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Validated HPLC/MS/MS assay for CI-1011 in rat plasma and a comparison with an HPLC/UV assay

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Abstract

A liquid chromatographic/mass spectrometric (LC/MS/MS) method to quantitate CI-1011 in rat plasma has been validated and compared to an LC/UV assay. The analyte and internal standard were isolated from the plasma matrix by using liquid/liquid extraction with diethyl ether. The ether layer was evaporated to dryness and the residue reconstituted in acetonitrile–water (70:30, v/v). A 2.1 × 150 mm × 5 μm Zorbax RX-C18 column with a mobile phase of acetonitrile–ammonium acetate (pH 8.0; 5 mM)–triethylamine (70:30:0.03, v/v/v) delivered at a flow rate of 0.2 ml min^{-1} was used for chromatography. Analyte and internal standard ion chromatograms were obtained by operating the mass spectrometer in the negative ion multiple reaction monitoring mode to detect the presence of a precursor–product ion pair for both the analyte and the internal standard. Samples were introduced into the mass spectrometer using electrospray ionization. Retention times of CI-1011 and of the internal standard (IS), [$^{13}C_6$]CI-1011, were approximately 4.2 min. No peaks interfering with the quantitation of CI-1011 were observed throughout the validation process. Mean recoveries of CI-1011 from rat plasma ranged from 98.2 to 105%. The recovery of the IS was 100%. Assay precision for CI-1011, based on the percent relative standard deviation of replicate quality controls, was less than or equal to 5.60% with an accuracy of ± 8.80%. The lower limit of quantitation for CI-1011 was 0.500 ng ml^{-1} for a 0.2-ml sample aliquot. CI-1011 is stable in rat plasma for 24 h at room temperature and for at least 34 days at −20°C. This assay has been proven suitable for routine quantitation of CI-1011 in rat plasma at concentrations from 0.500 (100 pg on-column) to 500 ng ml^{-1}. The applicability of this method to determine CI-1011 concentrations in rat plasma is reported in this manuscript. CI-1011 concentrations, in plasma samples from cholesterol- and chow-fed rats administered single daily oral doses of CI-1011 in a CMC/Tween suspension, obtained using a validated LC/UV assay were compared to concentrations obtained using the reported LC/MS/MS assay over the concentration range 0.0806–12.3 μg ml^{-1}. The concordance correlation coefficient determined for this comparison was 0.9977, suggesting that the CI-1011 concentrations obtained by the two assays are in excellent agreement.

Keywords: Antiathersclerotic agent; CI-1011; LC/MS/MS; Lipid regulator

1. Introduction

CI-1011 is being investigated as a lipid regula-

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tor and an antiatherosclerotic agent. Preclinically, CI-1011 functions as an inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT) [1]. ACAT catalyzes the intracellular esterification of cholesterol. ACAT inhibitors are regarded as potential antiatherosclerotic agents because the cholesterol ester enrichment of arterial walls is an integral process in the pathogenesis of atherosclerosis. CI-1011 inhibits the progression of atherosclerotic lesions in long-term studies of atherosclerotic activity in rabbits [2].

An assay more sensitive than an existing validated liquid chromatography–ultraviolet (LC/UV) assay was required to measure CI-1011 concentrations in rat plasma samples from pharmacology studies that were expected to be below the lower limit of quantitation (80 ng ml^{-1}) of the existing LC/UV assay. The method validation of the LC/UV assay for measuring CI-1011 in rat plasma has not been reported in the literature. The liquid chromatography–tandem mass spectrometry (LC/MS/MS) assay described in this manuscript is 160-fold more sensitive than the LC/UV assay.

A comparison study, described in the following sections, was executed to compare CI-1011 concentrations in plasma samples from dosed rats obtained using the validated LC/UV assay to those obtained using the validated LC/MS/MS assay.

All concentrations in this report are expressed as free acid equivalents. Chemically, CI-1011 is designated as a sulfamic acid phenyl ester. The structure of CI-1011 is shown in Fig. 1.

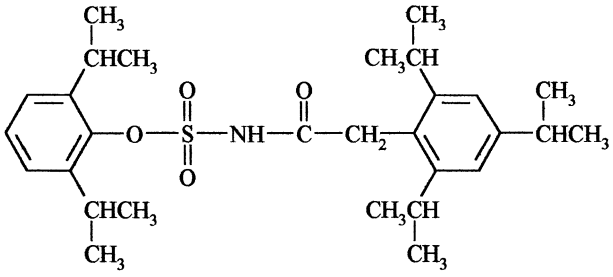

Fig. 1. Structure of CI-1011.

2. Materials and methods

2.1. Materials

CI-1011 and [$^{13}C_6$]CI-1011 were supplied by Parke-Davis Pharmaceutical Research Division, of the Warner-Lambert Company (Ann Arbor, MI). Acetonitrile and formic acid were obtained from Mallinckrodt (Paris, KY). Diethyl ether and water were purchased from EM Science (Cherry Hill, NJ). Ammonium formate was obtained from Kodak (Rochester, NY). All solvents were high-performance liquid chromatography (HPLC) grade and all reagents were analytical grade unless noted otherwise.

Heparinized rat plasma was supplied by Pel-Freez Biologicals (Rogers, AR).

2.2. Liquid chromatograpy–tandem mass spectrometry

The liquid chromatographic system consisted of a Series 200 pump and an autosampler from Perkin-Elmer (Norwalk, CT). The detector was a Quattro II Mass Spectrometer purchased from Micromass (Manchester, UK). A Zorbax RX-C18, 2.1 × 150 mm × 5 μm, column was supplied by MAC-MOD Analytical (Chadds Ford, PA). The mobile phase consisted of acetonitrile–ammonium acetate (pH 8.0; 5mM)–triethylamine (70:30:0.03, v/v/v) and was delivered at a flow rate of 0.2 ml min^{-1}. The sample injection volume was 0.025 ml. The retention time of the analyte and the internal standard was approximately 4.2 min.

The mass spectrometer was operated in the multiple-reaction monitoring (MRM) mode by using electrospray ionization with an ionization potential of 3000 V. The cone voltage was 40 V and the source temperature was 120°C. Ions were collisionally activated at an energy of 40 eV and at an indicated argon pressure of 2×10^{-3} torr. To assay CI-1011, the mass spectrometer was operated at unit mass resolution, and was set to select the $[M-H]^-$ ion of m/z 500 for CI-1011 and the $[M-H]^-$ ion of m/z 506 for the internal standard ([$^{13}C_6$]CI-1011) via the first quadrupole mass filter (Q1), while signals for product ions of

m/z 177 and 183 for analyte and internal standard, respectively, were monitored via the third quadrupole mass filter (Q3). The dwell time for each transition was 50 ms. Multiple-reaction monitoring data were acquired and integrated using the MassLynx version 2.2 software package (Micromass).

2.3. Preparation of calibration standards and quality controls

Stock solution I, containing 1.0 mg ml^{-1} CI-1011 in acetonitrile, was prepared for each batch run beginning with the weighing of the compound. Stock solution II, containing 0.1 mg ml^{-1} CI-1011, was prepared by diluting stock solution I with acetonitrile. A working standard of 1000 ng ml^{-1} CI-1011 in rat plasma was prepared by adding 0.1 ml of stock solution II to 9.9 ml with heparinized blank control plasma. Calibration standards were prepared by serial dilution of the 1000 ng ml^{-1} working standard.

Rat plasma quality controls (QCs) containing 1.25, 12.5 and 125 ng ml^{-1} CI-1011 were prepared by diluting 0.1-ml aliquots of standard solutions containing 0.15, 1.5 and 15 μg ml^{-1} CI-1011 with 11.9 ml blank rat plasma. These controls were subdivided into 0.3-ml aliquots and stored in 1.5-ml conical plastic tubes at -20°C. Dilution quality controls (DQC), 10000 ng ml^{-1}, and limit of quantitation standards (LOQ), 0.500 ng ml^{-1}, were prepared and stored in a similar fashion. Dilution quality controls were diluted 25-fold prior to analysis. Quality control, DQC and LOQ samples were prepared by weighings of CI-1011 which were independent of those for calibration standards.

Stock solution III, containing 1.25 mg ml^{-1} [$^{13}C_6$]CI-1011 in acetonitrile, was prepared fresh for each batch run beginning with the weighing of compound. Stock Solution III was diluted with acetonitrile to prepare stock solution IV, 50 μg ml^{-1}. Stock solution IV was diluted with blank heparinized rat plasma to prepare a working standard containing 250 ng ml^{-1} [$^{13}C_6$]CI-1011. A 0.1-ml aliquot of the working standard was added to all samples to produce an internal standard concentration of 125 ng ml^{-1} plasma.

2.4. Extraction procedure

Samples, QCs, DQCs and LOQ samples were thawed at room temperature for approximately 90 min, then vortexed for 1 min on a multitube vortexer, and a 0.2-ml aliquot was placed in two dram vials. Internal standard, 0.1 ml of the 250 ng ml^{-1} working standard, was added to all vials and the contents of each vial were vortexed for 1–3 s. Ammonium formate buffer (pH 3.0; 0.1 M), 0.1 ml, was added to all vials and the contents of each vial were vortexed for 1–3 s. Diethyl ether, 2 ml, was added to each sample vial and the vials were sealed with PTFE-lined screw caps. Vials were then shaken for 20 min. The solution was clarified by centrifugation for 20 min at $2200 \times g$. The aqueous layer was frozen in a dry ice/isopropanol cold bath for approximately 10 min. The top ether layer was decanted into 12×75 mm culture tubes and was evaporated to dryness in a water bath at 45–50°C under a steady stream of nitrogen for approximately 5–6 min. The residue was reconstituted in 0.1 ml acetonitrile–water (70:30, v/v) and vortexed for 1 min on a multitube vortexer. The reconstituted sample was centrifuged for 30 min at $2200 \times g$. The supernatant was transferred to 0.2-ml vials for injection into the LC/MS/MS system.

2.5. Quantitation

The assay method was validated over the concentration range 0.500–500 ng ml^{-1} by assaying 10 calibration standards in singlet and three quality control samples in quadruplicate in three separate batch analyses. The best-fit line was determined by least-squares linear regression of the calibration data from each batch run using a weighting factor of $1/\text{concentration}^2$ [3]. Rat plasma CI-1011 concentrations were determined using peak–area ratios of CI-1011 to IS and the regression parameters.

2.6. Selectivity

Assay selectivity was assessed by testing blank rat plasma from 10 different sources. The assay was considered adequately selective if no endoge-

nous rat plasma components, at the same m/z of the analytes of interest, eluted at the retention times of the analytes of interest.

2.7. Accuracy and precision

Quality control samples were assayed in quadruplicate at four concentrations in three separate batches. Assay accuracy was expressed as the percent relative error (%RE), the percentage of the deviation of the mean observed concentration from the nominal value. Assay precision was expressed as the percentage of the relative standard deviation (%RSD) of the mean observed concentrations.

2.8. Lower limit of quantitation

LOQ samples were assayed at least in triplicate at one concentration in three separate batches. The lower limit of quantitation is defined as the lowest concentration of the calibration standards with acceptable accuracy and precision. In order to meet acceptance, the LOQ samples were to have an intra-run accuracy of $\pm 20\%$ and an intra-run precision of less than or equal to 20%.

2.9. Recovery

Recovery of CI-1011 from rat plasma was determined using nine replicates at each of the concentrations used for the quality controls. Recovery of IS was determined at 125 ng ml^{-1} plasma. Recovery was assessed by comparison of the chromatographic responses obtained from extracted samples with the chromatographic responses of non-extracted controls prepared in solvent from reconstituted blank plasma extracts.

2.10. System repeatability

Repeatability of chromatographic response, given as percent relative standard deviation (%RSD) of mean peak–area ratios, was determined for nine replicate injections of extracted rat plasma quality control samples at three concentrations of CI-1011.

2.11. Stability

The stabilities of CI-1011 and IS, expressed, where appropriate, as a percentage of initial values, were evaluated in stock solution, injection solvent, biological matrix at room temperature and -20°C, and following three freeze–thaw cycles of the biological matrix. The acceptance criterion for stability was a difference from the initial chromatographic responses of less than or equal to 10%.

2.12. Applicability of method

The suitability of the method for investigating the pharmacokinetics of CI-1011 in rats was assessed by analyzing plasma samples from an efficacy study using chow- and cholesterol-fed male and female rats. Rats were administered single daily oral doses of CI-1011 in a CMC/Tween suspension for 14 days. The chow-fed rats were administered doses of 1, 10 and 30 mg kg^{-1} daily. The cholesterol-fed animals were administered doses of 0.03, 0.1 and 1 mg kg^{-1} daily. One blood sample was collected on day 1 and seven more samples were collected serially after dose administration on day 14. These samples were placed in heparinized tubes, centrifuged, and the resulting plasma was stored at -20°C until analysis.

2.13. Comparison of concentrations obtained from LC/MS/MS and LC/UV assays

Briefly, the LC/UV method used a 0.5-ml aliquot of acetonitrile to precipitate proteins from a solution containing 0.1 ml of internal standard and 0.2 ml of plasma sample. After centrifugation, the resulting supernatant was placed in a clean tube and diluted with 1 ml water. Extract Clean RC C18 solid-phase cartridges, 100 mg, from Alltech Associates (Deerfield, IL) were preconditioned with 2 ml acetonitrile followed by 2 ml water. The diluted supernatant was aspirated through the preconditioned cartridges and washed with 2 ml water. Analytes were eluted with 0.5 ml acetonitrile–water (60:40, v/v). A 120-μl aliquot of the eluent was placed in a polypropylene injection vial and 70 μl was introduced to the chro-

matography system. Chromatography was performed with a 4.6×250 mm Zorbax phenyl column (Chads Ford, PA) held at a temperature of approximately 40°C. The mobile phase consisted of acetonitrile–0.1% trifluoroacetic acid (67:33, v/v) delivered at a flow rate of 1.5 ml min^{-1}. Analyte concentrations were determined by using UV detection at 215 nm. The LC/UV assay was validated over the concentration range 0.100–40.0 μg ml^{-1} using the same acceptance criteria as the LC/MS/MS assay described in detail in this manuscript.

Thirty-three representative rat plasma samples, analyzed by the LC/MS/MS assay for CI-1011 for the efficacy study, were selected on the basis of common concentration ranges for analysis by the LC/UV assay and to cover as much of the common concentration range between the two assays as possible.

The CI-1011 UV and MS/MS assays were statistically compared via three statistics [4], namely, the Pearson correlation coefficient:

$$\rho = \frac{\sigma_{12}}{\sigma_1 \sigma_2},$$

the bias correction factor:

$$C_b = \frac{2}{\sigma_1/\sigma_2 + \sigma_2/\sigma_1 + (\mu_1 - \mu_2)^2/(\sigma_1\sigma_2)},$$

and the concordance correlation coefficient:

$$\rho_c = \rho C_b,$$

where μ_I is the mean of assay I and σ_i is the standard deviation of assay I, for $I = 1$, 2, and σ_{12} is the covariance between assays 1 and 2. These statistics were calculated using SAS (Release 6.08, SAS Institute Inc, Cary, NC). The Pearson correlation coefficient reflects precision, the degree to which points cluster about the regression line. The bias correction factor reflects accuracy, the degree to which the regression line adheres to the 45° line through the origin (the concordance line). The concordance correlation coefficient (ρ_c) reflects the degree to which points adhere to the concordance line. The concordance correlation coefficient and ρ assume values between -1 and 1; C_b assumes values between 0 and 1. Two assays are considered to be in perfect agreement if and only if $\rho_c = 1$. The value of ρ_c stemming from the LC/MS/MS assay and LC/UV assay comparison was compared to a benchmark value of ρ_c. This benchmark was derived from triplicate observations from a validated LC/MS assay of CI-1011 concentrations in 11 samples of dog plasma by the following process: for each plasma sample, two of the three observations were randomly chosen. One observation was randomly assigned to the horizontal axis, the other to the vertical axis. The concordance correlation coefficient was then calculated. This process was repeated 1000 times, yielding a maximum value of ρ_c of 0.9901, and giving a sense of how well the LC/MS assay agrees with itself. The concordance correlation coefficient for the rat plasma data was compared to this benchmark by testing

$$H_0: \quad \rho_c \leq \text{benchmark} \quad \text{vs.} \quad H_A: \quad \rho_c > \text{benchmark}$$

and reporting the P-value. A benchmark ρ_c using LC/MS/MS rat plasma CI-1011 concentrations was unavailable due to insufficient volume of the study samples for triplicate analysis.

3. Results and discussion

3.1. Quantitation

Peak–area ratios of calibration standards were proportional to the concentration of CI-1011 in rat plasma over the range 0.500–500 ng ml^{-1}. The calibration curve appeared linear and was well described by least-squares regression lines with a mean ($n = 3$) coefficient of determination of 0.999. A weighting factor of $1/\text{concentration}^2$ [3] was chosen to achieve homogeneity of variance. The inter-run (between-run) precision and accuracy were determined by pooling the individual assay results of the calibration standards over three separate batch runs. Inter-run accuracy and precision estimates for CI-1011 calibration standards were less than 4.20 and 4.22%, respectively. These data are tabulated in Table 1.

Table 1
CI-1011 concentrations in rat plasma calibration standards in three separate batch runs

Concentration (ng ml^{-1})	0.5	1.0	2.5	5.0	10	25	50	100	250	500	Slope	Intercept	R^2
Batch run 1	0.488	1.04	2.51	5.00	10.0	24.9	49.5	101	248	493	0.866	0.0676	0.9996
Batch run 2	0.498	0.967	2.68	5.31	9.96	25.2	50.1	99.9	238	473	0.839	0.0457	0.9978
Batch run 3	0.498	1.01	2.48	4.97	10.5	25.2	50.9	99.9	249	471	0.829	0.0705	0.9992
N	3	3	3	3	3	3	3	3	3	3	3	3	3
Grand mean	0.495	1.01	2.56	5.09	10.2	25.1	50.2	100	245	479	0.845	0.061	0.999
Inter-run SD	0.0058	0.0367	0.108	0.188	0.301	0.173	0.702	0.635	6.08	12.2	0.0191	0.0136	0.0009
Inter-run %RSD	1.17	3.63	4.22	3.69	2.95	0.689	1.40	0.64	2.48	2.54	2.26	22.3	0.09
Inter-run %RE	−1.00	1.00	2.40	1.80	2.00	0.40	0.40	0.00	−2.00	−4.20			

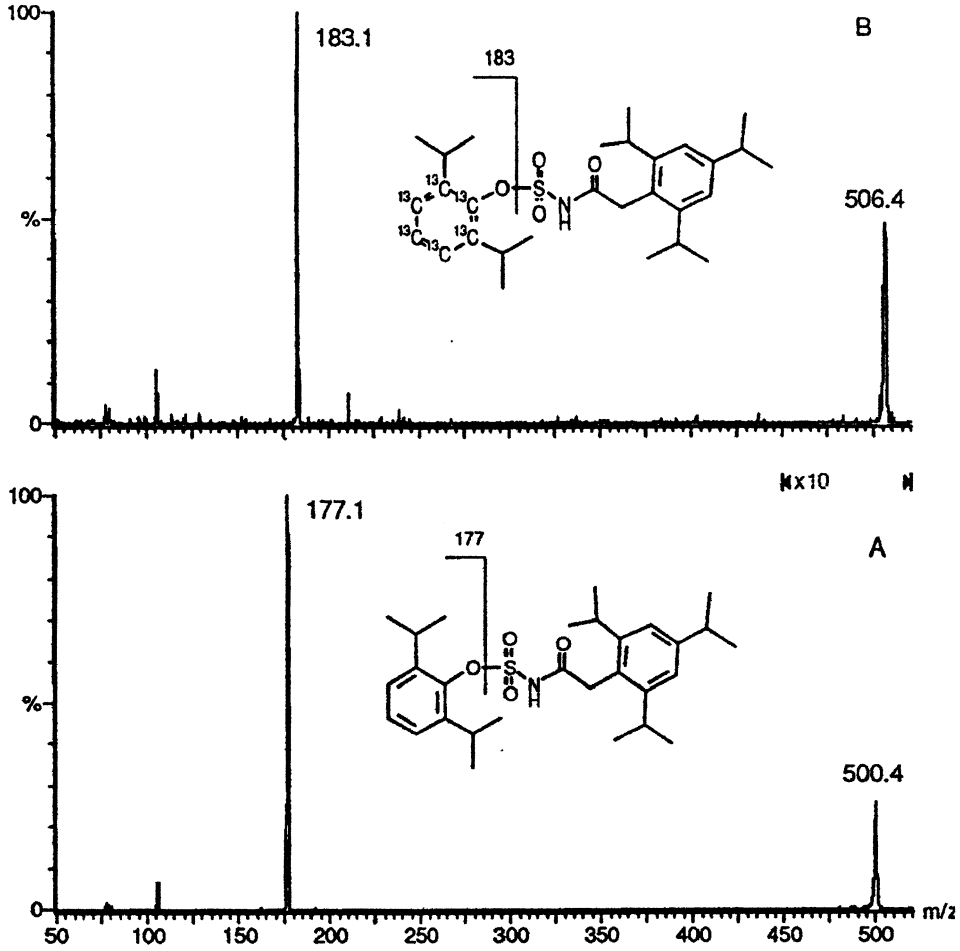

Fig. 2. MS/MS negative ion electrospray mass spectra of: (A) [$^{13}C_6$]CI-1011 ([M–H]$^-$ of m/z 506.4; and (B) CI-1011 ([M–H]$^-$ of m/z 500.4.

3.2. Assay selectivity

The product ion mass spectra of 200 ng ml^{-1} of pure standards of CI-1011 ([M–H]$^-$ of m/z 500.4) and [$^{13}C_6$]CI-1011 (IS, [M–H]$^-$ of m/z 506.4) are shown in Fig. 2(A) and (B), respectively. The predominant product ions of m/z 177.1 and m/z 183.1 are specific for the analyte and IS, respectively. The collision-induced decomposition transitions of m/z 500.4 → 177.1 and 506.4 → 183.1 were chosen for monitoring the analyte and IS, respectively, in the LC separation.

LC/MS/MS multiple-reaction monitoring (MRM) ion current chromatograms representing the separation of the analytes from matrix are shown in Figs. 3–5. MRM is the simultaneous monitoring of one or more collision-induced decomposition transitions of a precursor ion to product ion. Because unique ions were generated for each component, separate ion chromatograms were constructed for each injection. In each set of MRM ion chromatograms, the top trace (A or C) represents the relative abundance for ions corresponding to the transition 506.6 → 183.1 (IS), and the lower trace (B or D) represents the relative abundance for ions corresponding to the transition 500.6 → 177.1 (CI-1011). These transitions are marked in the upper right corner of each MRM

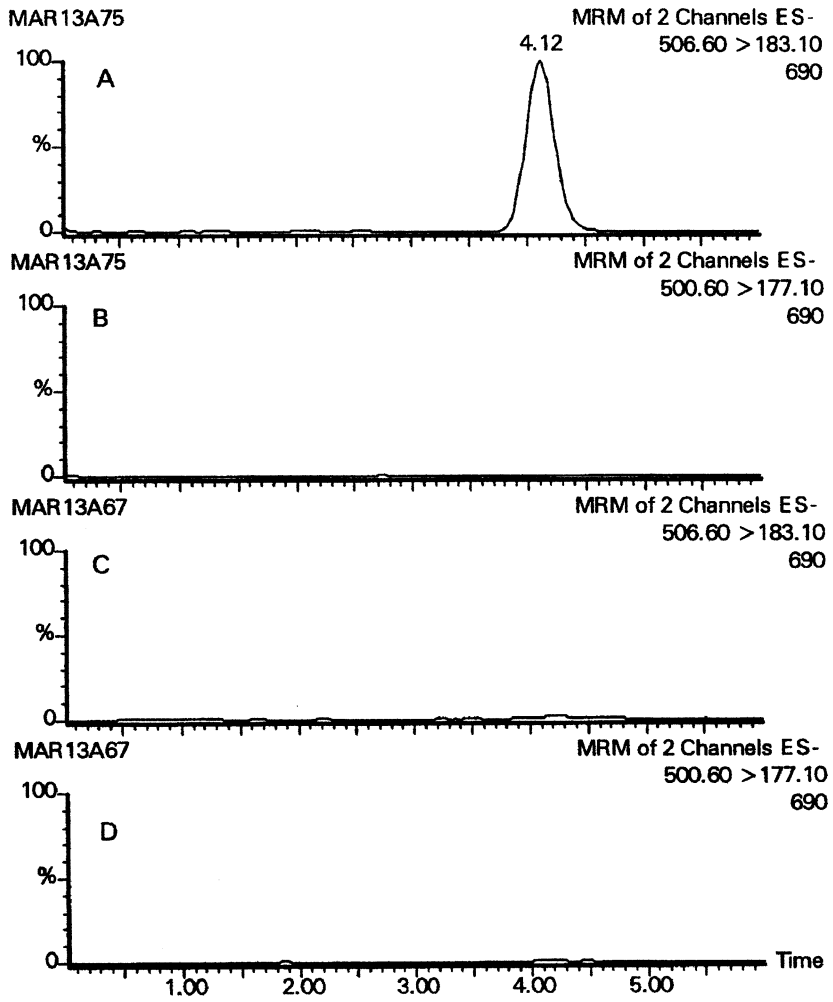

Fig. 3. LC/MS/MS MRM ion current chromatograms of extracted blank rat plasma (0.2 ml) with internal standard added (A and B) and without internal standard added (C and D) obtained by multiple-reaction monitoring of m/z 506.6 → 183.1 for the internal standard and m/z 500.6 → 177.1 for CI-1011.

chromatogram. The number below the transition is the total ion current and indicates the relative abundance of ions for the transition monitored. No apparent rat plasma components eluted at the retention time of CI-1011 (4.1 min) and IS at the m/z monitored, 177.1 and 183.1, respectively, in blank rat plasma samples from 10 independent sources. Fig. 3 is a MRM ion chromatogram of extracted blank rat plasma with (Fig. 3(A) and (B)) and without (Fig. 3(C) and 3(D)) internal standard. Fig. 4 is a MRM ion chromatogram of a plasma extract from a female rat taken prior to administration of CI-1011. Fig. 5 is a MRM ion chromatogram of an extracted rat plasma calibration standard containing 1.0 ng ml^{-1} CI-1011 and 125 ng ml^{-1} IS. These data indicate that the assay has adequate selectivity.

3.3. Intra-run CI-1011 accuracy and precision

Intra-run (within-run) precision and accuracy were determined by assaying quality controls in at least triplicate, at each of four levels, in three separate batch runs. The assay acceptance criterion for intra-run accuracy and precision esti-

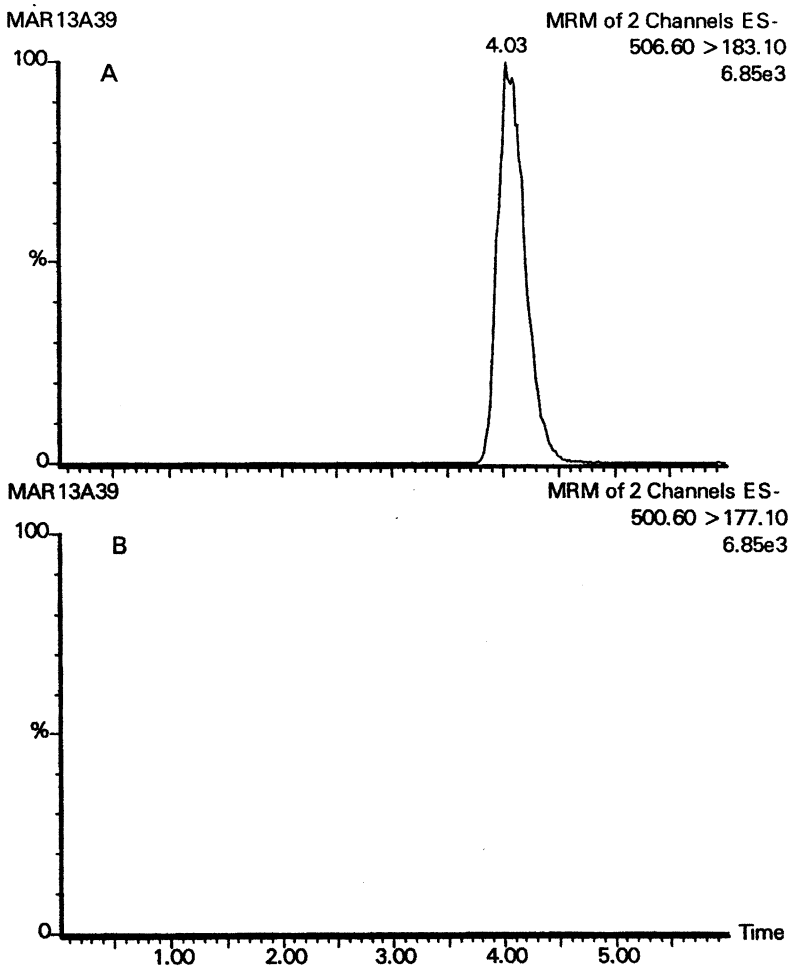

Fig. 4. LC/MS/MS MRM ion current chromatograms of a predose rat plasma (female chow-fed, 30 mg kg^{-1}) extract obtained by multiple-reaction monitoring of: (A) m/z 506.6 → 183.1 for IS (125 ng ml^{-1}); and (B) m/z 500.6 → 177.1 for CI-1011.

mates was 15% or less. The precision estimates for CI-1011 were less than 4.80, 1.76, 2.65 and 3.79% (%RSD) for quality control samples containing 1.25, 12.5, 12.5 and 10000 ng ml^{-1} CI-1011, respectively. Intra-run %RE estimates for these CI-1011 samples were −13.6, −11.2, −5.60 and −8.26%, respectively (Table 2).

These data indicate the assay has acceptable intra-run accuracy and precision.

3.4. Interrun CI-1011 accuracy and precision

Inter-run (between-run) precision and accuracy were determined by pooling all individual assay results of at least triplicate quality controls over three separate batch runs. The assay acceptance criterion for inter-run accuracy and precision estimates was 15% or less. Inter-run precision estimates for CI-1011 were 5.60, 3.94, 2.04 and 4.10% (%RSD) for quality controls containing 1.25, 12.5, 12.5 and 10000 ng ml^{-1} CI-1011, respectively. Inter-run %RE estimates were −8.80, −7.20, −4.00 and −5.05%, respectively (Table 2). These data indicate the assay has acceptable inter-run accuracy and precision.

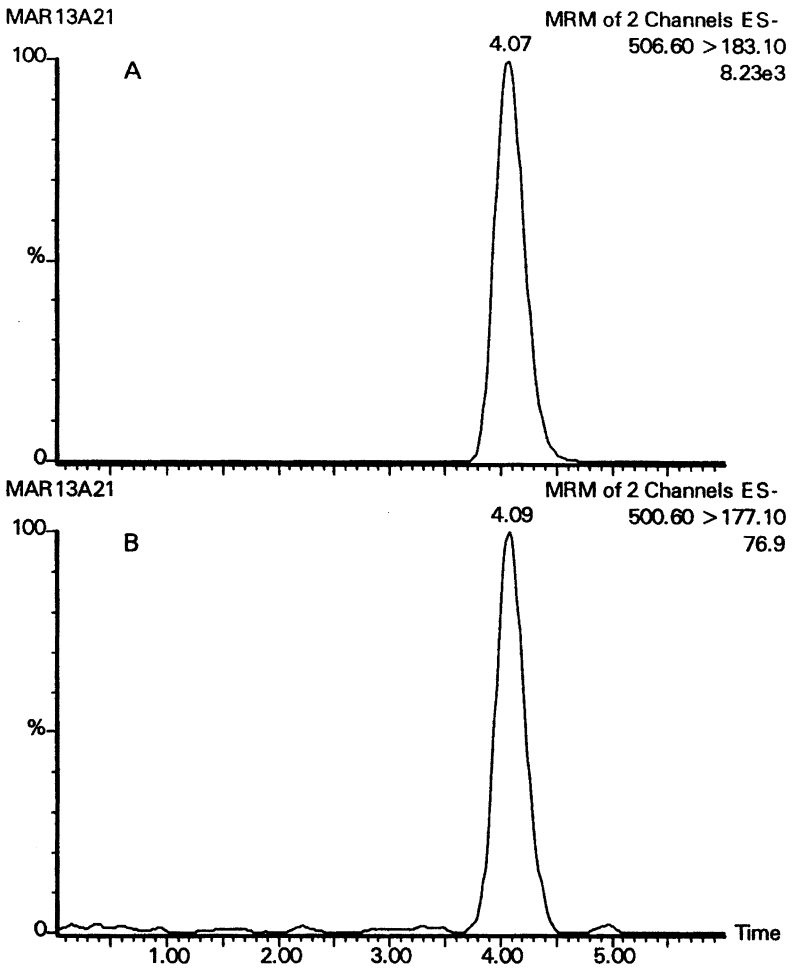

Fig. 5. LC/MS/MS MRM ion current chromatograms of an extracted 1.0 ng ml^{-1} rat plasma calibration standard obtained by multiple-reaction monitoring of: (A) m/z 506.6→183.1 for IS (125 ng ml^{-1}); and (B) m/z 500.6→177.1 for CI-1011.

3.5. Lower limit of quantitation

The LOQ in rat plasma, as determined by the intra-run accuracy and precision of replicate standards, was 0.500 ng ml^{-1} for CI-1011. The assay acceptance criterion for LOQ accuracy and precision estimates was 20% or less. CI-1011 LOQ samples had acceptable intra-run accuracy and precision of 17.8 and 10.5%, respectively.

3.6. Extraction recovery

The extraction efficiency of CI-1011 from rat plasma, expressed as percent recoveries, was determined by assaying at least nine samples at each of three concentrations and comparing the chromatographic responses to the mean of at least nine non-extracted standards at each of three concentrations. Mean (%RSD) CI-1011 recoveries from rat plasma were 105 (20.8), 98.2

Table 2
Rat plasma CI-1011 concentrations in lower limit of quantitation, quality control and dilution samples for three separate batch runs

Concentration added (ng ml^{-1}) (free acid equivalents)	0.500[a]	1.25	12.5	125	10000[b]
Batch run 1	0.445	1.10	11.2	118	9993
	0.364	1.08	11.0	117	10220
	0.433	1.10	11.1	119	9502
	0.397	1.04	11.2	117	9519
	0.416				9738
N	5	4	4	4	5
Intra-run mean	0.411	1.08	11.1	118	9794
Intra-run SD	0.0319	0.0283	0.0957	0.957	310
Intra-run %RSD	7.76	2.62	0.862	0.811	3.17
Intra-run %RE	−17.8	−13.6	−11.2	−5.60	−2.06
Batch run 2	0.532	1.17	11.9	121	9104
	0.481	1.19	12.0	119	9103
	0.463	1.22	11.6	122	9316
N	3	3	3	3	3
Intra-run mean	0.492	1.19	11.8	121	9174
Intra-run SD	0.0358	0.0252	0.208	1.53	123
Intra-run %RSD	7.28	2.12	1.76	1.26	1.34
Intra-run %RE	−1.60	−4.80	−5.60	−3.20	−8.26
Batch run 3	0.478	2.63[c]	11.9	125	8910
	0.508	1.14	12.1	119	9512
	0.412	1.22	12.2	120	9529
N	3	2	3	3	3
Intra-run mean	0.466	1.18	12.1	121	9317
Intra-run SD	0.0491	0.0566	0.153	3.21	353
Intra-run %RSD	10.5	4.80	1.26	2.65	3.79
Intra-run %RE	−6.80	−5.60	−3.20	−3.20	−6.83
N	11	9	10	10	11
Mean concentration found (ng ml^{-1})	0.448	1.14	11.6	120	9495
Inter-run SD	0.0501	0.0642	0.457	2.45	389
Inter-run %RSD	11.2	5.60	3.94	2.04	4.10
Inter-run %RE	−10.4	−8.80	−7.20	−4.00	−5.05

[a] Lower limit of quantitation samples.
[b] Dilution quality control samples.
[c] Rejected as outlier and omitted from all calculations.

(3.64) and 99.5 (1.47)% at concentrations of 1.25, 12.5 and 125 ng ml^{-1}, respectively. Mean (%RSD) recovery of internal standard from rat plasma was 100 (1.33)% at 125 ng ml^{-1}.

3.7. System repeatability

In order to assess the stability of the LC/MS/MS system during a typical analysis, the system repeatability experiment was deliberately performed for an analysis time 2 h longer than the typical batch analysis time of approximately 9 h. System repeatability was acceptable at 4.94, 0.92 and 0.91% for CI-1011, at concentrations of 1.05, 22.2 and 235 ng ml^{-1}, respectively, and indicated instrument stability over approximately an 11 h time span.

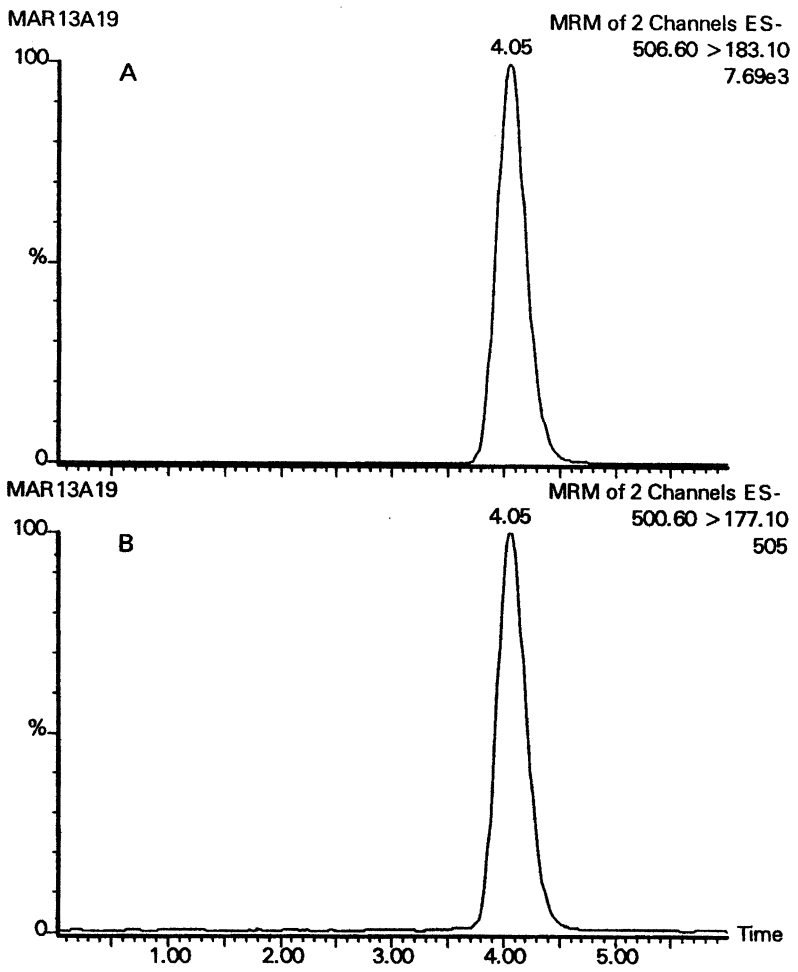

Fig. 6. LC/MS/MS MRM ion current chromatograms of a plasma extract from a chow-fed male rat 1 h after administration of a 14th consecutive single daily oral dose of 30 mg kg^{-1} of CI-1011. Chromatograms obtained by multiple-reaction monitoring of: (A) m/z 506.6→183.1 for IS (125 ng ml^{-1}); and (B) m/z 500.6→177.1 for CI-1011 (192 ng ml^{-1}).

3.8. Stability

The chromatographic responses of stock solution I of CI-1011 and IS were within 7.8% of initial values after 26 days at 4°C, indicating these compounds are stable in stock solution for at least this time period.

The peak–area ratios of CI-1011 in injection solvent after 7 days at 4°C were within 6.74% of initial values, indicating injection solvent stability for at least this time period.

CI-1011 concentrations in rat plasma quality controls after storage for 24 h at room temperature were within 8.3, 3.2 and 4.7% of initial concentrations for the low, mid and high QCs, respectively, indicating stability at room temperature for at least 24 h. The CI-1011 concentrations in rat plasma quality controls after 34 days of storage at −20°C were within 2.41, 1.55 and 1.22% of initial concentrations of the low, mid and high QCs, respectively, indicating stability frozen under for at least 34 days.

The mean responses of CI-1011, at assayed concentrations of 1.05, 22.2 and 235 ng ml^{-1}, after three freeze–thaw cycles, were within 1.90, 0.23 and 2.23% of initial mean control values, indicating CI-1011 is stable in rat plasma to three freeze–thaw cycles.

3.9. Applicability of method

Fig. 6 is a MRM ion chromatogram of a plasma extract, containing 192 ng ml^{-1} of CI-1011, taken from a chow-fed male rat 1 h after administration of a 14th consecutive single daily oral dose of 30 mg kg^{-1} of CI-1011. Fig. 7 shows the concentration–time profiles on day 1 and day 14 following single daily oral gavage administration of CI-1011 to chow-fed (Fig. 7(A)) and cholesterol-fed (Fig. 7(B)) female rats. These data indicate the successful application of this method. The pharmacokinetic parameters obtained from this study are reported in detail elsewhere [5].

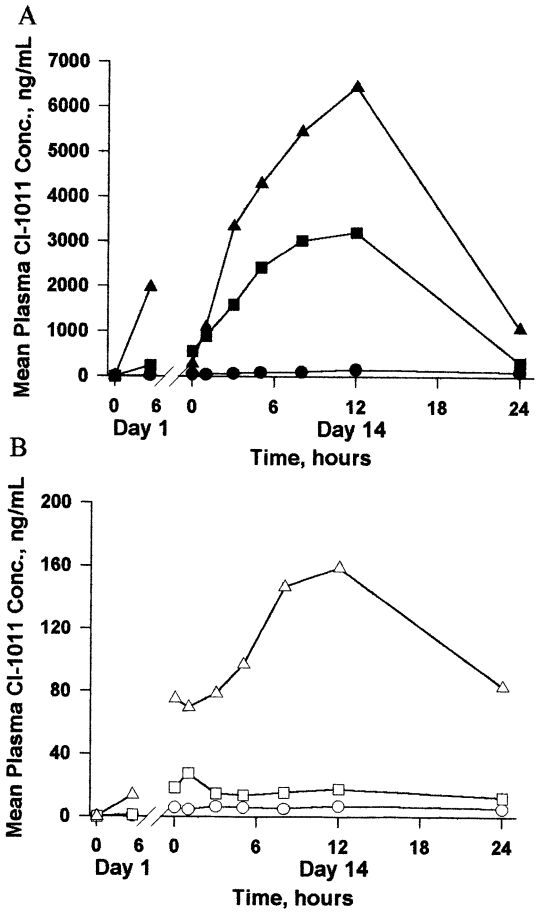

Fig. 7. Mean plasma CI-1011 concentration–time profile following single daily oral gavage administration of: 1 (●), 10 (■), or 30 (▲) mg kg^{-1} CI-1011 doses to chow-fed female rats (A); and 0.03 (○), 0.1 (□), or 1 (△) mg kg^{-1} CI-1011 doses to cholesterol-fed female rats (B).

3.10. Comparison of LC/MS/MS assay to LC/UV assay

Table 3 summarizes the individual plasma CI-1011 concentrations for each sample determined using the LC/UV and the LC/MS/MS assay. Fig. 8 is a plot of rat plasma CI-1011 concentrations determined by LC/MS/MS assay versus those determined using the LC/UV assay.

The correlation coefficient for the comparison of the LC/MS/MS assay to the LC/UV assay was 0.9979, suggesting good precision between the two assays. The C_b value of 0.9998 for the LC/UV assay with respect to the LC/MS/MS assay suggests negligible bias (high accuracy) in determining rat plasma CI-1011 concentrations between the two assays. The concordance correlation coefficient (ρ_c) value was 0.9977, which suggests excellent agreement between the paired observations that cluster around the 45° line of unity (Fig. 8). The ρ_c value (0.9977) for the comparison of the LC/MS/MS and LC/UV assays is a significantly ($P < 0.0001$) larger value than the largest benchmark ρ_c (0.9901) for the comparison of replicate concentration values determined using a validated LC/MS assay of CI-1011 in dog plasma. Because the agreement between LC/MS/MS and LC/UV assays for determining CI-1011 in rat plasma is better than the benchmark agreement between replicate dog plasma CI-1011 concentrations determined using a LC/MS assay, it is concluded that the LC/MS/MS and the LC/UV assays could be used interchangeably for concentrations ranging from 0.0806 to 12.3 μg ml^{-1}.

The authors would like to note that the ideal bench mark for ρ_c would be obtained by analyzing rat plasma CI-1011 concentrations determined in triplicate using LC/MS/MS and LC/UV assays. Because rat plasma CI-1011 concentrations from LC/MS/MS data were not available in triplicate, the authors used dog plasma CI-1011 concentrations determined using LC/MS to generate the bench mark for ρ_c.

The assay proved very efficient and robust for sample analysis with as many as 76 study samples processed routinely per day in a 96-sample batch

Table 3
Rat plasma CI-1011 concentrations (μg ml^{-1}) determined by a liquid chromatographic–mass spectrometric (LC/MS/MS) and a liquid chromatographic–ultraviolet (LC/UV) assay

Rat	Day of dosing	Time (h)	Rat plasma CI-1011 concentrations (μg ml^{-1})	
			LC/MS/MS	LC/UV
10	0	Predose	BLQ	BLQ
14	0	Predose	BLQ	BLQ
42	0	Predose	BLQ	BLQ
45	0	Predose	BLQ	BLQ
63	0	Predose	BLQ	BLQ
7	14	5	0.0806	0.0848
62	14	3	0.116	0.122
15	14	0	0.159	0.175
14	14	24	0.259	0.392
10	14	12	0.318	0.324
13	14	12	0.519	0.554
9	14	1	0.627	0.635
15	14	8	0.777	0.892
45	14	24	0.832	1.07
41	14	0	0.867	0.960
14	14	3	0.930	1.08
13	14	8	1.03	1.03
41	14	1	1.12	1.13
46	14	1	1.29	1.29
13	14	3	1.56	2.00
42	14	3	1.72	1.72
45	1	5	1.86	2.09
41	14	8	2.39	2.71
41	14	3	2.69	2.85
15	14	3	2.88	2.84
43	14	12	3.13	2.86
41	14	12	3.30	3.37
45	14	12	3.73	3.73
46	14	5	3.93	4.00
42	14	8	4.70	4.15
46	14	8	4.95	4.93
48	14	3	5.97	5.83
48	14	5	7.42	7.45
48	14	8	8.83	8.95
48	14	12	12.3	12.8

analysis. The ease of sample preparation even permitted two validation attempts in 1 day.

4. Conclusion

A liquid chromatographic–mass spectrometric method to quantitate CI-1011 in rat plasma was validated. No rat plasma components interfered with the quantitation of CI-1011 or IS. The method proved suitable for routine quantitation of CI-1011 in rat plasma at concentrations from 0.500 to 500 ng ml^{-1}. The CI-1011 concentrations, in plasma samples from cholesterol- and chow-fed rats administered single daily oral doses of CI-1011 in a CMC/Tween suspension, obtained by using a validated LC/UV assay, are in excellent agreement with those obtained using a validated LC/MS/MS assay over the concentration range tested, 0.0806–12.3 μg ml^{-1}.

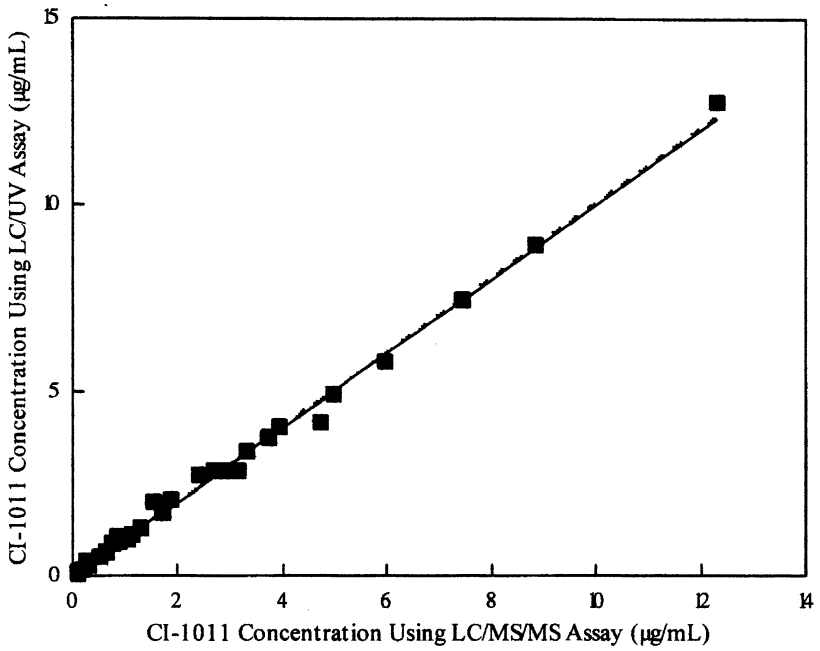

Fig. 8. Rat plasma CI-1011 concentrations as determined by a liquid chromatographic–mass spectrometric (LC/MS/MS) vs. concentrations determined by a liquid chromatographic–ultraviolet (LC/UV) assay: observed concentration data (■), regression line (- - -), and line of unity (——).

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