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The development and cross-validation of methods based on radioimmunoassay and LC/MS-MS for the quantification of the Class III antiarrhythmic agent, MK-0499, in human plasma and urine

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Abstract

An analytical method based on radioimmunoassay (RIA) has been developed for the determination of the antiarrhythmic agent, MK-0499, in plasma and urine. Owing to the potency of the drug, the specificity of this assay in human plasma could not be adequately determined using conventional RIA procedures. A highly specific procedure, based on LC/MS-MS, was developed to cross-validate the RIA. The lower quantifiable limits of the RIA and LC/MS-MS-based methods were 0.05 and 0.013 ng ml^{-1}, respectively. Cross-validation data, compared using paired student's *t*-test regression analysis, showed excellent correlation between methods. The mass spectrometric assay was also used to simultaneously measure plasma concentrations of unlabeled and ^{14}C-labeled MK-0499 following administration of the drug at high specific activity to volunteers.

Keywords: Analysis; Class III antiarrhythmics; Cross-validation; LC/MS-MS; Plasma; Radioimmunoassay; Radioisotopes; Urine

1. Introduction

The compound MK-0499 (methanesulfonamide, *N*-[1′-(6-cyano-1,2,3,4-tetrahydro-2-naphthalenyl)-3,4-dihydro-4-hydroxyspiro[2*H*-1-benzo-pyran-2,4′-piperidinyl]-6-yl] monohydrochloride) is a selective Class III antiarrhythmic agent [1,2] designed for the treatment and prevention of ventricular tachyarrhythmias and potentially for the prevention of sudden cardiac death [3].

Pharmacological studies on MK-0499 and its pro-drug ketone (L-702,958) have recently been described [4–6]. An analytical method was needed to support both preclinical and clinical phases of the drug development program. Radioimmuno-assay (RIA) was selected since the potency of MK-0499 required high sensitivity (≈ 0.05 ng ml^{-1}) and the procedure needed to be efficient and convenient. Additionally, a differential RIA had previously been successfully applied to the determination in body fluids of L-691,121, an earlier ketonic spirobenzopyran Class III agent and its alcohol metabolite [7]. In the present study the alcohol MK-0499 was selected as a drug candidate in preference to its ketone, L-702,958. Rabbit antisera were raised against several immunogens prepared by conjugating drug derivatives to bovine serum albumin (BSA). One specific antiserum was selected as affording best specific-

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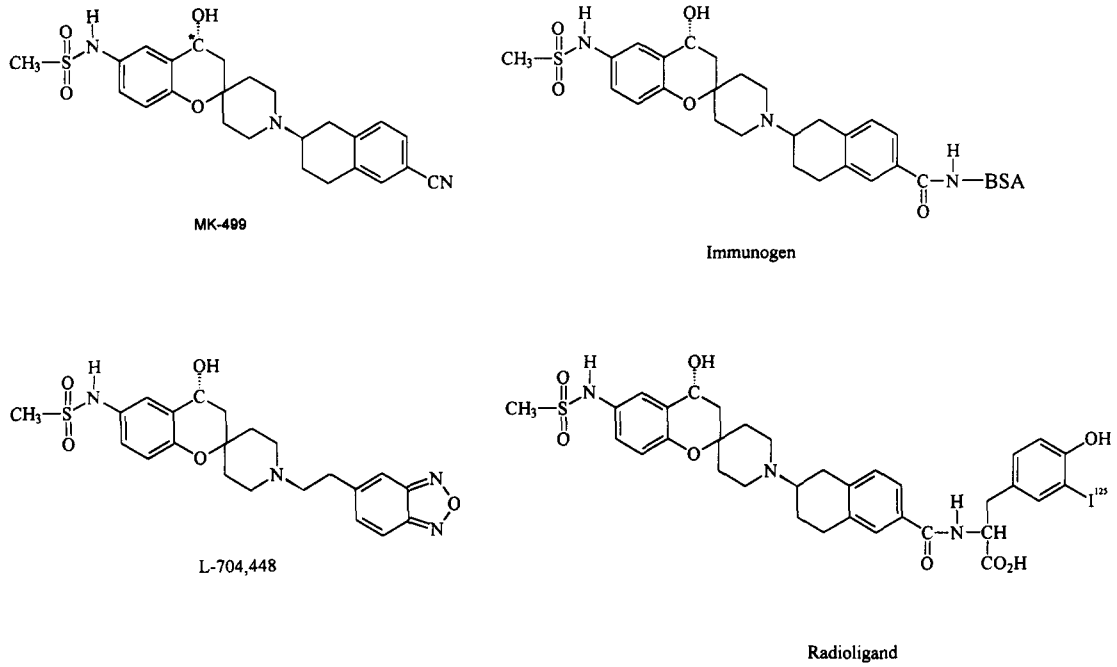

Fig. 1. Structures of MK-0499, L-704,448 (the internal standard used in the LC/MS-MS-based assay), and the immunogen and radioligand used for development of the RIA.

ity and adequate sensitivity, and the corresponding ^{125}I-radioligands were prepared from the same derivative. The RIA proved completely satisfactory through both preclinical and clinical development. However, the high potency of the drug obviated the use of standard techniques for establishing specificity in human plasma. Accordingly, a method based on LC/MS-MS was developed to cross-validate and thus ensure the integrity of the RIA. The high specificity of the LC/MS-MS-based assay, faced with analysis of blood after administration of ^{14}C-labeled drug of high specific activity, gave rise to an unanticipated complication which was resolved by separate monitoring of the ^{12}C- and ^{14}C-labeled species of the drug.

2. Experimental

2.1. Materials

MK-0499, ^{14}C-labeled MK-0499 (47 μCi mg^{-1}), the internal standard L-704,448 and the di-sodium and di-lithium salts of L-706,942 (the 6-carboxy derivative of MK-0499) used to prepare the immunogen and radioligand (Fig. 1) were synthesized within Merck Research Laboratories.

The following reagents were purchased from the indicated vendors: BSA and rabbit gamma-globulin from Calbiochem (San Diego, CA); Crotein SPC® from Croda, Inc. (Parsipany, NY); carrier-free ^{125}I-labeled sodium iodide from Amersham (Arlington Heights, IL); *N*-hydroxysuccinimide, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide, α-cyano-4-hydroxycinnamic acid and sodium metabisulfite from Aldrich (Milwaukee, WI); chloramine T, L-tyrosine, 8-anilino-1-naphthalenesulfonic acid (ANSA) and trifluoroacetic acid from Sigma (St. Louis, MO); acetic acid and sodium hydroxide from Mallinckrodt (Paris, KY); anhydrous dimethylformamide, acetonitrile, methanol, methyl-*t*-butyl ether, potassium phosphate, disodium ethylenediamine tetraacetic acid (EDTA), sodium azide, sodium tetraborate and phosphoric acid from Fisher (Fair Lawn, NJ); sheep anit-rabbit gamma globulin from Arnel Products Co. (New York, NY); air (hydrocarbon-free), nitrogen (99.999%) and argon (99.999%) from West Point Supplies (West Point, PA).

2.2. Radioimmunoassay

Preparation and characterization of the immunogen

To 10.7 mg (0.02 mmol) of L-706,942 di-sodium salt in 0.406 ml dimethylformamide

were added 2.07 mg (0.02 mmol) of *N*-hydroxysuccinimide and 8.38 mg (0.04 mmol) of 1-(3-dimethyl-aminopropyl)-3-ethyl carbodiimide both in 0.2 ml dimethylformamide. The mixture was stirred overnight at room temperature. Examination by TLC on silica layers using a mobile phase of chloroform–methanol–water–acetic acid (90:10: 1:1, v/v) showed >95% conversion of L-706,942 ($R_f = 0.05$) to its *N*-hydroxysuccinimide ("active") ester ($R_f = 0.15$).

To 26 mg (0.4 μmol) of BSA in 2.0 ml potassium phosphate (pH 8.89; 0.125 M) was added, in portions over a period of 4 h, 684 μl (0.017 mmol) of the active ester solution. An equivalent quantity of BSA, dissolved in buffer and treated with 684 μl of dimethylformamide, served as a control. Both the immunogen and control solutions were carefully placed in 8-cm lengths of dialysis tubing and exhaustively dialyzed in the same beaker of distilled water (2 l) for 72 h with water changes every 24 h. After dialysis the protein solutions were diluted to concentrations of 1 mg ml^{-1} with distilled water.

The degree of incorporation of hapten into the immunogen was determined by measurement of the differences in mass between the immunogen and the control BSA, using matrix-assisted laser desorption time-of-flight mass spectrometry MALD-TOF/MS [8].

To 100 μl of the test solutions was added 10 μl of trifluoroacetic acid (1%) in water. The stock solution of UV-absorbing matrix, α-cyano-4-hydroxycinnamic acid, was prepared at a concentration of 10 mg ml^{-1} in a 1:1 mixture of acetonitrile and water. The immunogen (13.5 pmol) and BSA (4.5 pmol) were deposited on the sample slide with 63 nmol of matrix and dried under vacuum prior to introduction into the spectrometer.

The samples were analyzed on a Kratos Kompact MALDI III time-of-flight laser desorption mass spectrometer. Analysis was performed in the linear high-mass mode using a laser power of 47. The spectrum (Fig. 2) is that of a mixture of the immunogen and control BSA (added as calibrant), and is an average of 1000 laser shots, smoothed with a 20-point average. The immunogen, which contained no unreacted BSA, showed a median incorporation of 24.8 moles of hapten per mole of protein (range 21–32).

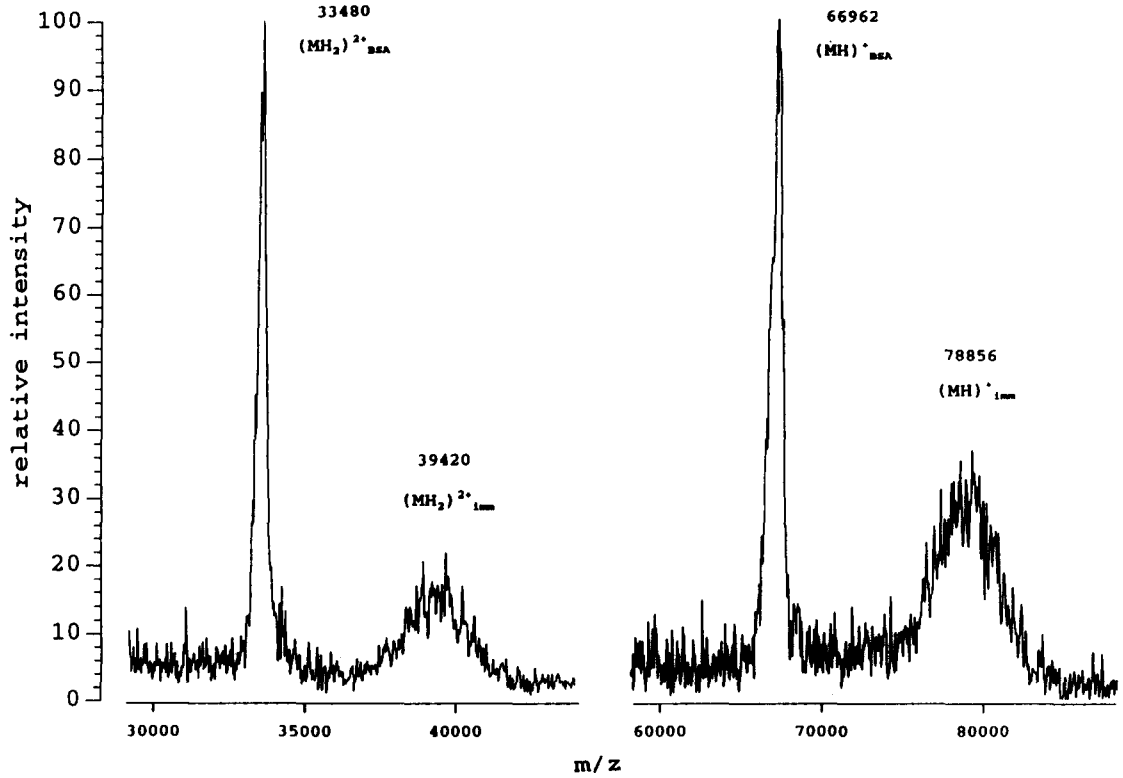

Fig. 2. MALD-TOF mass spectrum of a mixture of control BSA and the immunogen prepared by coupling L-706,942 via its *N*-hydroxysuccinimide ester to BSA. Both singly and doubly charged species are shown. Masses cited are the approximate centroid positions of the peaks.

Immunization

Immunogen solutions were emulsified with an equal volume of Freund's complete adjuvant. Female New Zealand white rabbits were immunized with approximately 1 mg of protein by multiple intra-dermal injections plus single intramuscular (i.m.) and subcutaneous (s.c.) injections. Boosting with 0.5 mg of protein was performed in incomplete adjuvant (i.m. and s.c. only) at 1, 3 and 7 months post-initiation. Antisera were stored at −70 °C.

Preparation of radioligand

The radioligand was prepared by reaction of ^{125}I-labeled L-tyrosine with L-708,983 (the dilithium salt of L-706,942). To 0.2 μg (0.00107 μmol) of L-tyrosine in 5 μl of 0.05 M borate buffer (pH 8.5) was added 10 μl of 0.5 M potassium phosphate (pH 7.5), 20 μl (2 mCi) of carrier-free Na^{125}I (100 mCi ml^{-1}), and 5 μl of a freshly prepared solution of chloramine T (2.5 mg ml^{-1}) in water. The reaction was stopped after 60 s by addition of 15 μg sodium metabisulfite in 10 μl of 0.5 M potassium phosphate. To the mixture was added 10 μl (0.25 μmol) of a solution of the *N*-hydroxysuccinimide ester of L-708,983. The mixture was allowed to react overnight at room temperature. The radioligand was purified by HPLC on a Waters (Milford, MA) μBondapak C-18 column (3.9 × 300 mm, 10 μm). Gradient elution was used with a mobile phase consisting initially of 20% methanol in 0.1% acetic acid, increasing to 50% and 70% methanol after 30 min and 60 min, respectively. Radioactivity was monitored using a flow-through radioisotope detector (Beckman, Model 170, Irvine, CA). Peaks corresponding to the radioligand were collected using a fraction collector (Gilson Medical Electronics Inc., Model 203, Middleton, WI). A radiochromatogram is shown in Fig. 3. The mono- and di-iodinated radioligands eluting at retention times of about 36 and 43 min were tested for binding in the assay and then stored at −20 °C, under which conditions they had a useful life of approximately 8 weeks. The mono-iodinated radioligand (specific activity ≈ 2.8 mCi μg^{-1}) was used preferentially since it was more stable to autoradiolysis.

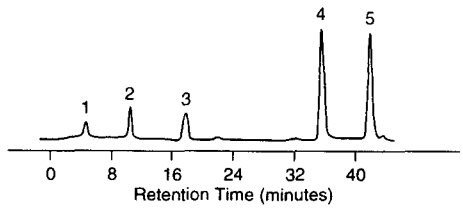

Fig. 3. Radiochromatogram of the products of the reaction of the *N*-hydroxysuccinimide ester of L-706,942 with mono- and di-^{125}I-labeled tyrosine. Peak 1 is iodide, peaks 2 and 3 correspond to mono- and di-iodotyrosine, and peaks 4 and 5 are the mono- and di-iodinated radioligands, respectively.

Assay procedure

The principal buffer was potassium phosphate (0.05 M; pH 7.0) containing 0.05 M EDTA, 2% Crotein SPC and 0.01% sodium azide. The buffer used in the immunoassay contained ANSA (142 μg ml^{-1}) to minimize non-specific protein binding. The stock standard consisted of 0.1-ml aliquots of MK-0499 in 0.01 M HCl at a concentration of 1 μg ml^{-1}, which were stored at −70 °C. The assay was configured to use 50-μl aliquots of test plasma or standard solutions. The standards were diluted in buffer containing 20% methanol to yield concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng ml^{-1} (0.0025 to 1 ng per assay tube).

Quality control samples consisted of normal (drug-free) plasma containing the drug at nominal concentrations of 0.2, 0.5 and 2 ng ml^{-1}. The rabbit antiserum ("first antibody") was diluted 1:180 000 in buffer containing 50 μg ml^{-1} of rabbit gamma globulin to provide bulk for the double antibody precipitation. For convenience in pipetting, the radioligand (200 000 cpm ml^{-1}) were pre-mixed with buffer containing sheep antirabbit globulin ("second antibody") serum at a dilution of 1:50 with buffer. In our experience with double antibody assays, the binding at equilibrium is similar whether the first and second antibodies are added separately or simultaneously.

Reagents were added to 12 × 75 mm glass culture tubes using an automatic pipetting station (APS, Micromedic, Horsham, PA) as follows for 50 μl standard solution, test plasma sample or quality control plasma. Normal plasma (50 μl) was added to standards and 50 μl buffer to test samples, 100 μl of the first antibody/gamma globulin reagent, 100 μl of the second antibody/radio-ligand (20 000 cpm per tube) and 700 μl of assay buffer. Non-specific binding was determined in tubes lacking the first antibody and analyte. After overnight incubation at room temperature (≈ 18 h), the tubes were centrifuged for 45 min at 800 g, and

the supernatants were decanted from the precipitates and discarded.

The tubes were inverted to drain (on paper towels) 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-0499 at concentrations in excess of 20 ng ml^{-1} were diluted as appropriate with control plasma and 50-μl aliquots taken for re-analysis. Calibration curves were constructed using a third-order polynomial as a variant of the conventional logit–log transformation. A calibration curve of net control binding ($100 \times (B - \text{nsb}/B_o - \text{nsb})$) versus plasma concentration was constructed. B_o is the observed binding (in counts per minute) in the absence of analyte and nsb is the non-specific binding. Appropiateness of fit was evaluated by reading standards as though unknowns. The concentrations of MK-0499 in test samples were determined by interpolation from the standard curve.

Fractionation of urine samples

Urine samples were fractionated to verify the specificity of the assay in urine. Two human clinical urine samples, collected in the intervals 2–4 h and 18–24 h after administration of MK-0499 (2.1 mg, p.o.), were assayed by RIA. The concentrations of the drug were 133 and 117 ng ml^{-1}, respectively. Aliquots (100 μl) of each urine were fractionated by HPLC using a Spherisorb 250 × 4.6 mm Cyano column (5 μ). A linear gradient of 5–35% acetonitrile in 0.1% aqueous tri-fluoroacetic acid was used at a flow-rate of 1 ml min^{-1}. Fractions, collected at 1-min intervals, were analyzed by RIA. MK-0499 eluted after 24 min.

LC/MS-MS

LC/MS-MS was performed on a Sciex (Thornhill, Ontario) Model API III Plus triple quadrupole mass spectrometer, interfaced via a Sciex heated nebulizer probe to a liquid chromatograph consisting of a Hewlett-Packard (Wilmington, Delaware) 1050 solvent delivery system and 1050 autoinjector equipped with a 150-μl loop. HPLC was performed using a 250 × 4.6 mm Zorbax RX C_8, 5 μm, from Dupont (Wilmington, DE). The mobile phase was acetonitrile–methanol–water (45:5:50, v/v) containing 0.1% v/v trifluoroacetic acid at a flow-rate of 1 ml min^{-1}. The volume of extract injected was 75 μl and samples were chromatographed in batches of 80–180 under the control of a Macintosh IIfx Computer running Sciex's RAD (routine acquisition and display) software. The nebulizer probe temperature setting was 500 °C. The nebulizing gas (air) pressure and auxiliary (make-up) gas settings were 80 p.s.i. and 1 l min^{-1} respectively. Atmospheric pressure chemical ionization was effected by a corona discharge needle (+3 μA) and positive ions were sampled into the triple quadrupole mass analyzer via a 0.0045-inch pinhole aperture. The curtain gas was nitrogen at 0.7 l min^{-1}. The mass spectrometer was programmed to admit the protonated molecules $[M + H]^+$ at m/z 468 and m/z 459 for MK-0499 and its internal standard, respectively, via the first quadrupole mass filter (Q1), with collision induced fragmentation in Q2 (collision gas argon at a density of 200×10^{12} atoms cm^{-2}) and monitoring of the product ions at m/z 253 (MK-0499) and m/z 244 (internal standard) via Q3. The orifice potential, electron multiplier and interface heater settings were 70 V, 3.8 kV and 50 °C, respectively. The dwell time was 200 ms. Peak area ratios obtained from multiple reaction monitoring of the analyte (m/z 468 → 253)/internal standard (m/z 459 → 244) were computed using Sciex's Mac Quan software. For quantification of ^{14}C-labeled MK-0499 the parent/product ion combination used was m/z 470 → 138. The calibration curves were constructed using a weighted (reciprocal of concentration) linear least-squares regression, and the concentrations of the drug in test samples were calculated by interpolation from the standard curves.

Extraction procedure for LC/MS-MS assay

Standards were prepared by adding solutions of MK-0499 to 0.5-ml aliquots of control human plasma to yield a series of concentrations in the range 0.05–10 ng ml^{-1}. Separate quality control samples were prepared at 0.05, 0.2 and 2 ng ml^{-1}. An aliquot of plasma (test sample, standard or quality control) was placed in a 13 × 100 mm borosilicate glass screw-cap tube. After the addition of internal standard (50 μl, 0.5 ng) and 0.5 ml of potassium phosphate solution (0.1 M; pH 7.0), the tube was briefly vortexed. Methyl *t*-butyl ether (5 ml) was added, the mixture was shaken for 10 min and the phases separated by centrifugation. The organic layer was transferred to a fresh culture tube and the solvent removed by evaporation under a stream of nitrogen at 40 °C. The extract was

reconstituted in the mobile phase (125 μl) and the tube was briefly vortex mixed then placed in an ultrasonic bath for 10 min. After centrifugation (5 min) the extract was transferred to an autosampler vial just prior to analysis.

The absolute recovery of MK-0499 was determined by spiking, the duplicate, aliquots of control plasma (0.5 ml) with the drug at three different concentrations, adding the internal standard (0.5 ng) *after* extraction, and comparing the peak area ratios with those of unextracted reference samples to which the same quantity of internal standard had been added. The recoveries at 0.2, 2 and 20 ng ml^{-1} were 91.6, 90.8 and 87.8%, respectively.

Statistical analysis of data

Statistical comparisons of data resulting, for example, from drug stability studies in plasma, determinations of accuracy by standard addition and assay cross-validation experiments were undertaken using the paired student's *t*-test and regression analysis programs supplied in the statistical analysis package of Excel, Version 4.0 (Microsoft Corporation, Redmond, WA). Fixed and proportional errors were determined from the 95% confidence limits around the slope and intercept functions of the linear regression. Random error was assessed from the relative standard deviation (RSD) of the individual test data/reference data ratios.

The reproducibility between different analytical methods was calculated using a modification of the relationship proposed by Caulcutt and Boddy [9]:

$$R_C = tn^{1/2}(\sigma_{C1}^2 + \sigma_{C2}^2)^{1/2} \quad (1)$$

where R_C is the predicted reproducibility of methods at plasma concentration C, and t is the Student's t-statistic ($P = 0.05$) at the combined degrees of freedom used to compute the precision of the individual assays. n defines the number of methods being compared (two here), and σ_{C1}^2 and σ_{C2}^2 are the variances of assays 1 and 2 at concentration C. Should there be no significant differences between the methods (i.e. the reproducibility of results obtained by the two methods is defined only by the precision of the individual assays), then 95% of results obtained by the test method should be within the range $C \pm R_C$ obtained by the reference method.

3. Results

The objective of this research was the development of a rapid and reliable method for the determination of MK-0499 in plasma. The high potency of this substance predicted the need for assays sensitive to at least 0.1 ng ml^{-1}. Our acceptance criteria required that assays be both accurate and specific with intra-assay coefficients of variation, at the lower quantifiable limit, not exceeding 10%. For a previous drug of the same class and potency, RIA had proved highly satisfactory [7] and was thus selected as the method of choice in this instance.

3.1. Radioimmunoassay

Antisera

Specific antisera were raised in four rabbits. All antisera were tested three months after immunization. The antiserum was selected partly on the basis of its titer against the radioligand, but principally according to its specificity with respect to the analogous ketone (L-702,958) and the corresponding *N*-desalkylated substances considered to be potential metabolites. Antisera from animal # 72 appeared superior, yielding 50% binding (B_0 per total number of counts = 0.5) of radioligand at a dilution of 1:180 000. Binding was optimal at pH 7.0, at which the assay was run routinely. The I_{50} was 35 pg per tube. Scatchard [10] analysis (bound/free) versus molar concentrations (M) of bound antigen showed the anticipated curved relationship indicating a mixed population of antibodies in this polyclonal antiserum. Deconvolution of the curves, using the method of Hunston [11], indicated the pressure of two principal antibodies with affinity constants (K_a) of 4.4×10^{10} and 1.5×10^9 M^{-1}.

The antiserum was stored diluted as small aliquots in assay buffer (1:300) at −70 °C and was used in all subsequent evaluations and assays.

Assay sensitivity, precision

Using a 10% maximum for intra-assay RSD, the lowest measurable concentration for the RIA of plasma was 0.05 ng ml^{-1}. The upper quantifiable limit of the RIA was 20 ng ml^{-1} ($B/B_o > 5\%$). Samples with concentrations in excess of this were diluted with control plasma and re-assayed. The non-specific binding was 0.90%. The inter-assay accuracy and precision were determined from quality control data (from spiked control plasma) obtained during separate analyses of clinical samples. At concentrations of 0.2, 0.5, 2 and 10 ng ml^{-1}, the mean

values ($n = 16$) for percentage recovery (RSD) were 94.7% (6.0%), 97.9% (4.3%), 98.9% (3.7%) and 107.2% (2.6%), respectively.

To accommodate the higher concentrations of MK-0499 in urine it was necessary to dilute samples of urine 1:50 with buffer. A 50-µl aliquot of this diluted sample was taken for analysis. This volume of urine (1 µl per assay tube) had no measurable effect on the assay binding. The lower quantifiable limit for the assay of urine was 2.5 ng ml^{-1}, which was adequate to enable calculation of urinary excretion for up to 8 days after oral or intravenous administration to man.

Parallelism

Parallelism experiments are conducted on RIAs in an attempt to detect unknown drug-related substances which might compete with the antigen for binding sites on the antibodies. Such substances, generally metabolites, frequently show cross-reactivities with respect to the antigen, which are not constant but which increase with increasing dilution.

Parallelism was conducted on samples of plasma and urine collected from patients who had received MK-0499 orally and intravenously. The concentrations of the drug were first measured on the undiluted sample, which was subsequently diluted 1:2, 1:4, 1:8, 1:16 and 1:32 (urine only) with normal human plasma or urine as appropriate and then re-assayed.

Plots were constructed to the normalized found concentration versus dilution factor. There was no significant trend to raise or lower concentrations in either plasma or urine, and hence no indication of interfering metabolites.

Accuracy (recovery) in patient's plasma and urine using the method of standard addition

The effect of potential unknown drug-related substances on the accuracy of the assay was assessed using the method of standard addition. Aliquots of 10 samples of plasma from patients were assayed for MK-0499. The plasma concentrations ranged from 0.5 to 5 ng ml^{-1}. Subsequently, additional drug (equivalent to 2.18 ng ml^{-1} of plasma) was added and the samples were re-assayed. The "expected" data were the intrinsic plasma concentrations plus the value of the spike. The "measured" data were those concentrations obtained by analysis after addition of the spike. The paired Student's t-statistic (Table 1) suggests a small but consistent reduction in the measured/expected ratios. However, the average ratio is 0.97 (RSD = 2.57%) and the regression analysis showed no significant fixed or proportional errors.

A similar experiment was conducted in six samples of patients' urine with intrinsic concentrations of MK-0499 ranging from 50 to 150 ng ml^{-1}. To these a spike equivalent to 86.5 ng ml^{-1} was added. The average recovery (measured/expected) was 1.06 (RSD 2.88%). Student's t-test and regression analyses were regarded as inappropriate on such a small sample set over such a narrow range of concentrations.

In both matrices the accuracy of the assay was regarded as satisfactory.

Stability studies

Stabililty of the stock standard solution. Reference solutions of MK-0499 in 0.01 M HCl at concentrations of 1.0 and 10.0 µg ml^{-1} were stored at −70 °C for 14 months. The standards were then assayed in duplicate against freshly prepared solutions. The measured concentrations in the stored standards were 1.002 ± 0.005 and 10.15 ± 0.193 ng ml^{-1}.

Sample stability under storage at −20 °C. Sixteen samples of plasma collected from subjects dosed orally and intravenously with MK-0499 were assayed on receipt from the clinical site. The concentrations of MK-0499 ranged from 0.56 to 5.7 ng ml^{-1}. The samples were stored at −20 °C for 4 months and re-assayed. Statistical data, given in Table 1, showed a mean ratio (repeat assays/original assays) of 1.02 (RSD = 6.73%). The student's t-statistic revealed no significant differences and the regression analysis showed no biases between the data sets. It was concluded that clinical plasma containing MK-0499 showed satisfactory stability.

Stability under freeze–thaw cycles. Three samples of plasma collected after oral administration to volunteers were assayed and subjected to three freeze–thaw cycles (−20 °C → 25 °C). The initial concentrations were 2.11, 1.01 and 10.02 ng ml^{-1}, respectively. The corresponding concentrations at completion of the experiment were 2.05, 1.00 and 10.29 ng ml^{-1}, respectively. The results suggest no appreciable change in plasma concentrations after three freeze–thaw cycles.

Light and room temperature stability. The stability of MK-0499 in plasma when exposed to light

Table 1
Statistical treatments (ratios, Student's t-test and regression) of analytical data generated in support of RIA accuracy, drug stability and assay cross-validation experiments. The +95% and −95% values associated with slope and intercept are the upper and lower confidence intervals

Experiment	Test data	Reference data	No. of samples compared	Mean ratio (test/ref)	RSD of ratio (%)	t-found	t-critical	slope (m)	m		Intercept (c)	c	
									+95%	−95%		+95%	−95%
RIA accuracy by standard addition to plasma	Measured	Expected	10	0.970	2.57	−2.88	2.26	0.992	1.076	0.908	−0.105	0.328	−0.539
RIA plasma stability	Repeat	Original assays	16	1.020	6.73	0.13	2.13	0.924	1.004	0.843	0.225	0.486	−0.036
RIA vs. RIA cross-validation	Results at Phoenix	Results at Merck	33	0.996	5.55	−1.90	2.04	0.924	0.941	0.907	0.031	0.048	0.014
RIA vs. LC/MS-MS cross-validation A (unlabeled)	Results by RIA	Results by LC/MS-MS	40	1.013	5.85	1.16	2.02	1.017	1.027	1.008	−0.022	0.021	−0.065
RIA vs. LC/MS-MS cross validation B (labeled)	Results by RIA	Sum of ^{12}C- and ^{14}C-results by LC/MS-MS	34	0.991	5.36	−0.50	2.03	1.043	1.068	1.019	−0.064	0.023	−0.152

at room temperature was examined. The test plasma was a composite of those collected from patients receiving the drug orally at time points close to T_{max}. Samples were exposed to normal laboratory fluorescent light at room temperature (≈ 25 °C) for up to 20 h. A control sample was stored alongside the test sample in darkness. No appreciable loss of MK-0499 was noted from the test sample (0.456 ng ml^{-1}) with respect to the control (0.426 ng ml^{-1}). A similar series of experiments conducted using MK-0499 in urine showed satisfactory stability at room temperature and 4 °C for up to 48 h.

Specificity

The variability of the assay when applied to the assay of pre-dose (i.e. drug-free) plasma from 10 human volunteers was determined. No significant variations in B/B_0 were observed using 50 μl of test plasma.

Several substances structurally related to MK-0499 were tested to assess their potential for interference in the immunoassay. The cross-reactiv- ities, expressed as the ratios of the concentration of the test substance to that of MK-0499 necessary to cause 50% displacement, were approximately 100, 1000 and 10 000 for L-702,958, and the corresponding *N*-desalkylated alcohol and ketone derivatives of MK-0499, respectively. Of these, only the alcohol has been identified as a (urinary) metabolite of MK-0499. L-702,958 is pharmacologically active, being converted in vivo to MK-0499, but the reverse biotransformation is not significant.

The potential interferences of ten common prescription drugs were similarly tested. Lovastatin, simvastatin, metoprolol, captopril, diltiazem, furosemide, flurezepam, amitriptyline and nitroglycerine showed cross-reactivities in excess of 200 000, while that for warfarin was approximately 36 000.

To check for the presence of any unknown cross-reacting urinary metabolites, urine obtained from two subjects dosed orally with MK-0499 (2.5 mg) were first assayed directly and subsequently fractionated by HPLC, the immunoreactivity in the fractions being determined by RIA. The recoveries of immunoreactive species, determined by comparing the direct assays of the untreated urines (133 and 117 ng ml^{-1}) with the sum of the fractions corresponding in chromatographic retention time to MK-0499, were 100.9 and 94.9% for urine collected 2–4 h and 18–24 h after dosing. The only immunoreactive species in the fractionated urine corresponded to the parent drug. There was no evidence for the presence of cross-reacting metabolites.

Ruggedness

The ruggedness of the RIA for MK-0499 in plasma was tested by its establishment and re-validation in a contract laboratory (Phoenix International, Montreal, Canada), and cross-validation by analysis of a series of clinical plasma samples both at Merck and in the contract facility.

The assay at Phoenix proved satisfactory in terms of both intra- and inter-assay accuracy and precision over the range 0.077–7.7 ng ml^{-1}. Thirty-three samples of plasma from subjects dosed intravenously with MK-0499 were assayed in both laboratories. Twenty samples agreed within 5%, twelve within 10% and one within 12%. The paired Student's *t*-test (Table 1) showed no significant inter-laboratory differences in results. Regression analysis revealed no fixed bias, but a proportional bias of between −5.9 and −9.3%. The mean of the ratios (Phoenix/Merck) was 0.996. The random error (RSD) was 5.5%. The results were interpreted as indicating equivalent results in both laboratories.

3.2. LC/MS-MS

Mass spectra and selection of parent/product ion combinations

The product mass spectra of MK-0499, ^{14}C-labeled MK-0499 and the internal standard, L-704,448 are shown in Fig. 4. The internal standard shows a diagnostic fragment ion at *m/z* 244. The analogous fragment ion for both ^{12}C- and ^{14}C-labeled MK-0499 is at *m/z* 253. The complementary fragments of *m/z* 216 (^{12}C) and *m/z* 218 (^{14}C) have the general formula

$$CH_3SO_2NHC_6H_4OH\overset{*}{C}H=\overset{+}{O}H - CH_3SO_2H$$

$$HN=C_6H_3O\overset{*}{C}H=\overset{+}{O}H$$

where * indicates the site of a ^{14}C-label. Subsequent loss of methyl sulfonic acid yields the secondary fragment ions at *m/z* 136 and 138, respectively.

The cross-reactivity of unlabeled (^{12}C-)MK-0499 on several experimental parent/product combinations for the determination of the ^{14}C-labeled drug was examined. Specifically, the combination of *m/z* 468 → 253, used for mea-

surement of the unlabeled drug, was investigated for its effect on the measurements of the ^{14}C-labeled species using three potentially useful parent/daughter combinations. The amount of signal generated by the mass combination m/z 468→253 on the m/z 470→253 channel was 6.1%, i.e. 10 ng of unlabeled MK-0499 produced an apparent quantity of 0.61 ng of the ^{14}C-labeled drug. The cross-reactivities on the m/z 470→297 and 470→138 combinations were 5.8 and 1.0%, respectively. The reduced sensitivities to interference using the latter combination of parent/product ions could be anticipated from the fragmentation patterns (Fig. 4). It was anticipated that the $^{12}C/^{14}C$ ratios in plasma would not differ greatly from that of the dosing solution, so the error due to this type of "cross-talk" would not exceed 2%. Accordingly, measurement of the ^{14}C-labeled drug used the parent/daughter combination m/z 470→138.

Multiple reaction monitoring (MRM) chromatograms of extracts of plasma containing 0 and 0.013 ng ml^{-1} of (unlabeled) MK-0499 are shown in Fig. 5.

Calibration

Calibration curves for both MK-0499 and ^{14}C-labeled MK-0499 are shown in Fig. 6. Peak area ratios gave good fit to the weighted (reciprocal of concentration) regression lines. Calibration standards of plasma containing no drug showed peak area ratios that were essen-

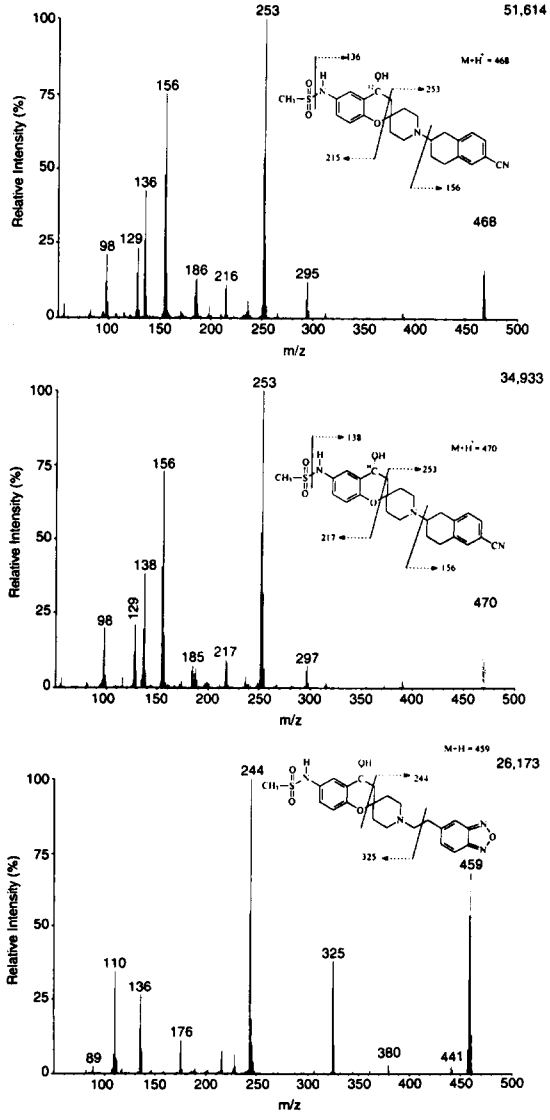

Fig. 4. Positive product ion mass spectra (background subtracted) of the protonated molecular ions of (top) MK-0499 (m/z 468), (center) ^{14}C-labeled MK-0499 (m/z 470) and (bottom) internal standard L-704,448 (m/z 459).

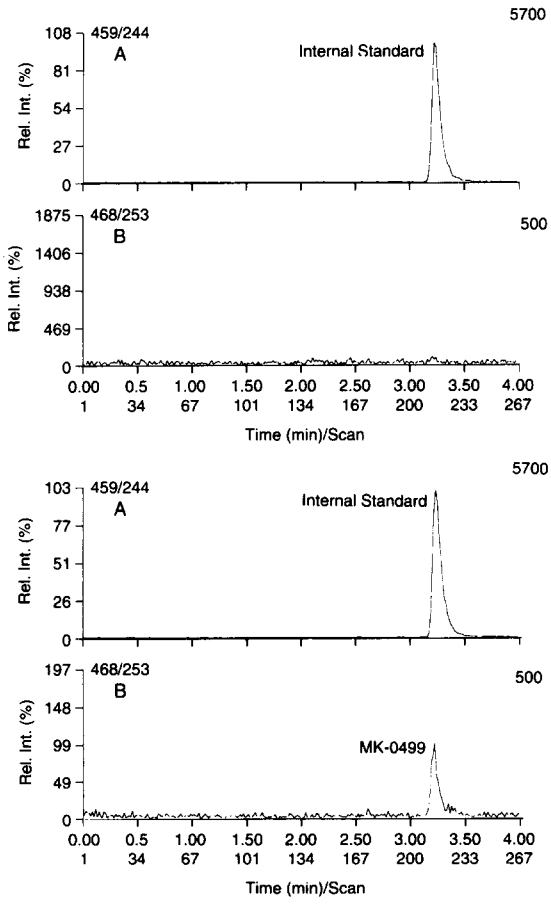

Fig. 5. Chromatograms obtained by multiple reaction monitoring of extracts of plasma. Retention times are shown in minutes. Channel A, m/z 459→244, internal standard; channel B, m/z 468→253, MK-0499. Top: extract of plasma containing no drug. Bottom: extract of plasma containing MK-0499 at a concentration of 0.013 ng ml^{-1}.

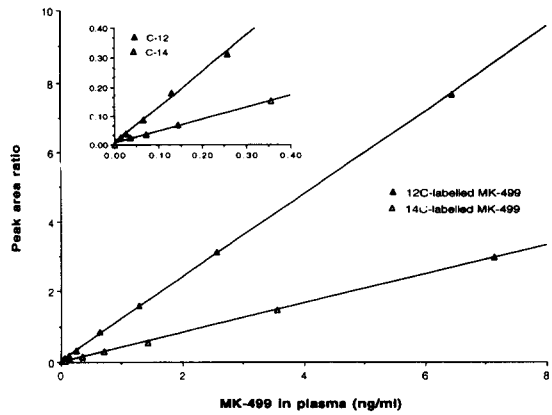

Fig. 6. Calibration curves for the determination of the unlabeled ^{12}C- and ^{14}C-labeled MK-0499 in human plasma by LC/MS-MS. The low ends of the curves (0–0.4 ng ml^{-1}) are shown in the insert.

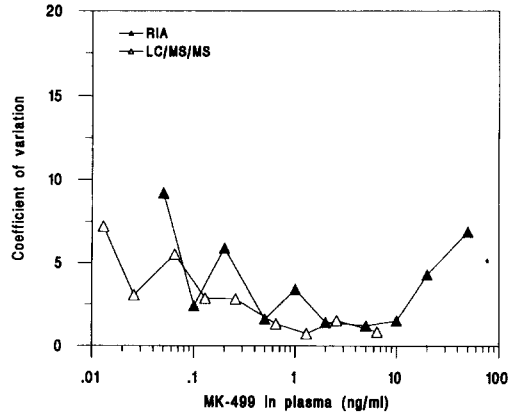

Fig. 7. Comparison of intra-assay precision profiles for the determination of MK-0499 in human plasma by RIA and LC/MS-MS.

tially zero. There was no interference from either endogenous plasma components or metabolites with the determination of the parent drug or its internal standard.

Precision and accuracy

Intra-assay precision was determined by extraction of five sets of standards, each set consisting of plasma containing MK-0499 at concentrations of 0, 0.013, 0.026, 0.064, 0.129, 0.258, 0.664, 1.288 and 6.44 ng ml^{-1}. A comparison of the intra-assay precision profiles of the RIA and LC/MS-MS-based assays is shown in Fig. 7. The inter-assay accuracy and precision were determined by the analysis of quality control plasma samples containing the drug at concentrations of 0.050, 0.2 and 2.0 ng ml^{-1} included in successive analyses. Data are shown in Table 2. The lower quantifiable limit for unlabeled MK-0499 using the present calibration range was 0.013 ng ml^{-1} (RSD = 7.2%). Owing to the need to use a different (and less sensitive) combination of parent/product ions for monitoring of ^{14}C-labeled MK-0499, its lower quantifiable limit was approximately three times greater (0.036 ng ml^{-1}). Isotopically pure ^{14}C-labeled MK-0499 was not available, so quality control samples were prepared using a mixture of the ^{14}C- and ^{12}C-labeled drug. Recoveries and RSDs are included in Table 2.

3.3. Cross-validation of the LC/MS-MS and RIA methods

The assays were cross-validated by analysis of plasma obtained from clinical studies. Two cross-validation experiments were performed. The first used plasma obtained from two patients who received the drug (700 μg) by intravenous infusion. Twenty plasma samples were collected from each patient from 10 min to 6 days post-dose. The statistical data are shown in Table 1. The mean ratio was 1.013 with an RSD of 5.9%. 35% of the samples agreed within 2%, 67.5% within 5%, 92.5% within

Table 2
Intra- and inter-assay accuracy and precision for the determination of MK-0499 in plasma by LC/MS-MS

	MK-0499 concentration (ng ml^{-1})	Mean % recovery	RSD (%)
Intra-assay (n = 5)	0.013	90.1	7.21
	0.026	93.8	3.06
	0.064	99.9	5.52
	0.129	107.9	2.87
	0.258	101.0	2.82
	0.644	108.2	1.31
	1.288	101.1	0.73
	2.576	99.1	1.52
	6.440	99.2	0.83
Inter-assay (n = 5)	0.05	104.0	2.51
	0.20	103.1	3.18
	2.00	101.4	3.45
	^{14}C-labeled MK-0499 concentration (ng ml^{-1})	Mean % recovery	RSD (%)
Inter-assay (n = 3)	0.036	101.5	1.39
	0.071	101.8	5.18
	0.714	106.7	1.96
	7.140	97.8	1.60

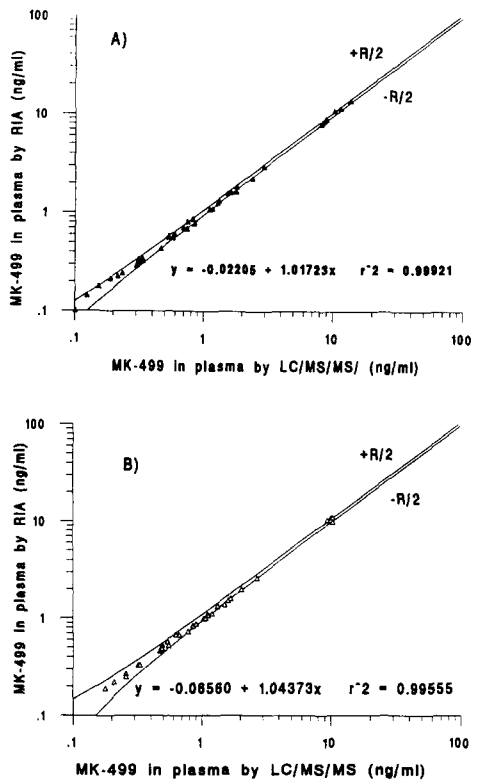

Fig. 8. Cross-validation of assays for the determination of MK-0499 in human plasma by RIA and LC/MS-MS. (A) Comparison of analyses obtained on plasma following intravenous administration of unlabeled MK-0499 to two volunteers. (B) Comparison of measurements made of plasma following oral and intravenous administration of ^{14}C-labeled MK-0499 of high specific activity to a single volunteer. The data obtained by LC/MS-MS are the sum of the ^{12}C- and ^{14}C-labeled species.

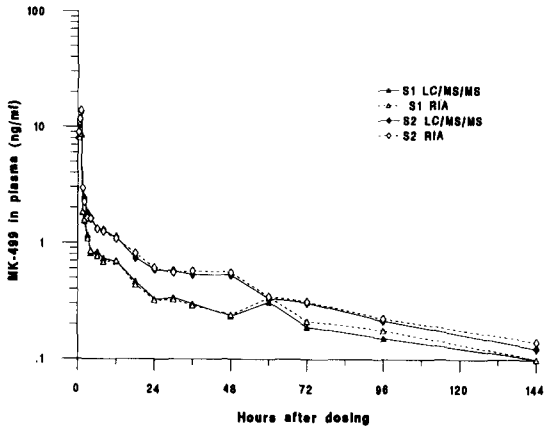

Fig. 9. Plasma concentration–time curves of MK-0499 following administration of the drug (700 μg) by intravenous infusion for 1 h to two human volunteers (S1 and S2). Determinations of plasma concentrations were made by both RIA and LC/MS-MS.

10%, 97.5% within 15% and 2.5% (1 sample) exceeded 15%. The observed value of t (−1.16 with 39 degrees of freedom) when compared with t-critical (2.02 for $p = 0.05$) showed no significant difference in assay results generated using the two different methods. Linear regression analysis (Fig. 8(A)) showed an overall assay bias (mean LC/MS − mean RIA) of −0.9%. The intercept was −0.022 with confidence intervals ranging from −0.065 to +0.0207, indicating no fixed bias. The slope was 1.017 (1.0078–1.0266), showing the RIA to have a small positive bias of between 0.8 and 2.7%. Plasma concentration–time profiles are shown in Fig. 9.

In the second cross-validation experiment, plasma was obtained from a single patient dosed both orally and intravenously with ^{14}C-labeled MK-0499 (47 μCi mg^{-1}). At this specific activity, 35.7% of the drug molecules in the dosing solution were labeled. A total of 34 samples was assayed. Cross-validation necessitated separate measurement of the ^{14}C- and ^{12}C-labeled species by LC/MS-MS and comparison of the sum of their concentrations with the results obtained by RIA.

Good agreement of data obtained by LC/MS-MS and RIA were observed (Table 1). Sixty percent of the samples agreed within 5% and all were within 10%. The mean ratio (RIA/total LC/MS) was 0.991. The observed value of t (−0.50 with 33 degrees of freedom) when compared with t-critical (2.03 for $p = 0.05$) showed no significant differences between and assays. The overall bias was −2.0%. Regression showed no significant fixed bias, but a positive proportional bias of the RIA of between 1.9 and 6.8%. The random error (RSD) was estimated as 5.4%. The regression line is presented in Fig. 8(B). Plasma concentration–time curves showing the labeled and unlabeled species determined by LC/MS-MS, MK-0499 determined by RIA, and the total measured radioactivity after oral administration are shown in Fig. 10.

4. Discussion

RIA is a preferred analytical technique for small molecules in our laboratories owing to its sensitivity, simplicity and ability to batch-process large numbers of clinical samples, and the relative inexpensiveness of the equipment. The principal drawback of RIA is its potential lack of specificity with respect to closely related

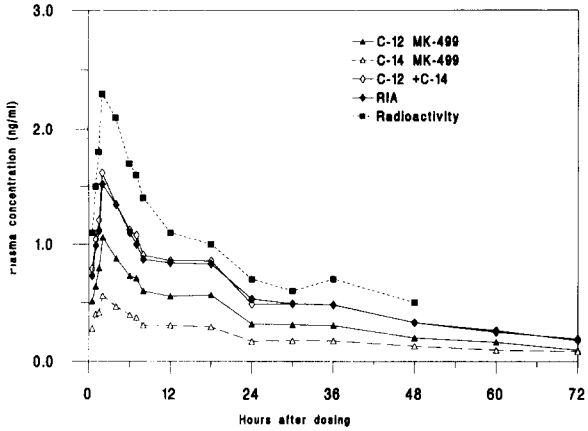

Fig. 10. Plasma concentration–time curves of unlabeled (^{12}C-) MK-0499, ^{14}C-labeled MK-0499, and their sum (all determined by LC/MS-MS), MK-0499 determined by RIA, and the total radioactivity profile after oral administration of the ^{14}C-labeled drug to a human volunteer.

substances, generally metabolites, where biochemical modification of the test drug has occurred close to the bridge between the hapten and the carrier protein. Such modifications are in the "blind-spot" of the antibodies and will generally result in overestimation of the analyte. Frequently, application of an immunoassay to the analysis of a drug in biological fluids must be implemented with little or no knowledge of the nature of metabolites in the species in question. Thus, a very sensitive immunoassay can be developed which ultimately proves non-specific. Achievement of specificity for biological fluids from one species, say rats, is no guarantee that the assay will prove successful in another, e.g. dogs, monkeys or man. In the past few years our group has had to abandon several RIAs specific to the analysis of test drugs in animals, but not so in humans, even with the precaution of raising antisera against three or four different immunogens in which the carrier protein was linked to a derivative of the test molecule in different ways.

We recently reported an assay for L-691,121, an antiarrhythmic of similar class to MK-0499 in which the parent drug, a ketone, was very extensively converted to the corresponding hydroxyl metabolite in vivo [7]. The cross-reactivity of the metabolite was only 0.8%, but the high efficiency of conversion gave metabolite/drug ratios in excess of 50:1, resulting in unacceptable overestimation of the drug. A differential immunoassay using two antibodies with different specificities was ultimately successful, although tedious. With these experiences in mind, an RIA for the determination of MK-0499 in plasma and urine was developed in response to a need for a procedure of sufficient sensitivity to measure parent drug in plasma several days after single oral doses of 1–4 mg. The assay proved satisfactory in terms of sensitivity (0.05 ng ml^{-1}) and precision. The accuracy of an RIA cannot, however, be determined solely from analysis of spiked drug-free plasma, so additional tests of parallelism and accuracy in patient's plasma, using the method of standard addition, were performed and indicated satisfactory performance of the assay.

A more definite demonstration of specificity is routine in our laboratories [12,13]. Plasma, urine, bile, etc., from a patient dosed with the drug are assayed. The sample, or its extract, is then fractionated by HPLC and the fractions are assayed by RIA. This enables construction of an immunochromatogram in which it is hoped that, firstly, there is no chromatographic peak other than the analyte which cross-reacts in the RIA, and, secondly, the recovery of the analyte, defined by its retention time, agrees closely (95–105%) with that determined in the unextracted material.

For the RIA of MK-0499 such recovery experiments, conducted in rat and dog plasma and also in human urine, demonstrated satisfactory specificity. They were not feasible in human plasma, however, owing to the low concentrations of the drug. The need to ensure specificity of the RIA in human plasma was the sole reason for establishing the alternative procedure based on LC/MS-MS. The latter procedure proved nearly four times more sensitive than the immunoassay.

The criteria for satisfactory cross-validation of bioanalytical methods are somewhat subjective. We chose to use regression analysis as a measure of fixed and relative bias, the RSD of the ratios being an indication of random error and a paired Student's t-test assessing overall assay acceptability. These statistics show considerable interaction and it is unwise to draw conclusions from a single parameter without consideration of the others. Strictly, the use of simple linear regression assumes no error in the independent variables. This is not the case with cross-validation experiments, where use of a much more complex errors-in-variables regression has been recommended [14]. However, for the comparison of plasma drug concentration data spread over several orders of magnitude, the differences in confidence intervals obtained using both types of regression are minimal [15].

Ultimately, only the analyst can establish criteria for the satisfactory cross-validation of assays using objective common sense aided perhaps by several simple statistical treatments. Both cross-validation experiments described here show non-significant t-statistics, absence of fixed error, random errors of less than 6%, and a positive proportional error for the RIA of 0.7–2.7% in one case and 1.8–6.8% in the other. Finally, with one exception in a total of 75 pairs, the measured reproducibilities of the determinations were within the limits predicted from the individual variances of the assays.

Based on these results, we concluded that the RIA and LC/MS-MS-based assays were essentially equivalent and, accordingly, that the immunoassay demonstrated satisfactory specificity.

LC/MS-MS has, in the past few years, evolved into an invaluable quantitative bioanalytical tool, largely because of its sensitivity and unparalleled specificity [16–20]. The latter, however, proved disadvantageous for the analysis of plasma after administration of radiolabeled MK-0499 of high specific activity, necessitating the development of separate assays for the ^{12}C- and ^{14}C-labeled species. Analytical laboratories need to carefully consider their strategies for the analysis of biological samples following administration of very potent ^{14}C-labeled drugs if their principal analytical technique is based on mass spectrometry.

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