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Determination of MK-383, a non-peptide fibrinogen receptor antagonist, in human plasma and urine by radioimmunoassay

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Abstract: MK-383 is a novel, non-peptide fibrinogen receptor antagonist. A sensitive and specific radioimmunoassay has been developed for the determination of this drug candidate in plasma and urine. The immunogen was prepared by coupling to albumin via the N-hydroxysuccinimide ester from which the radioligand was also prepared by reaction with [I^{125}]iodotyrosine. The method was specific and no immunoreactive material other than the parent drug was detectable in plasma and urine from dosed volunteers. This direct assay, using 5 μl of plasma or 0.5 μl of urine, is sensitive to 1 and 10 ng ml^{-1}, respectively, without matrix interference and has sufficient sensitivity, specificity, accuracy, and precision for the analysis of clinical samples.

Keywords: *MK-383; fibrinogen receptor antagonist; radioimmunoassay (RIA); antithrombotic drug; plasma; urine.*

Introduction

Platelet activation and aggregation play a major role in thrombosis during vaso-occlusive disorders such as acute myocardial infarction, unstable angina pectoris, and re-occlusion following thrombolytic therapy. Anti-thrombotic therapy can be attained through pharmacological antagonism of platelet aggregation [1–4]. It has been shown that the final step in platelet aggregation involves the binding of plasma fibrinogen to an activated platelet membrane-bound glycoprotein complex, GPIIb/IIIa [5–7]; the tripeptide sequence Arg-Gly-Asp represents the minimal amino acid sequence necessary for fibrinogen binding and this interaction presents an opportunity to inhibit platelet aggregation with small molecules that interfere with fibrinogen binding [8].

MK-383 is a specific, non-peptide fibrinogen receptor antagonist of nanomolar potency [9]. A sensitive method for the quantitation of MK-383 in biological fluids was required to support the preclinical and clinical studies necessary to develop this drug candidate. This report describes the development and performance of a competitive binding radioimmunoassay (RIA) and its application to human plasma and urine.

Experimental

Materials

MK-383, N-(*n*-butanesulphonyl)-O-[4-(butane-4-piperidinyl)]-L-tyrosine (Fig. 1), and two potential metabolites, L-708,350 (N-*n*-butanesulphonyl-L-tyrosine) and 4-piperidinyl-butan-1-ol, were synthesized by the Medicinal Chemistry Department, Merck Research Laboratories, West Point, PA.

Mono- and di-basic sodium phosphate, sodium azide, disodium ethylenediamine tetra-acetic acid (EDTA), L-tyrosine, hydrochloric acid, acetonitrile, N,N-dimethylformamide (DMF), phosphoric acid, triethylamine, and sodium tetraborate were obtained from Fisher (Fair Lawn, NJ). Bovine serum albumin (BSA, catalogue no. A7638), sodium heparin, and 8-anilino-1-naphthalenesulphonic acid (ANSA) were purchased from Sigma (St Louis, MO); sodium hydroxide and acetic acid were from Mallinkrodt (Paris, KY); sheep anti-rabbit gamma globulin serum was obtained from Arnel Products (New York, NY); rabbit gamma globulin and the BSA used to prepare the immunogen were supplied by Calbiochem (La Jolla, CA); ^{125}I-labelled sodium iodide was purchased from Amersham (Arlington Heights, IL); chloramine-T, sodium meta-bisulphite, N-hydroxysuccinimide and dicyclo-

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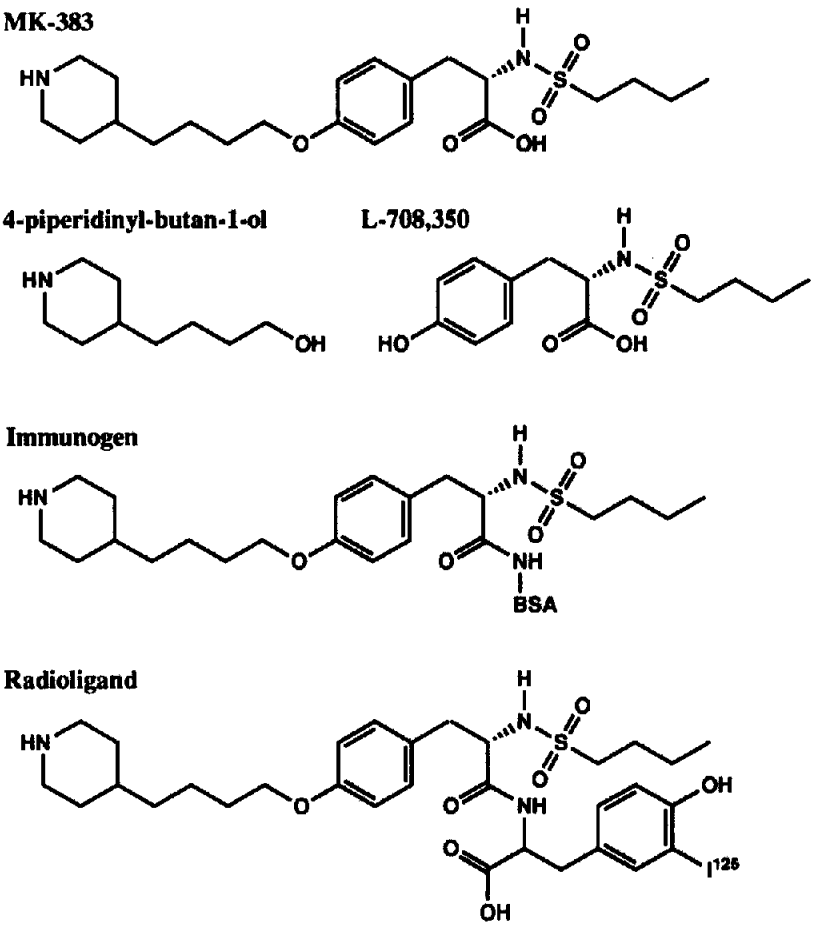

Figure 1
Structures of MK-383, its putative metabolites, L-708,350 and 4-piperidinyl-butan-1-ol, and the immunogen and radiotracer used in development of the immunoassay.

hexylcarbodiimide were obtained from Aldrich (Milwaukee, WI).

Preparation of the immunogen

The N-hydroxysuccinimide ester (NHS active ester) was prepared by reacting 0.033 mmol MK-383, 0.033 mmol N-hydroxysuccinimide, and 0.10 mmol dicyclohexylcarbodiimide in 2.0 ml dry N,N-dimethylformamide at room temperature for 48 h. Without isolation, 1.46 ml of the active ester solution was added to bovine serum albumin (BSA, 20 mg, 0.31 μmol) in 0.77 ml 0.125 M potassium phosphate, pH 8.5. DMF, 1.46 ml was added to a control solution of BSA. Additions were made in small (0.1 ml) aliquots with stirring at 0–4°C over a 4-h period. The solutions were diluted with 1.0 ml distilled water and stirred overnight at 0–4°C. 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-383 into the immunogen was approximately 25 mol/mol of BSA.

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, im and sc). Antisera were collected just prior to the 3- and 6-month boost and were stored at −20°C prior to use.

Preparation of the radioligand

$Na^{125}I$ (20 μl, 2 mCi) and aqueous chloramine-T (25 μg in 5 μl) were added to L-

tyrosine (1.1 nmol in 5 μl 0.16 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 metabisulphite (25 μg, 10 μl). Two hundred nmol of the N-hydroxysuccinimide ester of MK-383 in 5 μl DMF and 40 μl DMF were added and the mixture was incubated overnight. Purification of the radioligand was carried out by LC on a (μBondapak) C_{18} column (10 μm, 3.9 × 300 mm, i.d.) (Waters, Milford, MA), eluting with a 50-min linear gradient of 15–50% acetonitrile in 0.15 M phosphoric acid, adjusted to pH 3.5 with triethylamine, with a flow rate of 1 ml min^{-1}. 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). A representative radiochromatogram is shown in Fig. 2. 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 approximately 3 months. Although the mono-iodinated radioligand was generally used, the di-^{125}I-labelled species is also satisfactory.

Fractionation of plasma samples

To verify the specificity of the assay, human clinical samples obtained 1 and 3 h after commencement of a 1-h infusion of MK-383 (0.4 μg kg^{-1} min^{-1}) were first assayed directly.

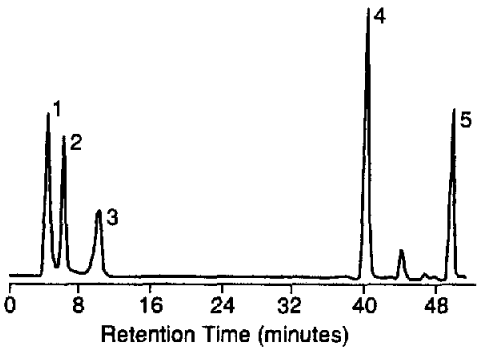

Figure 2
Radiochromatogram of the products of the reaction of the N-hydroxysuccinimide ('active') ester of MK-383 with ^{125}I-labelled tyrosine. Chromatographic conditions are described in the text. Peak 1 corresponds to iodide, peaks 2 and 3 are mono- and di-iodotyrosine, and peaks 4 and 5 correspond, respectively, to the mono- and di-iodinated radioligands.

Then further aliquots were deproteinated with two volumes of acetonitrile, precipitated protein was removed by centrifugation, and the supernatant reduced to dryness in a SpeedVac Model SVCZ001-1 (Savant, Farmingdale, NY). The residue was reconstituted in mobile phase and fractionated by LC using a Beckman Ultrasphere C_{18} column (5 μm, 25 cm × 4.6 μm). A linear gradient of 15–50% acetonitrile in 0.15 mM phosphoric acid, pH 3.5, was used at a flow rate of 1 ml min^{-1}. Fractions, collected at 1-min intervals, were dried under vacuum and reconstituted in assay buffer for determination of immunoreactivity. MK-383 was eluted after 28 min.

Immunoassay

The assay buffer was 0.05 M phosphate containing 0.05 M EDTA, 0.05% sodium azide, and 0.1% BSA, pH 7.5. The stock standard consisted of 0.25-ml aliquots of MK-383 in assay buffer at a concentration of 100 ng ml^{-1}. The standard curve, diluted from this stock, covered the range of 1–200 ng ml^{-1}. The buffer used to dilute the standards contained 10% normal human plasma. Quality control samples consisted of normal human plasma containing the reference at concentrations of 5, 10 and 50 ng ml^{-1}. The rabbit antiserum was diluted according to titre in assay buffer containing rabbit gamma globulin (0.05 ng ml^{-1}) to provide bulk for the double antibody precipitation. For convenience in pipetting, the radioligand was mixed with the anti-rabbit globulin. Plasma samples and quality controls were diluted 1:10 in buffer. Reagents were added to 12 × 75 mm glass culture tubes using an automatic pipetting station (APS, Micromedic Systems, Horsham, PA) as follows: 0.05 ml standard solution, diluted quality control or diluted sample, 0.1 ml first antibody/gamma globulin reagent, 0.1 ml second antibody/radioligand (20,000 cpm $tube^{-1}$), and 0.75 ml buffer containing ANSA at 66 μg ml^{-1}. Non-specific binding (nsb) was determined in tubes lacking first antibody but containing the carrier gamma globulin. After overnight incubation at room temperature (*ca* 18 h), the tubes were centrifuged for 45 min at 800***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 the conventional logit–log transformation, a calibration curve of net control binding [100 (B − nsb)/(B_0 − nsb)] versus concentration was constructed. The concentration of MK-383 in test samples was calculated by interpolation from the calibration curve. Using 1:10 dilutions of plasma, the quantifiable limits ranged from 1 to 200 ng ml^{-1}. Data were expressed as the hydrochloride salt.

The method was readily adapted to the analysis of urine. Test urines were diluted from 1:100 to 1:10,000 in buffer and assayed against a standard curve prepared in buffer. The addition of ANSA was unnecessary. Buffer quality controls were prepared at 100 times nominal concentration and diluted along with the test urines. Using 50-μl aliquots of urine diluted 1:100, the quantifiable limits ranged from 10 to 2000 ng ml^{-1}.

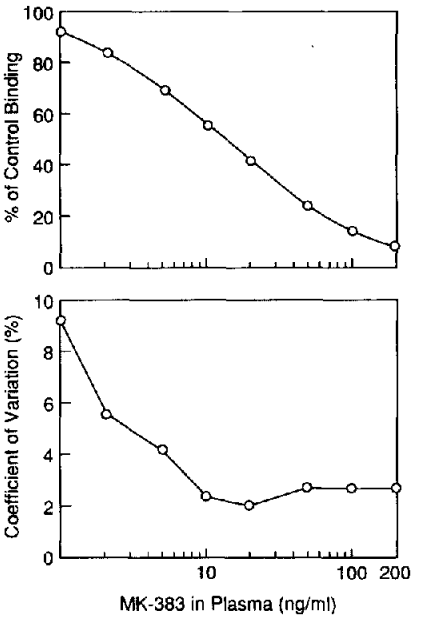

Figure 3
Typical standard curve (top) and intra-assay precision (bottom) profile for the determination of MK-383 in human plasma.

Results

Antisera

Titration of rabbit antiserum no. 39 collected 3 months after immunization demonstrated 50% binding (B_0/total counts) at a dilution of 1:24,000. A stock solution was stored in aliquots diluted 1:300 in assay buffer at −70°C and further diluted prior to each analysis. Binding was independent of pH in the range 7–8, and the assay was run routinely at pH 7.5.

Effect of plasma volume

Plasma samples were routinely diluted 1:10 in assay buffer, while standards were diluted in buffer containing 10% normal human plasma. Small differences in binding that were observed when control plasma from different human subjects was tested were eliminated by the addition of 50 μg ml^{-1} ANSA to the assay buffer. The mean per cent of control for drug-free plasma from 37 subjects was 99.6 ± 1.5% (range 97–102% of control).

Assay sensitivity and precision

A standard calibration curve and precision profile is shown in Fig. 3. The assay was configured such that 50% of the total radioactivity was bound in control (drug-free) plasma. Adopting a 10% maximum for intra-assay relative standard deviation (RSD) as a reliability criterion, the lowest measurable concentration is 1 ng ml^{-1} using 5 μl of plasma per assay tube. The upper limit is 200 ng ml^{-1}, plasma containing higher concentrations being diluted prior to analysis. The non-specific binding was 1% of total counts. Inter-assay accuracy and precision were determined from quality control data obtained during separate assays. Both intra- and inter-assay precision are shown in Table 1.

To accommodate high drug concentrations in urine, it was necessary to dilute samples 1:100 and higher. At 1:100 there was no matrix effect upon the assay. Absolute sensitivity is 5 pg $tube^{-1}$.

Effect of heparin

Normal drug-free human plasma was harvested from blood collected in tubes containing sodium heparin. The concentration of heparin in such plasma was ~30 units ml^{-1}.

Aliquots of plasma were assayed after addition of further quantities of heparin in buffer to the assay tubes to yield concentrations equivalent to 500, 5000 and 50,000 units per ml of plasma. Slight loss of assay accuracy was only apparent at the highest heparin concentration.

Parallelism and accuracy

Three samples of plasma taken at different times from a volunteer receiving MK-383 by intravenous infusion were diluted with control

Table 1
Assay accuracy and intra-day precision for the determination of MK-383 in control plasma to which accurately known quantities of drug had been added. Intra-assay 'accuracy' was determined by back calculation from the corresponding standard curve. Inter-assay accuracy was determined by the analysis of independently prepared quality control samples

	Plasma conc. (ng ml^{-1})	No of determinations*	Mean accuracy† (%)	RSD (%)
Intra-assay	1	9	100.0	9.0
	2	9	98.0	5.6
	5	9	101.0	4.2
	10	9	95.2	2.4
	20	9	100.0	2.1
	50	9	101.4	2.8
	100	9	101.0	2.8
	200	9	98.4	2.8
Inter-assay	5	9	102.2	6.6
	10	9	101.0	2.4
	50	9	101.2	8.9

*Each determination in triplicate.
†Found/added × 100.

Table 2
Proportionality of the measured concentrations in spiked control and patients' plasma, collected 30, 120 and 240 min after intravenous administration as a function of sample dilution with drug-free control human plasma

	Found concentration of MK-383 (ng ml^{-1})				
Dilution factor	Control plasma 1	Control plasma 2	Patient plasma (30 min)	Patient plasma (120 min)	Patient plasma (240 min)
Initial assay	47.3	50.4	39.2	19.8	6.3
1:2	46.3	48.5	36.6	19.1	6.0
1:4	45.5	48.2	37.2	18.4	5.9
1:8	43.0	45.5	36.6	18.2	6.1
1:16	44.7	45.2	34.6	17.6	6.6

Table 3
Accuracy: clinical samples from different subjects were assayed with and without addition of a 'spike' of MK-383 (2.8 ng ml^{-1})

Endogenous conc. of MK-383 (ng ml^{-1})	Added MK-383 (ng ml^{-1})	Measured MK-383 (ng ml^{-1})	Recovery of added drug* (%)
1.6	2.8	4.4	100.0
3.0	2.8	5.8	100.0
4.0	2.8	6.9	103.6
1.4	2.8	4.1	96.4
0.5	2.8	3.1	92.4

*((Measured − Endogenous)/Added) × 100.

human plasma to yield stocks whose concentrations fell within the calibration range. These stocks were serially diluted with control human plasma and aliquots (5 μl) were taken for assay. Two quality control samples consisting of drug-free plasma containing MK-383 at a concentration of 50 ng ml^{-1} were diluted and assayed concurrently. The measured concentrations of MK-383 were multiplied by the appropriate dilution factors, yielding the results shown in Table 2.

Accuracy was assessed by adding small quantities of analytical standard to five clinical samples whose endogenous concentrations were close to the assay's detection limit (1 ng ml^{-1}), followed by re-assay. The results in Table 3 show resulting analytical data that are not significantly different (<10%) from the theoretical values, and therefore, that the assay's accuracy is not compromised by the matrix.

Specificity

Cross-reactivities, expressed as the ratios of the concentration of the test material to that of MK-383 necessary to cause 50% displacement

of the radioligand, were greater than 1000 for both the potential metabolites, L-708,350, and 4-piperidinyl-butan-1-ol.

To check for the presence of any unknown cross-reacting metabolites, plasma obtained from a patient receiving MK-383 by intravenous infusion was first assayed directly, and subsequently fractionated by LC, the immunoreactivity in the fractions being determined by radioimmunoassay. The recoveries of the immunoreactive species, determined by comparing the direct assay of the untreated plasma (48.9 and 14.0 ng ml^{-1}) with the sum of the fractions, were 111% and 95% for samples collected at 1 and 3 h post dose. The reconstructed chromatograms are shown in Fig. 4. The only immunoreactive component in the fractionated plasma corresponded in chromatographic retention time to the parent drug and there was no evidence for the presence of cross-reacting metabolites.

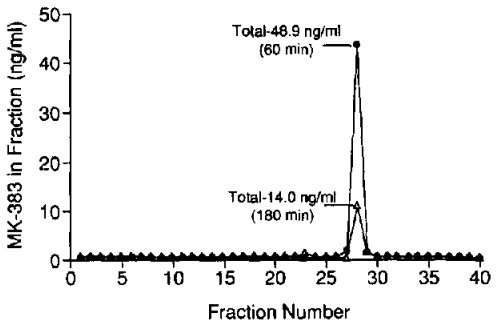

Figure 4
Reconstructed liquid chromatograms of the immunoreactive components of a deproteinated preparation of plasma obtained from a human volunteer dosed intravenously with MK-383. Chromatographic conditions are described in the text. The only immunoreactive peak in the chromatograms corresponds in retention time to the parent drug.

Table 4
Concentrations (ng ml^{-1}) of MK-383 in clinical plasma samples stored frozen at −20°C and assayed on two occasions, 9 months apart, to assess stability

Sample no	Initial assay (ng ml^{-1})	Re-assay (ng ml^{-1})	Ratio
1	8.1	7.8	0.96
2	27.1	32.0	1.18
3	27.3	27.8	1.02
4	15.3	16.9	1.11
5	10.8	10.6	0.98
6	6.6	5.4	0.82
7	4.4	4.9	1.11
8	2.2	2.5	1.14
9	1.6	1.5	0.94
		Mean	1.03
		SD	0.11

The analysis of control plasma containing acetylsalicylic acid and its metabolites, salicylic acid and salicyluric acid, at plasma concentrations up to 100 μg ml^{-1} showed no displacement of the radioligand.

Stability

Clinical samples were stored at −20°C. Reanalysis of a set of samples 9 months after initial assay indicated that the storage conditions maintain satisfactory stability (Table 4).

Discussion

The immunogen was prepared via the active ester of MK-383's carboxyl function 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 the radioligand. A 5-μl plasma aliquot was the selected sample size based on the concentrations of drug found in subjects receiving therapeutic doses. For convenience, samples were diluted 1:10 in assay buffer and a 50-μl aliquot of the solution taken for analysis. To render the matrix of the standards identical to that of test plasma, the standards were diluted in assay buffer containing 10% (v/v) normal human plasma. ANSA, at a concentration of 50 μg ml^{-1}, was included in the assay buffer to eliminate inter-subject depression of binding.

Administration of MK-383 to patients concurrently receiving heparin or acetylsalicylic acid was anticipated in early trials. Neither substance nor the principal metabolites of acetylsalicylic acid had any significant effect on assay performance. In the absence of an alternative analytical method of sufficient specificity and sensitivity (e.g. LC–MS), the accuracy of the assay was determined both by analysis of spiked control human plasma (Table 1) and by using the method of standard addition to clinical samples containing drug at concentrations close to the assay detection limit (Table 3). The latter is an important test designed to detect abnormalities in binding caused by differences in the endogenous plasma matrix.

Parallelism experiments were conducted (with patients' plasma) to detect both matrix effects (indicated by an apparent decrease in plasma concentration with dilution) and any cross-reacting metabolite with a non-parallel displacement curve which generally show

apparent increases in plasma concentration with dilution. The assay satisfactorily passed both of these tests.

Assay specificity was demonstrated by directly assaying plasma samples from patients receiving the drug and then fractionating the plasma components by preparative LC and assaying the fractions by RIA. None of the fractions other than those corresponding in chromatographic retention time to MK-383 showed any immunoreactivity. Equally important, the recoveries of MK-383 in the sum of these latter fractions were 95 and 111% of the quantities measured in the original plasma. Hence, no cross-reacting metabolites of MK-383 exist in the plasma of patients receiving the drug. The lack of significant (i.e. <0.1%) cross-reactivity of the potential metabolites L-708,350 and 4-piperidinyl-butan-1-ol is of interest in that the lack of efficient binding of these complementary substances suggests epitopes on either side of MK-383's phenyl ether linkage. Although the specific epitopes are unknown, both must be present to provide maximum binding to the antibodies.

The assay-acceptance criteria in this laboratory require that the intra-assay relative standard deviation does not exceed 10%. The assay in plasma was, accordingly, satisfactory over the range 1–200 ng ml^{-1}, the relative standard deviation at the lower and upper quantifiable limits being 9.0 and 2.8%. At the lower quantifiable limit in urine the relative standard deviation also met this criterion. The stability of the test substance in plasma was satisfactory for up to 9 months when stored at −20°C. The method has been applied to the analysis of samples from clinical studies [10]; plasma concentration–time curves obtained after subjects received single doses of MK-383 by intravenous infusion are shown in Fig. 5.

In conclusion, a simple, sensitive and specific direct radioimmunoassay has been developed in our laboratories which is suitable for the determination of the pharmacokinetics of MK-383 in animals and in man.

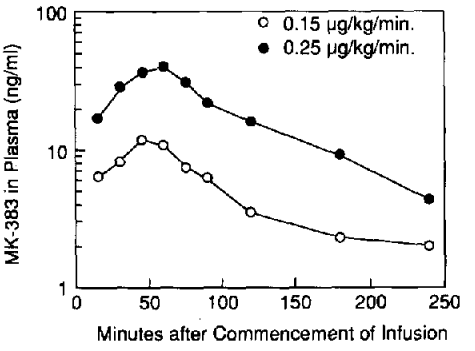

Figure 5
Concentration–time profiles of MK-383 in the plasma of two volunteers receiving the drug by intravenous infusion at rates of 0.15 and 0.25 µg kg^{-1} min^{-1} for 1 h.

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