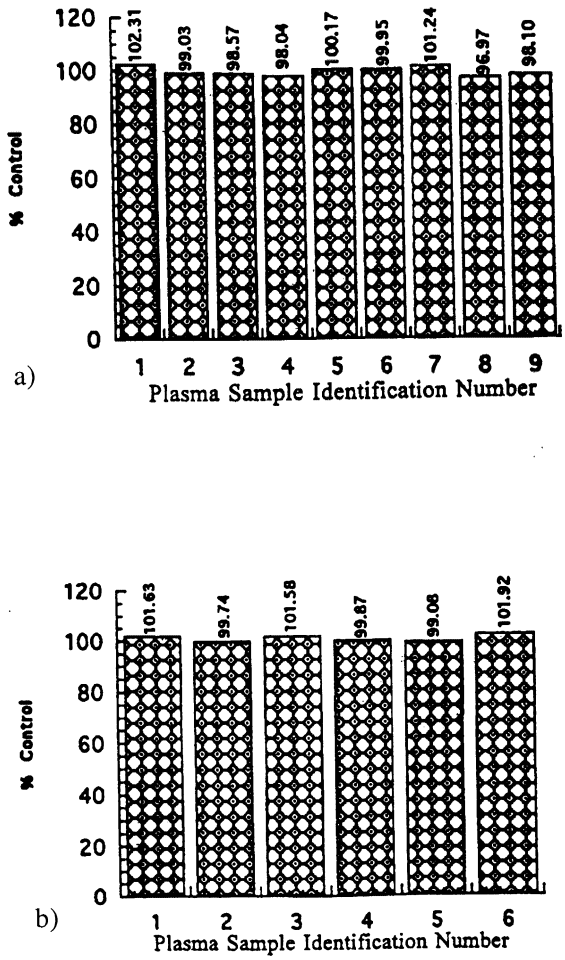


Fig. 4. Results of the effect of rat plasma (a) and monkey plasma (b) from different animals on percent of control binding.

2.5. Immunoassay

The assay buffer was 0.05 M potassium phosphate containing 0.05 M EDTA, 0.05% sodium azide, w/v and 0.1% BSA, (pH 7.5). The stock standard was dissolved in distilled water at a concentration of 1 mg/ml. Working standards were stored as 10 μg/ml solutions in assay buffer. The standard curve (Fig. 2) diluted from this stock, covered the range of 0.5–200 ng/ml. The buffer used to dilute the standards contained 0.02% w/v Triton X-100. The rabbit antiserum was diluted according to titre in assay buffer containing rabbit γ globulin (0.05 ng/ml) to provide bulk for the double antibody precipitation. Plasma samples and quality controls were diluted 1:10 in 0.01% Triton buffer. Reagents were added to 12×75 mm glass culture tubes using an automatic pipetting station (APS, Micromedic Systems, Horsham, PA) as follows: 0.025 ml standard solution, diluted quality control or diluted sample, 0.1 ml specific antibody/γ globulin reagent, 0.1 ml radioligand (20000 cpm per tube) and 0.3 ml buffer containing Triton X-100 at 0.01%. Non-specific binding (nsb) was determined in tubes lacking the specific antibody but containing the carrier γ globulin. After 3 h incubation at room temperature, 0.1 ml of goat anti-rabbit antibody was added to the assay mixture followed by an overnight incubation at room temperature (ca 18 h), the tubes were centrifuged for 45 min at $800 \times g$, supernatants were decanted and the tubes were inverted to permit draining. Radioactivity in the pellets was determined by counting for 3 min in a Micromedic Model Apex 10/600 multidetector gamma counter. All samples were assayed in triplicate. Using a third-degree polynomial as a variant of conventional logit–log transformation, a calibration curve of net control binding $[100(B - nsb)/(Bo - nsb)]$ versus concentration was constructed. The concentration of MK-0991 in test samples was calculated by interpolation from the calibration curve.

3. Results

3.1. Antisera

Titration of rabbit antiserum (No. 137) collected 3 months after immunization demonstrated 50% binding (B/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. Binding was independent of pH in the range 6–8 and the assay was run routinely at pH 7.5.

Table 1
Intra-assay precision for the determination of MK-0991 in diluted (1:10) monkey plasma

Target concentration (ng/ml)[b]	B/Bo[a]	Readback concentration (ng/ml)						Mean $n=6$	Recovery (%) found/actual	C.V.[c] (%)
		Curve 1	Curve 2	Curve 3	Curve 4	Curve 5	Curve 6			
0.4	98.03	0.59	0.54	0.46	0.42	0.58	0.47	0.51	127.5	13.7
1	92.35	1.19	1.00	0.88	0.93	1.03	0.93	0.99	99.0	11.1
2	80.22	2.13	1.95	2.19	2.61	1.81	2.03	2.12	106.0	12.9
4	65.86	3.93	4.01	4.08	3.71	4.04	3.57	3.89	97.2	5.2
10	40.89	10.0	9.87	9.75	9.91	9.89	9.83	9.88	98.8	0.8
20	23.72	21.5	21.5	21.2	20.9	21.5	20.7	21.2	103.6	1.6
40	12.99	39.4	40.0	40.6	39.1	39.9	39.9	39.8	99.6	1.2
100	5.42	100.5	91.1	101.2	96.8	94.0	86.9	95.1	95.1	5.8
200	1.95	191.3	209.1	211.9	191.8	198.8	194.9	199.6	99.8	4.4

[a] B/Bo = normalized binding.
[b] Concentrations are expressed as ng/ml of diluted plasma.
[c] Coefficient of variation.

3.2. Effect of plasma volume

The binding of the radioligand to the antibodies was critically dependent on the volume of plasma used. The assay was configured to use 0.025 ml of a mixture of plasma diluted 1:10 with buffer (equivalent to 2.5 μl undiluted plasma) per assay tube (Fig. 3). This plasma volume defined the useful concentration range of 10 to 1000 ng/ml of test plasma and is a compromise between the need for sensitivity and the adverse effect of test plasma on binding. The lower limit of control plasma radioligand binding was at 30% (B/T) of total radioactivity. Results of the plasma matrix effect from rats and monkeys on percent of control binding are shown in Fig. 4. No significant variation in percent control binding was found with individual rat or monkey plasma.

3.3. Assay precision and working range

The intra-assay precision was determined from seven sets of standards at a concentration of 0.4–200 ng/ml of diluted plasma (1–10 in assay buffer). All samples were assayed in one batch. Samples from Sets 1 through 6 were read as unknowns against the curve generated from the reference curve (Table 1).

The inter-assay precision was determined by the analysis of quality control samples prepared at

Table 2
Determination of inter-day accuracy and precision for the determination of MK-0991 in rat and monkey plasma by radioimmunoassay

Actual concentration (ng/ml)[b]	Found concentration (ng/ml)[b]				Mean $n=4$	Accuracy (%) found/actual	C.V.[c] (%)
Rat plasma							
2	2.28	2.11	2.1	1.75	2.06	103.00	10.80
10	9.88	10.66	10.79	10.41	10.44	104.40	3.85
40	40.17	46.09	38.48	45.91	42.66	106.65	9.18
Monkey plasma							
2	ns[a]	2.09	2.10	1.80	2.00	100.00	8.50
10	9.54	9.12	10.00	9.31	9.49	94.90	4.00
40	38.79	39.82	36.89	40.92	39.11	97.78	4.38

[a] ns, No sample due to dilution error.
[b] Concentrations are expressed as ng/ml of diluted plasma; equivalent to 20, 100 and 400 ng/ml in undiluted plasma, over a period of 10 days in rat plasma and 33 days in monkey plasma.
[c] Coefficient of variation.

Table 3
Determination of assay accuracy using the method of standard addition to test rat and monkey plasma

Sample (h)	Unspiked	Spiked	Expected (E)	Actual (A)	% (A/E)
		Concentration (ng/ml)[a]			
Rat					
A-2	8.41	20.62	29.03	28.78	99.1
A-48	5.44	20.62	26.06	24.52	94.0
F-2	8.38	20.62	29.00	27.68	95.4
F-48	8.55	20.62	29.17	29.55	101.3
Monkey					
M1-72	11.37	5.00	16.37	16.11	98.4
M1-240	1.61	5.00	6.61	7.03	106.3

[a] Concentrations are expressed as ng/ml of diluted (1:10) plasma.

Table 4
Parallelism of MK-0991 in test plasma samples

Sample	Time (h)	Initial	1:2 Dilution	1:4 Dilution	1:8 Dilution	1:16 Dilution
			Concentration (ng/ml)[b]			
Monkey plasma						
1	0.08	60.5	57.4	62.3	64.9	59.9
1	0.25	64.2	74.8	77.2	68.7	76.7
1	8	54.7	61.2	63.8	62.3	55.7
1	24	[a]	90.4	91.8	86.8	87.2
Control plasma	–	[a]	78.2	88.6	86.8	89.3
Rat plasma						
A	0.5	84.7	78.0	81.1	81.2	76.8
A	32	18.0	24.4	23.5	26.3	22.7
F	0.5	89.1	85.3	86.6	86.3	91.2
F	32	31.7	33.1	35.1	36.9	46.7
Control plasma	–	88.1	95.9	99.2	88.2	94.0
Human plasma						
013	6	763.3	835.1	797.1	749.4	697.3
014	4	836.2	857.4	846.5	767.7	789.8
018	2	845.6	936.9	873.1	804.5	767.5
Control plasma	–	365.4	377.2	395.2	356.0	384.8

[a] Out of assay range.
[b] Concentrations are expressed as ng/ml of diluted (1:10) plasma.

Table 5
Comparison of plasma concentrations of MK-0991 obtained by RIA and HPLC after intravenous administration to rats

Time (h)	Plasma concentration (μg/ml)									
	Rat A		Rat B		Rat C		Rat D		Rat F	
	RIA	HPLC	RIA	HPLC	RIA	HPLC	RIA	HPLC	RIA	HPLC
0.08	13.04	11.75	13.91	13.21	14.54	11.63	17.38	16.65	11.42	10.81
0.25	9.35	9.28	8.64	7.80	9.95	7.76	11.82	11.43	9.56	8.33
0.5	8.80	8.35	7.97	7.30	8.58	8.52	9.67	10.30	8.58	9.18
1	6.67	7.19	7.19	6.98	7.17	6.59	8.68	8.12	5.38	5.72
2	5.51	6.16	6.43	4.67	6.19	6.36	6.95	6.43	5.78	5.51
4	4.21	4.50	4.54	4.18	4.58	4.58	4.38	4.48	4.21	4.25
8	2.91	2.71	2.99	3.13	2.98	2.99	3.60	3.57	2.97	2.77
24	0.37	0.33	0.72	0.42	0.47	0.39	0.62	0.48	0.54	0.47

concentrations equivalent to 2, 10 and 40 ng/ml of diluted plasma. The data (Table 2) showed acceptable values for precision of rat and monkey plasma samples.

A representative calibration curve and the precision and accuracy profiles are shown in Fig. 3. The diluted plasma assay binding was considered to be satisfactory over the range 1–100 ng/ml. Although the precision values at 0.4 and 200 ng/ml were acceptable, the percent of control binding was regarded as too low or high to be reliable. The average non-specific binding was 8.5%. The assay precision at the lower quantifiable limit (10 ng/ml) was 11.1%. Test plasma, containing MK-0991 at concentrations in excess of 1000 ng/ml, were further diluted prior to analysis.

3.4. Accuracy and parallelism

The assay accuracy was determined by the analysis of quality control samples (Table 2). Aliquots of four rat plasma and two monkey plasma samples collected following intravenous administration of MK-0991 were assayed both before and after the addition of a spike of standard MK-0991. Representative samples of early and late times post-dose were examined. The results in Table 3 show analytical data that are not significantly different ($< 10\%$) from the theoretical values and therefore, the assay's accuracy is not compromised by the matrix.

Parallelism was conducted on plasma from rats, monkeys and humans that had received MK-0991 intravenously. Samples were assayed following sequential dilution in drug free matrices. The measured concentrations of MK-0991 were multiplied by the appropriate dilution factor, yielding the results shown in Table 4. No significant trend to higher or lower concentrations with dilution was observed.

3.5. Selectivity

The selectivity of the immunoassay was determined by cross-validation with a selective HPLC based method [7]. Plasma samples, obtained serially from five rats receiving MK-0991 intravenously, were assayed by both methods (Table 5 and Fig. 5). The average ratio of RIA/HPLC data was at 1.05 with a variation coefficient $< 10\%$. The regression analysis indicated a small positive bias in the RIA method. This is within the criterion of accuracy and it is concluded that RIA is in good agreement with the HPLC method. The small bias of the RIA data might indicate interference from metabolites but the effect is insignificant. Human clinical study plasma samples were also compared in both methods (data on in-house file). The average ratio (RIA/HPLC) was 0.999 with a variation coefficient of 14%. Analytical data were further compared using a paired t-test and regression analysis. The observed t-value (-0.776 with 48 dof) when compared with t-critical (2.0101) showed no significant difference between the methods. Similarly, the linear regression showed the absence of a proportional bias. Hence, the methods are essentially identical and the specificity of the RIA is confirmed.

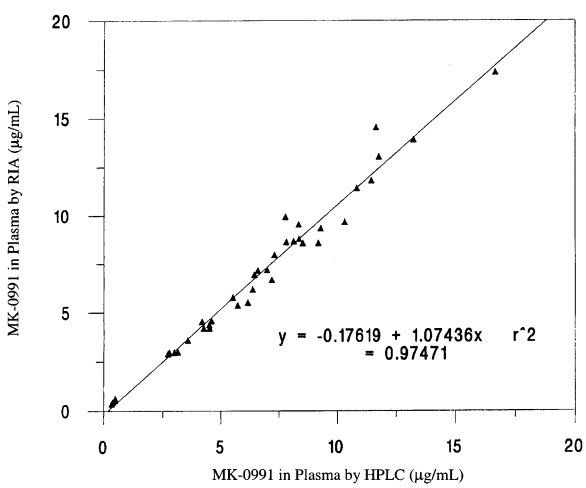


Fig. 5. Cross-validation of assays for the determination of MK-0991 in plasma by RIA and HPLC.

4. Discussion

The immunogen was prepared by coupling MK-0991 to BSA via a dinitrophenyl bridge to specifically avoid the non-specific coupling between hapten and the carrier protein. Two amino groups on the MK-0991 structure 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-labeled Bolton–Hunter reagent with MK-0991 may also yield multiple products as the result of conjugation at one or both amino group on the molecule. Individual radioligand product peaks were screened for immunoreactivity in the RIA and subsequently pooled for assay use.

Parallelism experiments were conducted on immunoassays in an attempt to detect unknown drug related substances that might compete with the antigen for binding sites in the antibodies. Such substances, generally metabolites or degrades, frequently show cross-reactivity with respect to antigen, which are not constant but

which increase with increasing dilution. Plots of parallelism were constructed to the normalized found concentration versus dilution factor. There was no significant trend to raise or lower concentrations in plasma and hence no indication of interfering substances.

The criteria for satisfactory cross-validation of bioanalytical methods [8,9] are somewhat subjective. We chose to use regression analysis as a measure of fixed and relative bias, the R.S.D. of the ratio being an indication of random error and the paired Student's t-test assessing the overall assay acceptability [10,11]. Both cross-validation experiments show non-significant t-statistics, absence of fixed error and random error for RIA. A small positive proportional error between 1.5 and 13.3% was observed in rats plasma. The mean ratio of RIA/HPLC results of rat and human plasma samples was satisfactory. The measured reproducibility of the determinations was within the limits predicted from the individual variances of the assays. Based on these results, we concluded that the RIA and HPLC based assays were essentially equivalent and accordingly, that the immunoassay demonstrated satisfactory selectivity. The advantage of using the RIA over the HPLC method is that RIA requires very small sample volume and provides a convenient direct assay without the complicated sample extraction. This is particular important at the preclinical animal studies. Small sample volume precludes the use of HPLC method.

In summary, the MK-0991 radioimmunoassay showed satisfactory accuracy and precision along with good selectivity and demonstrated its usefulness as a means of quantitation of this drug in mammalian plasma.

Acknowledgements

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