Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 6, pp. 427–434, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

Determination of MK-852, a new fibrinogen receptor antagonist, in plasma and urine by radioimmunoassay

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Abstract: MK-852 is a novel 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 a dinitrophenylene bridge and the radioligand by reaction of the drug with the ^{125}I-labelled Bolton–Hunter reagent. The method was specific and no immunoreactive material other than parent drug was detectable in plasma from dosed volunteers. The direct assay using 0.05 ml of plasma is sensitive to 0.2 ng ml^{-1} without matrix interference and has sufficient sensitivity, precision, accuracy, and selectivity for the analysis of clinical samples. The lower quantifiable limit in (diluted) urine is 50 ng ml^{-1}.

Keywords: *MK-852; fibrinogen receptor antagonist; radioimmunoassay (RIA); antithrombotic drug.*

Introduction

Recent progress toward understanding the biochemistry of platelet aggregation has demonstrated that the final step involves the binding of fibrinogen to an activated membrane-bound glycoprotein complex termed Gp IIb/IIIa [1–3]. A minimal amino acid sequence necessary for this interaction has been identified as Arg-Gly-Asp in fibrinogen [4] and it is possible to design synthetic analogues based on this model which are capable of blocking the interaction competitively. The disulphide-linked cyclic peptide MK-852 (Ac-Cys-Asn-Dmt-Amf-Gly-Asp-Cys-OH) is such an inhibitor with nanomolar potency [5]. (The abbreviations Dmt and Amf represent β,β-dimethylthioproline and *p*-aminomethyl-L-phenylalanine, respectively.) It displays antithrombotic efficacy in animals and would be expected to be clinically useful for acute therapy in conjunction with, for example, angioplasty or thrombolytic therapy [6–9].

A sensitive method for quantitation of MK-852 in biological fluids was necessary to support the preclinical and clinical studies needed to develop this drug candidate. The absence of a good chromophore and limited opportunity for derivatization was the stimulus for an assay other than a chromatographic [10] one requiring conventional detection techniques. Radioimmunoassay (RIA) was an obvious and readily available choice and this report describes the development and performance of the assay.

Experimental

Materials and methods

MK-852 (Fig. 1), its *N*-desacetyl and reduced (disulphydryl) derivatives, and *p*-aminomethyl-L-phenylalanine hydrochloride were synthesized by the Medicinal Chemistry Department, Merck Research Laboratories (West Point, PA, USA). Methanol (HPLC grade) and sodium phosphate (mono- and dibasic) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Sodium hydroxide and acetic acid were of analytical grade and were purchased from Mallinkrodt (Paris, KY, USA). Disodium ethylenediaminetetraacetic acid (EDTA), sodium azide, and sodium heparin (116.9 units mg^{-1}) were obtained from Sigma (St Louis, MO, USA).

Bovine serum albumin was obtained from several sources, viz., RIA-grade (cat. No. A-7888) and crystallized (cat. No. A-7638) from Sigma; RIA-grade (cat. No. 10868) and crystallized (cat. No. 10856) from United States Biochemical (Cleveland, OH, USA); and Microbiological grade (cat. No. 12660) from Calbiochem (La Jolla, CA, USA). Sheep

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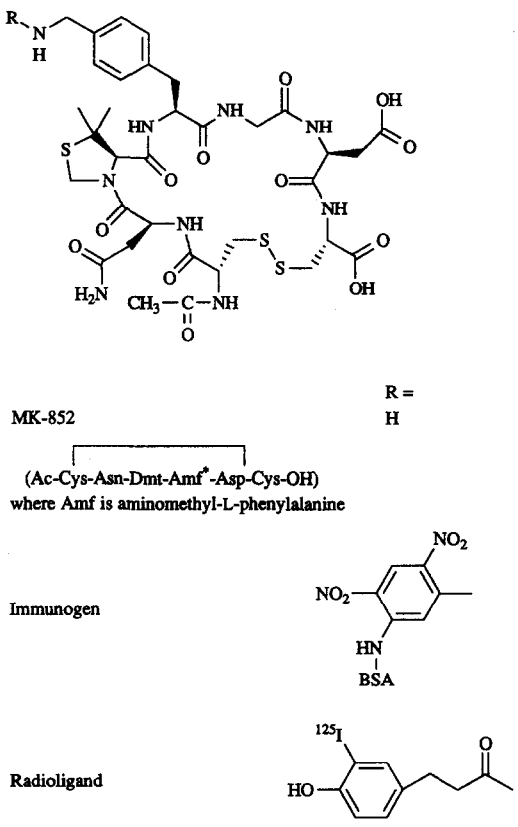

Figure 1
Structures of MK-852 and related substances used for development of the radioimmunoassay.

anti-rabbit gamma globulin serum was purchased from Arnel Products (New York, NY, USA) and ^{125}I-labelled Bolton–Hunter reagent [11] (5700 μCi μg^{-1}) in anhydrous benzene from New England Nuclear (Wilmington, DE, USA).

Thin-layer chromatography (TLC) was performed on pre-scored glass plates, (2 × 10 cm) coated with silica-gel GF-254, layer thickness 0.25 mm (Analtech, Newark, DE, USA). The developing solvent was ethyl acetate–*n*-butanol–acetic acid–water (1:1:1:1, v/v/v/v). Spots were visualized under irradiation at 254 nm. High-performance liquid chromatography (HPLC) was performed using a system comprising a Tosohass TSK-6010 dual solvent delivery system (Montgomeryville, PA, USA), a Beckman Model 170 radioisotope detector (Irvine, CA, USA), and a Gilson Model FC203 fraction collector (Middleton, WI, USA).

Preparation of the immunogen

MK-852 (17.4 mg, 0.02 mmol) was dissolved in 0.8 ml *N*,*N*-dimethylformamide and 1,5-difluorodinitrobenzene (DFDNB, 3.8 mg, 0.019 mmol) was added in 0.067 ml methanol. The reaction was performed with stirring and protection from light for 18 h at room temperature, and analysis by TLC (ethyl acetate–*n*-butanol–acetic acid–water, 1:1:1:1, v/v/v/v) indicated the absence of DFDNB (R_f, 0.9) and conversion of the peptide (R_f, 0.5) to a product with R_f = 0.7. Without isolation, the bulk of this (0.7 ml) was reacted with 26 mg BSA (Calbiochem) in 2 ml phosphate buffer (0.125 M, pH 9.75) by the addition, with stirring, of 0.1 ml aliquots to the BSA over a period of 5 h at room temperature. After reacting overnight in the dark, the mixture was dialysed exhaustively against water at 4°C.

The degree of incorporation of hapten into the immunogen was determined spectrophotometrically using, as a reference, the 2,4-dinitro-5-(2-aminoethylamino)phenyl derivative of MK-852. The latter was prepared by coupling the fluorodinitrophenyl derivative of MK-852 with ethylenediamine in dimethylformamide at room temperature for 3 h. The product, purified by column chromatography on silica gel, was isolated as a yellow solid (71% yield) with a molecular weight of 1093 determined by FAB–MS, and a purity of >95% as determined by TLC. The molar extinction coefficient (measured at 342 nm in water) was 16,250. The extent of incorporation of the dinitrophenyl derivative of MK-852 into the immunogen was calculated to be 19.5 moles per mole of BSA.

Immunization

The immunogen solution was emulsified with an equal volume of Freund's complete adjuvant (Sigma) and female New Zealand White rabbits were immunized with approximately 1 mg protein by multiple intradermal injections plus single intramuscular (i.m.) and subcutaneous (s.c.) injections. Boosting with a one-half quantity of protein was performed with emulsions in incomplete adjuvant, Sigma, (i.m. and s.c. only) at 1, 3 and 7 months post initiation and antisera were obtained prior to each of the latter two injections. These were stored at −70°C.

Preparation of the radioligand

Benzene was removed from the vial of ^{125}I-labelled Bolton–Hunter reagent by evaporation under a stream of dry nitrogen immediately prior to use. To the residue (1 mCi, 0.45 nmoles) was added 10 μg (11.5 nmoles) of

MK-852 in 10 μl borate buffer (0.1 M, pH 8.5). After incubation in an ice bath for 1 h, the mixture was stored at 5°C overnight. The reaction mixture (*ca* 9 μl) was fractionated by HPLC using a 4.6 × 150 mm column packed with Microbondapak, C_{18} 5 μm (Waters, Milford, MA, USA). The mobile phase consisted of mixtures of aqueous acetic acid (0.1% w/v, pH 2.9) and methanol. For 10 min, the mobile phase contained 20% methanol, with a subsequent gradient to 50% methanol after 60 min, and finally to 100% at 80 min. No unreacted Bolton–Hunter reagent remained in the reaction mixture and the product radioligand eluted between 76 and 78 min, well resolved from unreacted MK-852 at *ca* 29 min. Chromatograms are shown in Fig. 2. Four fractions of the eluent were collected in 0.5 ml aliquots as the tracer eluted. Two fractions containing the bulk of the radioactivity were tested for immunoreactivity, pooled and stored at −20°C under which conditions the radioligand had a useful life of approximately 8 weeks.

The structure of the radioligand (not further characterized) was, based on the chemistry of reaction, presumed to be that indicated in Fig. 1.

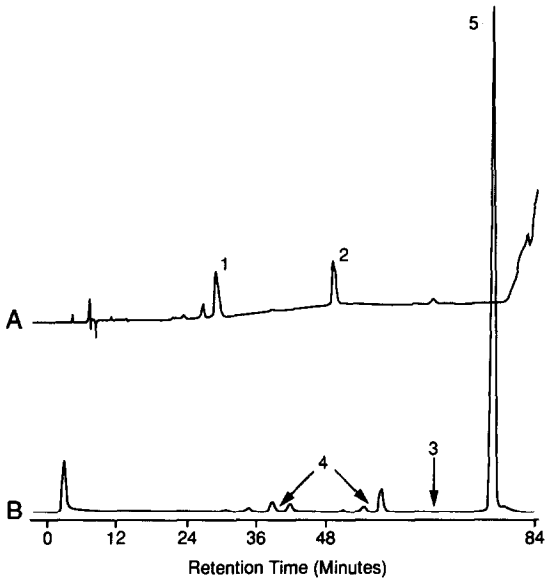

Figure 2
Liquid chromatograms of reagents and product of the coupling of MK-852 with ^{125}I-labelled Bolton–Hunter reagent. Chromatographic conditions are described in the text. Chromatogram A: ultraviolet detection at 254 nm; Chromatogram B: radiochemical detection. Peaks 1 and 2 correspond, respectively, to unreacted MK-852 and an unidentified impurity in the reagent. The retention time of the Bolton–Hunter reagent is indicated at 3. The series of radioactive peaks (4) are the hydrolysis products of the reagent while the product radioligand is peak 5.

Fractionation of plasma samples

Human clinical samples obtained 60 and 180 min after commencement of a 1-h intravenous infusion of MK-852 (0.5 μg kg^{-1} min^{-1}) were deproteinated with 2 volumes of acetonitrile and the supernatant was separated by centrifugation and evaporated to dryness in a SpeedVac Model SVCZ001-1 (Savant, Farmingdale, NY, USA). The residue was reconstituted in mobile phase and fractionated by HPLC using a Vydac protein and peptide C-18 column (4.6 × 150 mm). A linear gradient of 5–30% acetonitrile in 0.1% aqueous trifluoroacetic acid was used at a flow rate of 1 ml min^{-1}. Fractions collected at 0.5-min intervals were dried in the SpeedVac and reconstituted in assay buffer for immunoreactivity determination. MK-852 eluted after 23 min.

Immunoassay

The assay buffer was 0.05 M phosphate containing 0.05 M EDTA, 0.05% sodium azide, and 0.5% BSA (Sigma crystalline), pH 7.5. The working standard consisted of 0.1 ml aliquots of MK-852 at a concentration of 1.0 μg ml^{-1} in assay buffer stored at −70°C. For use, this was diluted in buffer to yield an appropriate series of concentrations (0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng ml^{-1}). Quality control samples consisted of normal drug-free plasma containing the reference at 0.5, 1 and 5 ng ml^{-1} stored at −70°C. The rabbit antiserum ('first antibody') was diluted according to titre in assay buffer containing rabbit gamma globulin (0.05 mg ml^{-1}) to provide bulk for the double antibody precipitate (5 μg tube^{-1}), and for convenience in pipetting, the radioligand was mixed with the anti-rabbit globulin. The binding at equilibrium was similar whether the primary and secondary antibodies were added simultaneously or separately. The final dilution of second antibody in this reagent was 1:40 to 1:100 (depending on the batch). The radioligand was added to provide about 20,000 cpm per assay tube.

Reagents were added to 12 × 75 mm glass culture tubes using an automatic pipetting station (APS, Micromedic, Horsham, PA, USA) as follows: 0.05 ml standard solution, plasma samples or quality control; 0.05 ml normal plasma was added to standards and 0.05 ml buffer to samples and quality control

samples. To all tubes were added 0.1 ml first antibody/gamma globulin reagents, 0.1 ml second antibody/radioligand (20,000 cpm tube^{-1}), and 0.7 ml buffer. Nonspecific 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* and supernatants were decanted from the precipitates and discarded. The tubes were inverted to drain and the radioactivity in the consolidated pellets was determined by counting for 3 min in a Micromedic Model Apex 10/600 multi-detector gamma counter. All samples were assayed in triplicate. Plasma containing MK-852 at concentrations in excess of 10 ng ml^{-1} were diluted as appropriate with buffer and 0.05 ml aliquots taken for re-analysis. In such cases, an appropriate dilution of control plasma in buffer was added to the standards to maintain a constant matrix in all assay tubes. Calibration curves were constructed, using a third-order polynomial, by plotting the fraction of control binding, $B - NSB/B_0 - NSB$ against plasma concentration. B_0 is the observed binding (counts per minute) in the absence of the analyte, B is the binding in the presence of a specified quantity of analyte and *NSB* is the non-specific binding. Appropriateness of fit was evaluated by reading standards as though unknowns. The concentrations of MK-852 in test samples were determined by interpolation from the standard curve.

The assay was readily modified to accommodate the analysis of urine in which the concentrations of MK-852 exceeded those in plasma by two to three orders of magnitude. Urine was appropriately diluted (1:100 to 1:10,000) in buffer prior to assay against standards prepared in buffer.

Results

Antisera

Antisera collected 3 months after immunization yielded 50% binding (B_0/Total counts = 0.5) of radioligand at dilutions ranging from 1:2000 to 1:20,000. After 6 months, serum collected from Animal No. 68556 gave a titre of 35,000. This was stored as small aliquots of 1:100 dilution in assay buffer at −70°C and used in all subsequent evaluations and assays. Binding was essentially unchanged with pH in the range 7–9 and the assay was run routinely at pH 7.5.

Assay sensitivity and precision

A standard calibration curve and precision profile is shown in Fig. 3. Using a 10% maximum for intra-assay relative standard deviation (RSD) as a reliability criterion, the lowest measurable concentration is 0.1 ng ml^{-1} using 50 µl of undiluted human plasma per assay tube. The upper limit is 10 ng ml^{-1}, plasma containing higher concentrations being diluted prior to analysis. The non-specific binding was 0.7% of total counts. Inter-assay precision was determined from quality control data obtained during separate assays. Both intra- and inter-assay precision are shown in Table 1.

The lower quantifiable limit for the analysis of urine is limited by the dilution in buffer prior to assay. Using a 1:1000 dilution, the accuracy and inter-assay precision ranged from 96% (RSD = 5.6%) at 50 ng ml^{-1} to 99% (3.9%) at 500 ng ml^{-1}.

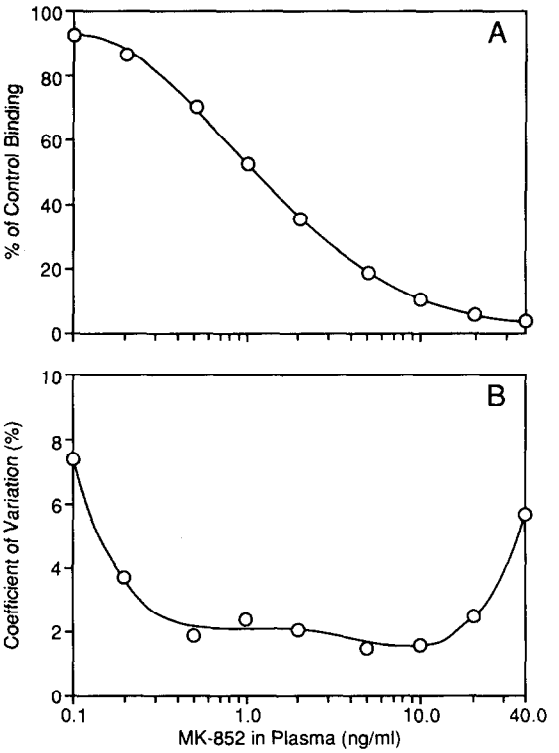

Figure 3
Typical standard curve (A) and intra-assay precision profile (B) obtained with Antiserum no. 68556 (dilution factor 35,000).

Table 1
Calculation of inter- and intra-assay accuracy and precision for the determination of MK-852 in plasma

Conc. (ng ml^{-1})	No. of replicates*	Mean % recovery (found/added)	RSD (%)
Intra-assay			
0.1	7	112.0	7.4
0.2	7	100.0	3.7
0.5	7	96.0	1.9
1.0	7	97.0	2.4
2.0	7	98.5	2.1
5.0	7	98.8	1.5
10.0	7	101.7	1.6
20.0	7	99.0	2.5
40.0	7	94.3	5.7
Inter-assay			
0.5	17	102.4	5.9
1.0	17	103.5	4.0
5.0	17	101.1	4.2

*Each determination in triplicate.

Source and concentration of BSA used in assay buffers in assay reproducibility

The effect on binding of using assay buffer containing BSA (0.1% w/v) from different sources was evaluated. In the absence of control human plasma, B_0/T was 56.6% with crystalline BSA from Sigma. In contrast, RIA grade BSA from Sigma and USB, and microbiological grade from Calbiochem reduced binding to unacceptably low levels (13–30%).

The introduction of human plasma (50 μl) into the assay mixture (using 0.1% crystalline BSA) reduced B_0/T to 50.0%. To compensate, control human plasma (50 μl) was added to the reference tubes. Some inter-subject variation in binding depression was apparent, but this was insignificant when the concentration of BSA in the buffer was increased to 0.5% (B_0/T 51.2%).

Drug-free plasma from 12 subjects was tested to verify the absence of inter-individual differences on B_0. The mean per cent of control relative to a single normal plasma was 101.0 ± 1.2% (range 99–103%).

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 ~15 units ml^{-1}. MK-852 was added to a portion of this control plasma to yield a concentration of 1.92 ng ml^{-1}. Aliquots of plasma (50 μl) were assayed after the addition of further quantities of heparin in buffer to the assay tubes yielding concentrations equivalent to 300, 3000 and 30,000 units per ml of plasma. A series of control plasma samples containing heparin at the same concentrations but with no added drug were assayed concurrently.

Results are shown in Table 2 and show significant loss of assay accuracy only after the addition of heparin at plasma concentrations equivalent to 30,000 units ml^{-1} (*ca* 9 mg per assay tube).

Parallelism and recovery

Two samples of plasma taken from a volunteer receiving MK-852 by intravenous infusion were diluted with control human plasma to yield stocks whose concentrations were measurable by the calibration curve.

These stocks were then serially diluted with control human plasma and aliquots (50 μl) were taken for assay. A quality control sample consisting of control plasma containing MK-852 at a concentration of 5 ng ml^{-1} was diluted and assayed concurrently. The measured concentrations of MK-852 in the samples were multiplied by the appropriate dilution factors. The results in Table 3 indicate reproducible analytical results in proportion to dilution.

Recovery was assessed by adding analytical standard to clinical samples in amounts that roughly doubled the endogenous concentrations. The results in Table 4 show resulting analytical data that are indistinguishable from theoretical values, and therefore, that accuracy is not compromised by the matrix.

Table 2
Analysis of control plasma spiked with *ca* 2 ng ml^{-1} of MK-852 after addition of sodium heparin

Heparin added per tube (units)	Heparin added per tube (mg)	Heparin added per ml of plasma equivalent (units ml^{-1})	0 ng ml^{-1} % of control binding	1.92 ng ml^{-1} found concentration (ng ml^{-1})
0.75	0.004	15	100	2.02
15.00	0.09	315	100	1.90
150	0.9	3015	100	1.92
1500	9.0	30,015	99	1.78

Table 3
Proportionality to dilution ('parallelism') of the assay using spiked control human plasma and plasma obtained from a clinical study. Samples were collected from a volunteer 70 and 180 min after commencement of a 1-h intravenous infusion of MK-852 at 0.33 μg kg^{-1} min^{-1}

Dilution factor	Found conc. of MK-852 (ng ml^{-1}) Control	Patient plasma (70 min)	Patient plasma (180 min)
Initial assay	4.90	173	55.4
1:2	4.72	169	62.6
1:4	4.68	178	60.8
1:8	4.80	180	61.6
1:16	4.96	184	65.5

Table 4
Recovery of MK-852 from clinical plasma samples with and without addition of reference solutions of the drug

Plasma sample (no.)	Original conc. of MK-852 (ng ml^{-1})	Added MK-852 (ng ml^{-1})	Measured MK-852 (ng ml^{-1})	% Measured/expected
1	0.00	2.00	2.04	102.0
2	0.00	0.50	0.54	108.0
3	0.91	2.00	2.77	95.2
4	1.43	2.00	3.22	93.9
5	1.70	1.00	2.85	105.6
6	1.00	2.00	2.91	97.0
7	2.65	2.00	4.52	97.2
8	1.63	2.00	3.62	99.7
9	0.28	0.25	0.57	107.5

Specificity

Cross-reactivity, expressed as the ratio of the concentration of the material to that of MK-852 necessary to cause 50% displacement of the radioligand, was greater than 200 for each of the two potential metabolites (*N*-desacetyl and the reduced disulphydryl form). *p*-Aminomethyl-L-phenylalanine (a urinary metabolite of MK-852 in dogs) was invisible to the assay except at mg ml^{-1} concentrations. To check for the presence of any cross-reacting metabolites, plasma obtained from a patient receiving MK-852 by intravenous infusion was assayed directly and subsequently fractionated by HPLC, the immunoreactivity in the fractions being determined by radioimmunoassay. The recoveries of immunoreactive species, determined by comparing the direct assays of the untreated plasma (96 and 28 ng ml^{-1}) with the sum of the fractions, were 105 and 96% for the samples collected at 60 and 180 min, respectively. 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. A parallel experiment conducted with patients' urine also showed quantitative recovery of immunoreactivity as a single chromatographic peak corresponding to MK-852.

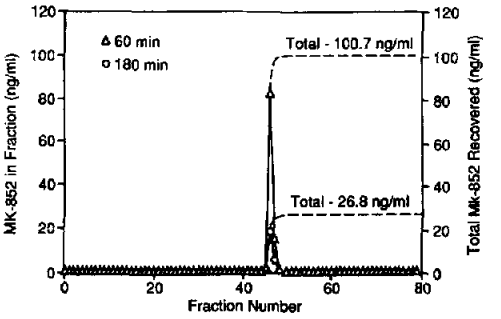

Figure 4
Reconstructed liquid chromatograms of the immunoreactive components of acetonitrile preparations of plasma obtained from a human volunteer dosed intravenously with MK-852. The chromatographic conditions are described in the text. The only immunoreactive peak in the chromatograms corresponds in retention time to unchanged drug.

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.

Table 5
Concentrations (ng ml^{-1}) of MK-852 in clinical plasma stored frozen at −20°C and assayed on two occasions, 15 months apart, to assess stability

Sample no.	First assay (ng ml^{-1})	Second assay (ng ml^{-1})	Ratio (%)
1	80.5	74.0	91.9
2	124	109	87.9
3	136	133	93.5
4	147	137	103.6
5	138	143	95.1
6	142	135	101.1
7	141	142	90.7
8	123	112	86.6
9	94	81	77.3
10	37.5	29.0	
			92.6
		Mean	7.6
		SD	

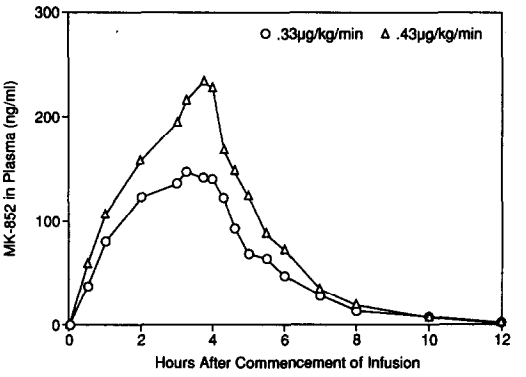

Figure 5
Concentration–time profiles of MK-852 in the plasma of two volunteers receiving the drug by intravenous infusion at rates of 0.33 and 0.43 μg kg^{-1} min^{-1} for 4 h.

Stability

Clinical plasma samples were stored at −20°C. Re-analysis of a set of samples 15 months after initial assay indicated that the storage conditions maintain satisfactory stability (Table 5).

Discussion

The effect of human plasma and the source and concentrations of BSA used in the preparation of assay buffer proved critical to the assay. In the absence of plasma, binding of the radioligand (B_0/T) was dramatically reduced when buffers containing RIA or microbiological grade BSA (0.1% w/v) from three separate vendors was used. Satisfactory binding was obtained using crystalline BSA from Sigma at concentrations in the buffer of 0.1% (w/v). The not unexpected reduction in binding, observed when 50 μl human plasma was added to the assay tubes, necessitated the addition of an equal volume of control plasma to the reference tubes, but the effect of human plasma was still variable. Variability was reduced to insignificance by the preparation of buffers containing crystalline BSA at 0.5%. As anticipated, the extensive dilution of urine prior to assay showed no bias in binding with respect to urine-free controls prepared in buffer.

Co-administration of MK-852 to patients concurrently receiving heparin or acetylsalicylic acid was anticipated in early clinical trials. Neither substance nor the principal metabolites of acetylsalicylic acid showed any significant effect on assay performance. Following intravenous administration to volunteers, plasma fractionation by HPLC showed no immunoreactive species other than the parent drug. The cross-reactivity of the putative *N*-desacetyl and sulphydryl metabolites was minimal; however, neither of these substances has ever been detected in the blood or urine of either animals or humans. In this context, it is of note that 85–90% of the intravenous dose is recovered unchanged in 0–72 h urine collections.

Our assay acceptance criteria require (1) the intra-assay RSD does not exceed 10%, and (2) the percentage of control binding (i.e. the quotient of B/B_0 expressed as a percentage) lies in the range 10–90%. Accordingly, the plasma assay is satisfactory over the range of concentrations 0.2–10 ng ml^{-1} and the RSDs at the lower and upper quantifiable limits were 3.7 and 1.6%, respectively. At the lower quantifiable limit in urine (50 ng ml^{-1}) the RSD is 5.6%. The stability of the test substance in plasma is satisfactory when stored at −20°C for up to 15 months. The method has been applied to the analysis of samples derived from early clinical studies. Plasma concentration–time curves obtained after subjects received single doses of MK-852 by intravenous infusion are shown in Fig. 5.

In conclusion, a simple, sensitive, and specific radioimmunoassay has been developed in our laboratories and has been successfully applied to elucidate the pharmacokinetics of MK-852 during early clinical studies.

Acknowledgements — The authors thank Mrs C.D. Colton, Dr S.F. Brady and Dr R.F. Nutt for synthesizing *p*-aminomethyl-L-phenylalanine and the *N*-desacetyl and

disulphydryl derivatives of MK-852, Dr T.D. Bjornsson and Mr D.S. Farrell for the provision of clinical samples of plasma and urine, and Ms Pat Schaible for her assistance in preparing the manuscript.

References

[1] G.A. Marguerie, E.F. Plow and T.S. Edgington, *J. Biol. Chem.* **254**, 5357–5563 (1979).
[2] J. Hawiger, S. Parkinson and S. Timmons, *Nature* **283**, 195–197 (1980).
[3] E.I. Peerschke, M.B. Zucker, R.A. Grant, J.J. Egan and M.M. Johnson, *Blood* **55**, 841–847 (1980).
[4] E. Ruoslahti and M.D. Pierschbacher, *Science* **238**, 491–497 (1987).
[5] R.F. Nutt, S.F. Brady, C.D. Colton, J.T. Sisko, T.M. Ciccarone, M.R. Levy, M.E. Duggan, I.S. Imagire, R.J. Gould, P.S. Anderson and D.F. Veber, in J.A. Smith (Ed.), *Proceedings of the 12th American Peptide Symposium*, Cambridge, MA, 16–21 June 1991, ESCOM, Leiden, pp. 914–916 (1992).
[6] B. Stein, V. Fuster, D.H. Israel, M. Cohen, L. Badimon, J.J. Badimon and J.H. Chesebro, *J. Am. Coll. Cardiol.* **14**, 813–836 (1989).
[7] D.J. Fitzgerald, L. Roy, F. Catella and G.A. Fitzgerald, *New Engl. J. Med.* **315**, 983–989 (1986).
[8] D.J. Fitzgerald, F. Catella, L. Roy and G.A. Fitzgerald, *Circulation* **77**, 142–150 (1988).
[9] C.W. Hamm, R.L. Lorenz, W. Bleifeld, W. Kupper, W. Wober and P.C. Weber, *J. Amer. Coll. Cardiol.* **10**, 998–1004 (1987).
[10] W.F. Kline, B.K. Matuszewski and J.Y.-K. Hsieh, *J. Chromatogr.* **578**, 31–37 (1992).
[11] A.E. Bolton and W.M. Hunter, *Biochem. J.* **133**, 529–539 (1973).

[*Received for review 13 October 1992; revised manuscript received 20 November 1992*]