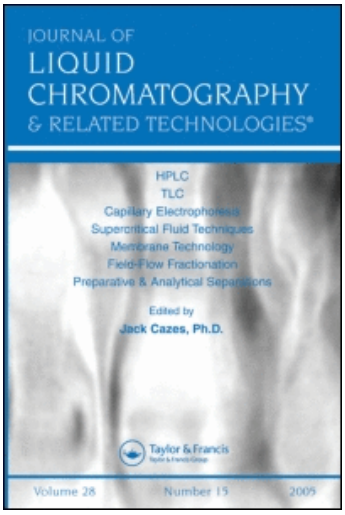

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QUANTITATIVE HPLC ANALYSIS OF 4-[4-4-(CHLOROPHENYL)PHENYL]-4-OXO-2S-(PHENYLTHIOMETHYL) BUTANOIC ACID (BAY 12-9566), A METALLOPROTEINASE INHIBITOR, AND ITS METABOLITES IN HUMAN PLASMA

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ABSTRACT

A High Performance Liquid Chromatographic (HPLC) method was developed for the analysis of Bay 12-9566 (4-[4-4-(chlorophenyl)Phenyl]-4-oxo-2S-(phenylthiomethyl) butanoic acid), a metalloproteinase inhibitor, and its three metabolites, M1 (sulfoxide), M2 (ρ-hydroxy) and M3 (reduction product) in plasma. Sample preparation involved precipitation of plasma proteins using acidified acetonitrile. A reverse phase chromatography with gradient elution, ultraviolet detection at 290 nm and internal standard were used for separation and quantitation of all analytes. The quantitation range was 0.10 μg/mL to 50 μg/mL for BAY 12-9566, and 0.10 μg/mL to 3.00 μg/mL for all three metabolites. The limit of detection for BAY 12-9566 and for all three metabolites was 0.05 μg/mL. Intra-day accuracy for BAY 12-9566 and the three metabolites ranged from 95.0 to 105.0% and precision (CV) ranged from 0.0 to 4.76%.

Inter-day accuracy and precision were based on quality control samples analyzed concurrently with subject plasma samples during a 15 month period. Accuracy ranged from 95.7 to 104.8% and precision ranged from 1.46 to 7.31%.

INTRODUCTION

BAY 12-9566 is a novel, non-peptidic biphenyl inhibitor of several matrix metalloproteinases (MMPs) which have been implicated in the pathophysiology of osteoarthritis and rheumatoid arthritis.[1,2] Consequently, BAY 12-9566 is under development as a potential treatment for osteoarthritis and as adjunct therapy for rheumatoid arthritis.[3] Since MMPs are also known to be involved in tumor growth and the spread of metastases,[4,5] BAY 12-9566 has also been tested in an array of anti-angiogenesis models and in murine models of tumor growth against murine and human tumor cell lines.[6,7,8] BAY 12-9566 is now under investigation in several clinical oncology trials.[9,10,11] The chemical structure of BAY 12-9566 is given in Figure 1.

An HPLC method for the quantitative analysis of BAY 12-9566 and its three metabolites in plasma was developed and used for the analysis of over four thousand plasma samples collected during Phase 1 and Phase 2 clinical studies.

EXPERIMENTAL

Materials and Reagents

Analytical grade sodium acetate, glacial acetic acid, phosphoric acid, and HPLC grade acetonitrile were purchased from J.T.Baker (Phillipsburg, NJ). Reference standard materials for BAY 12-9566 and three metabolites M1, M2, and M3 and for BAY 13-8825 (internal standard) were obtained from the Bayer Research Center (West Haven, CT). A Milli-Q reagent water system (Millipore, Bedford, MA) was used for the purification of water in the HPLC mobile phase. Protein precipitating solution (0.5% phosphoric acid in acetonitrile) was prepared by diluting 5.9 mL of phosphoric acid (85%) to 1000 mL of acetonitrile. Internal standard (BAY 13-8825) was added to this precipitating solution to obtain a concentration of 10 μg/mL. Reference standard stock solutions of BAY 12-9566, and metabolites were prepared at various concentrations in acetonitrile and stored at 4°C. The HPLC system consisted of a Perkin Elmer series 200 pump for solvent delivery, Perkin Elmer ISS 200 autosampler, and Perkin Elmer LC-95 UV/Visible spectrophotometer detector. All data were collected using the Turbochrom (Perkin-Elmer) data system.

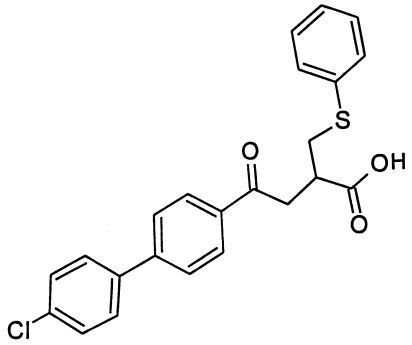

Figure 1. Chemical structure of BAY 12-9566.

Preparation of Plasma Calibration Standards and Quality Control Samples

Calibration standards and quality control samples were prepared by spiking blank plasma with the appropriate volume of stock solutions of BAY 12-9566 and the three metabolites. Separate stock solutions of BAY 12-9566 and metabolites were used to prepare calibration standards and quality control samples. The concentration of the BAY 12-9566 calibration standards ranged from 0.1 to 50 μg/mL, and concentrations of all three metabolites in each sample ranged from 0.1 to 3.0 μg/mL. The corresponding concentration ranges of BAY 12-9566 and metabolites in the quality control samples were 0.14 to 40.0 μg/mL and 0.15 to 2.80 μg/mL, respectively.

After blank plasma was spiked with appropriate concentrations of BAY 12-9566 and the three metabolites, the spiked plasma was equilibrated for about 30 minutes and shaken 2-3 times during that period. Aliquots of spiked plasma (500 μL) were transferred into 13 x 100 mm screw top tubes, capped, and stored at -20°C until analyzed.

Preparation of Plasma Samples

To each 0.5 mL aliquot of plasma at room temperature, 1.0 mL of precipitating solution containing internal standard (10 μg BAY 13-8825 per mL of acidified acetonitrile), was added with an Eppendorf repeat pipette. The precipitated plasma samples were vortexed for approximately 15 seconds, allowed to stand for a minimum of 5 minutes, and vortexed for another 10 seconds. The samples were then centrifuged for 10 minutes at 5000 rpm.

Table 1

HPLC Gradient for the Analysis of BAY 12-9566 and Metabolites in Plasma

Time (Minutes)	Flow Rate (mL/min)	% Solvent A (100 mM Acetate Buffer pH-3.5)	% Solvent B 10% Acetate Buffer, 90% Acetonitrile	Gradient
0.0	0.8	40	60	
15.0	0.8	10	90	Linear
7.0	0.8	10	90	
0.5	1.8	40	60	Linear
3.0	1.8	40	60	
0.5	0.8	40	60	

Note: If a standard centrifuge does not eliminate particulate matter, the precipitated plasma samples can be transferred to 1.5 mL microcentrifuge tubes and ultracentrifuged for 5 to 7 minutes at 10,000 rpm.) The resultant clear supernatant was transferred to a 1 mL amber injection vial, using a Pasteur pipet. The HPLC injection volume was 50 µL.

HPLC Analysis

A Beckman Ultrasphere C8, 5 µM, 100A, 250 x 4.6 mm analytical column at a temperature of 40°C was used for chromatography. A gradient elution was necessary to separate all analytes from the endogenous constituents of the plasma. Table 1 describes the gradient conditions used for the analysis. The UV detection was performed at 290 nm. Retention times of metabolites M1, M2 and M3, BAY 12-9566 and BAY 13-8825 (internal standard) were approximately 9, 12, 14, 16, and 20 minutes, respectively.

RESULTS AND DISCUSSION

Specificity of Chromatographic System

Figures 2 to 6 illustrate representative chromatograms of blank plasma containing internal standard; calibration standards containing BAY 12-9566 and the three metabolites; and pre-dose and post-dose clinical plasma samples.

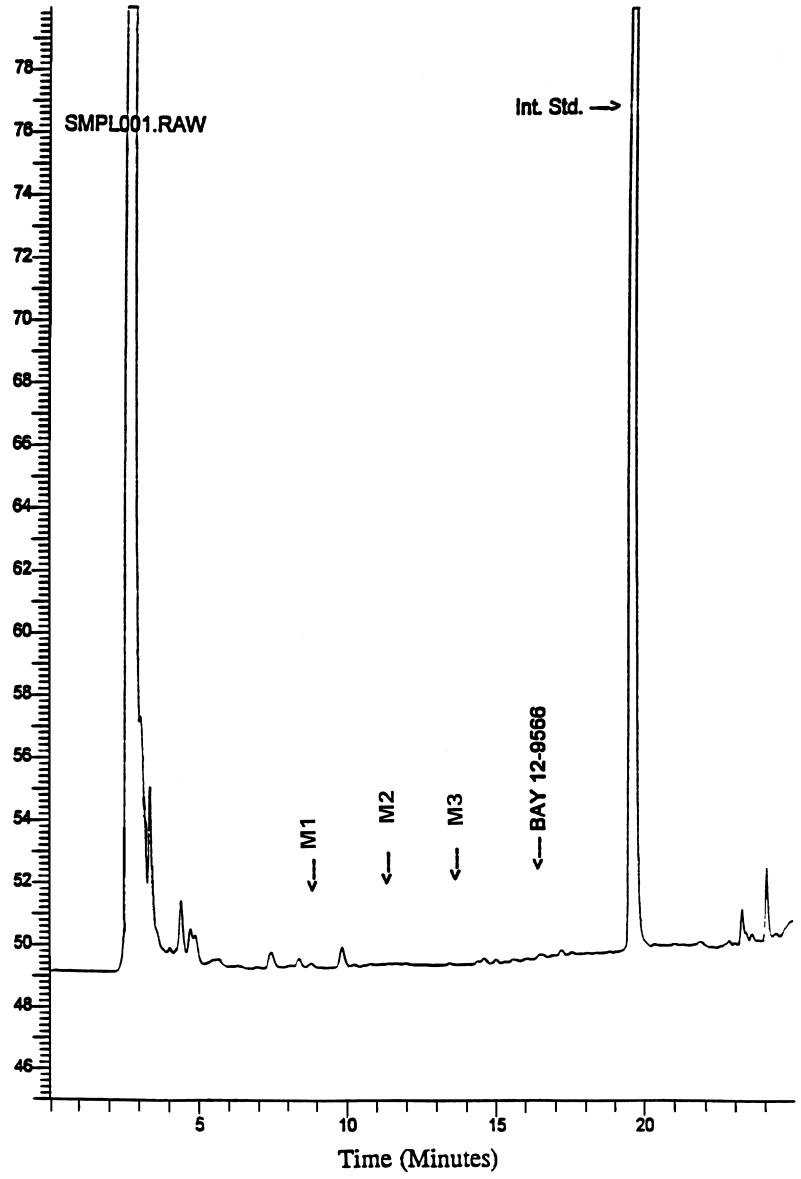

Figure 2. HPLC chromatogram of blank plasma supernatant containing internal standard.

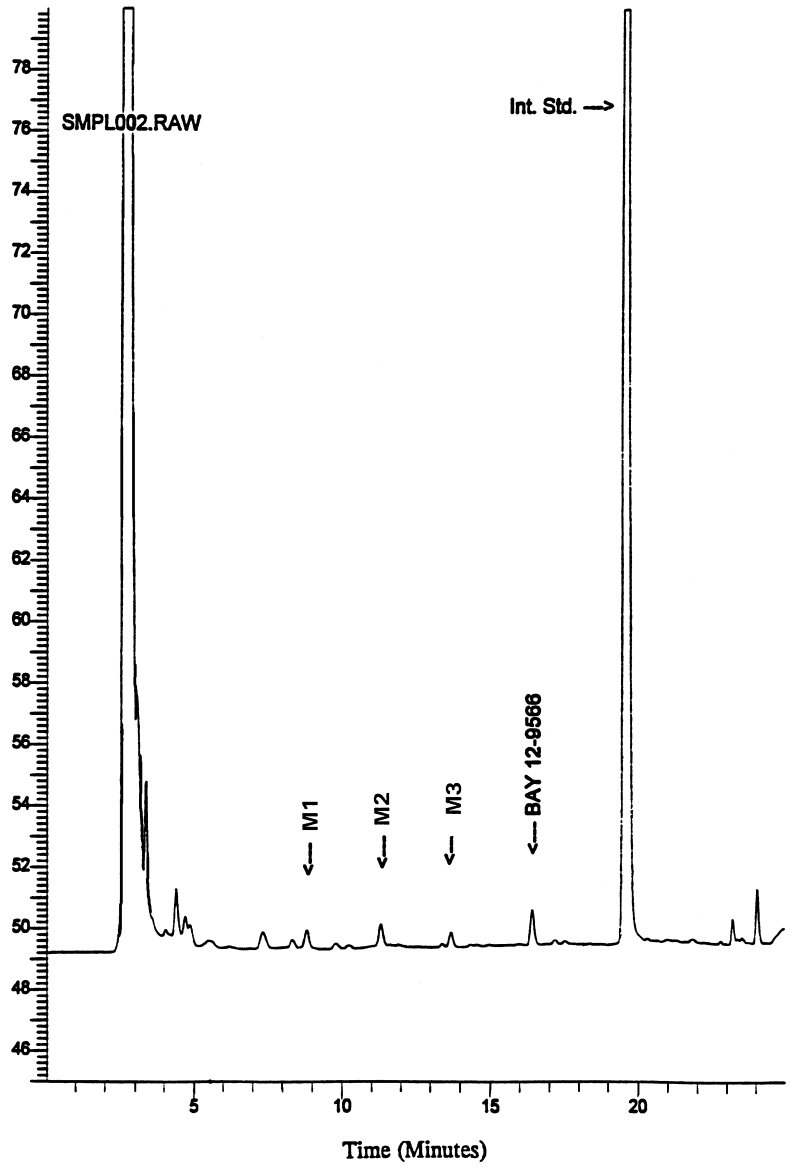

Figure 3. HPLC chromatogram of calibration standard plasma supernatant containing 0.1μg/mL of BAY 12-9566, and metabolites M1, M2, and M3.

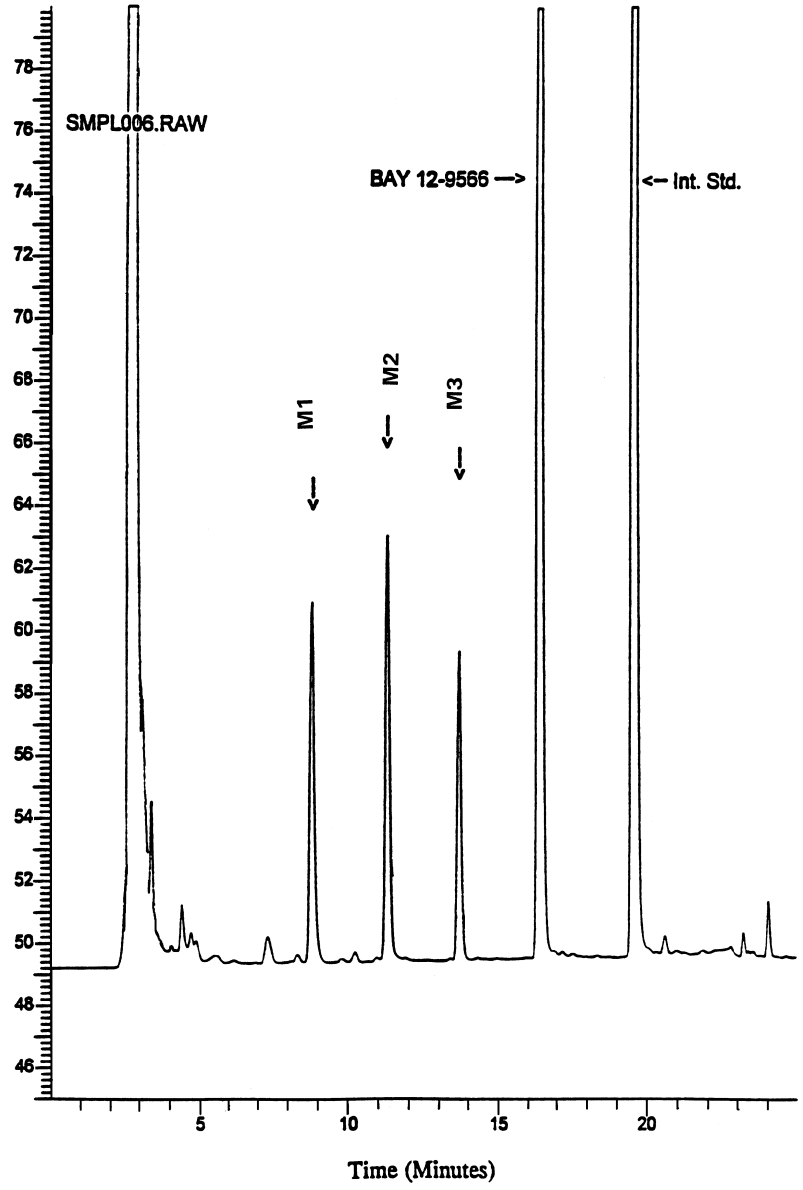

Figure 4. HPLC chromatogram of calibration standard plasma supernatant containing 15 μg/mL of BAY 12-9566, and 2 μg/mL of metabolites M1, M2, and M3.

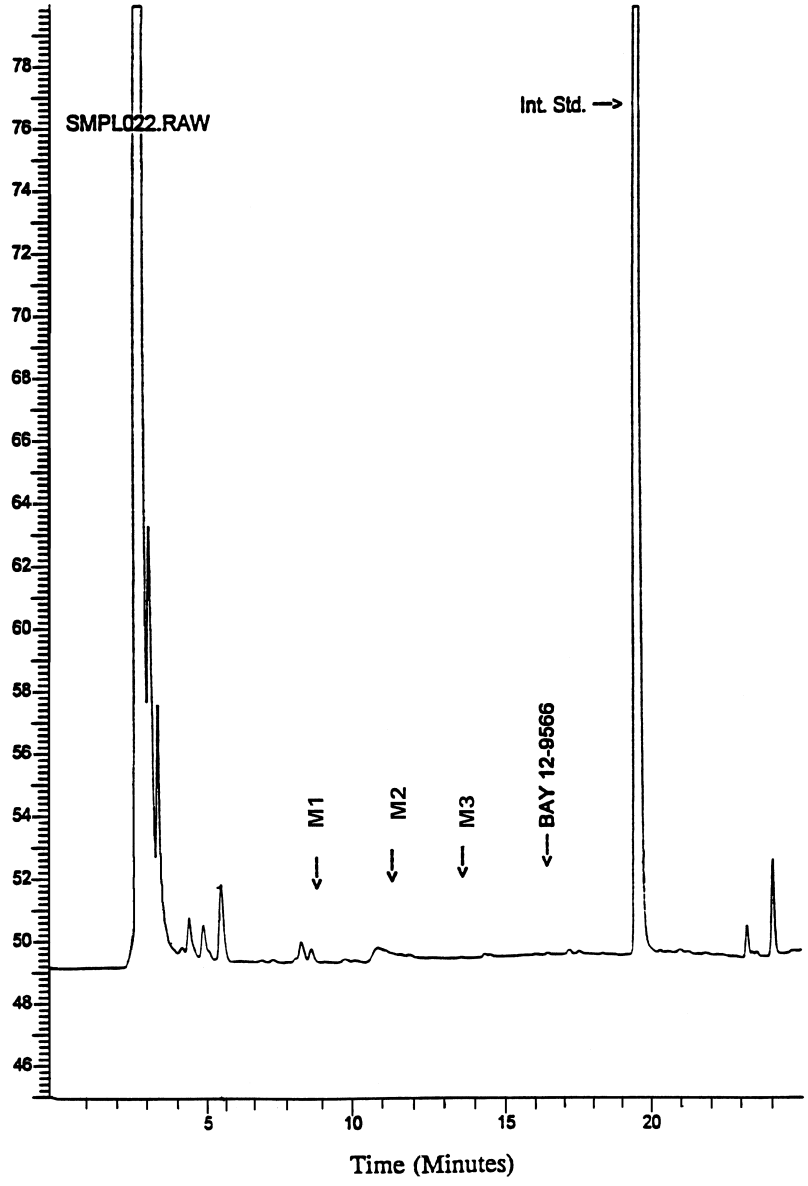

Figure 5. HPLC chromatogram of pre-dose subject plasma supernatant.

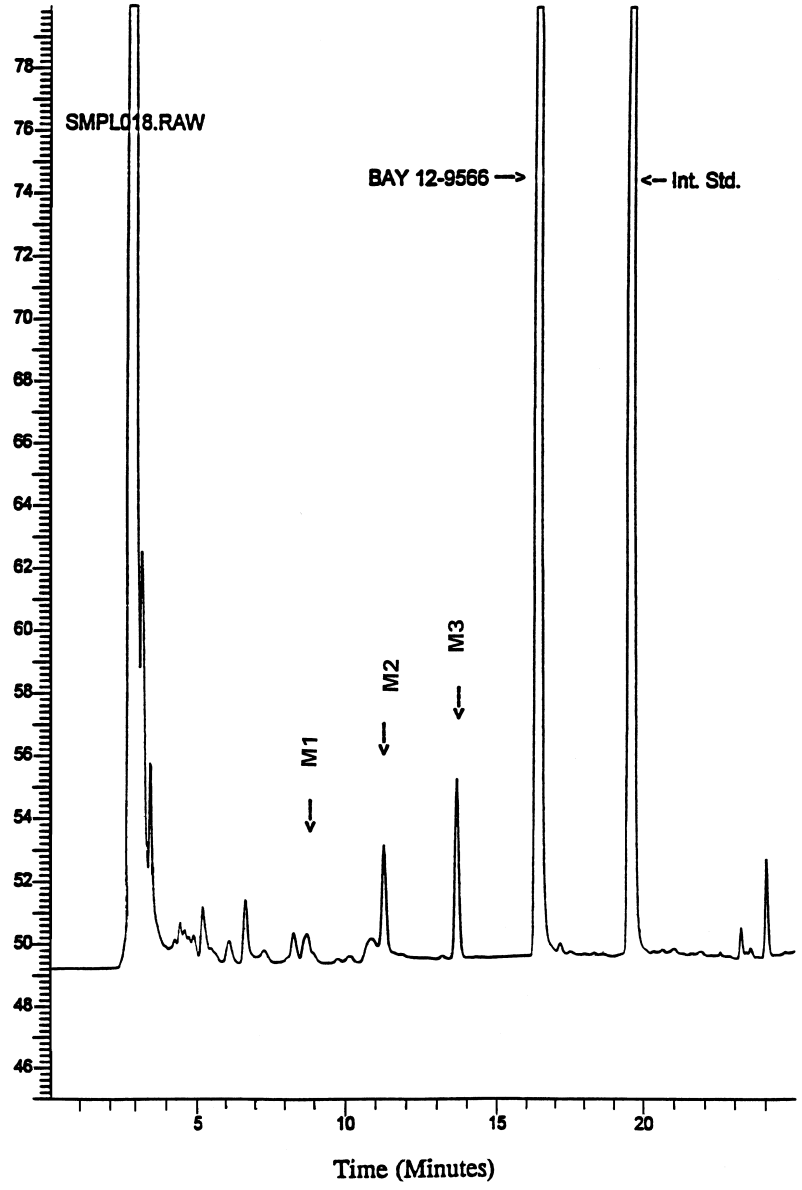

Figure 6. HPLC chromatogram of post-dose subject plasma supernatant.

The interferences from endogenous compounds at the retention times of BAY 12-9566 and its metabolites were negligible and did not affect quantitation.

Accuracy and Precision

The estimates of intra-day accuracy and precision for BAY 12-9566 and the three metabolites were based on the analysis of six replicates of calibration standards at seven concentration levels, and are summarized in Table 2. The intra-day accuracy ranged from 95.0 to 105.0%, and precision ranged from 0.0 to 4.76% for all analytes. Table 3 summarizes the inter-day accuracy and precision for BAY 12-9566 and the three metabolites observed with quality control samples which were analyzed concurrently with the clinical samples during a period of 15 months. Inter-day accuracy ranged from 95.7 to 104.8% and precision ranged from 1.46 to 7.31% for all of the analytes.

Quantitation

The quantitation of BAY 12-9566 and its metabolites in plasma was based on the linear correlation between the concentration and the relative peak height response ratio (PHRR) of each compound (BAY 12-9566, M1, M2 and M3) to the internal standard BAY 13-8825. The standard linear regression curves of concentration versus peak height response ratio were first order with a 1/y weighting factor. The correlation coefficients were greater than 0.9991 for all the analytes.

Concentrations of BAY 12-9566 and metabolites lower than 0.10 μg/mL but equal to or higher than 0.05 μg/mL were reported as not quantitated (NQ), and concentrations lower than 0.05 μg/mL were reported as non-detectable (ND). A lower quantitation limit of 0.05 μg/mL was possible when the injection volume was increased to 100 μL.

Stability of BAY 12-9566 and Metabolites Under Sample Storage and Experimental Conditions

The stability of BAY 12-9566 and metabolites in plasma was determined during storage at -20°C for one year and after four freeze-thaw cycles. The stability of BAY 12-9566 and metabolites in the precipitated plasma supernatant was determined after 48 hours at room temperature on the autosampler. All of the observed results were greater than 90% of the theoretical values, indicating no significant decomposition under these conditions.

Table 2

Intra-day Precision and Accuracy Observed with Plasma Calibration Standards Containing BAY 12-9566 and Metabolites in M1, M2 and M3

Analyte	Theoretical Conc. (μg/mL)	Mean Observed[a] Conc. (μg/mL)	Precision, Relative Standard Deviation (%)	Accuracy, Mean Observed/ Theoretical (%)
BAY12-9566	0.10	0.10	0.00	100.0
	0.50	0.50	0.74	100.3
	2.00	1.91	2.15	95.4
	5.00	5.07	0.32	101.4
	15.00	15.19	2.43	101.2
	25.00	25.10	1.33	100.4
	50.00	50.54	0.59	101.1
M1	0.100	0.10	3.79	98.3
	0.50	0.48	1.05	95.0
	1.00	0.99	2.40	98.5
	1.50	1.49	0.90	99.2
	2.00	1.97	2.74	98.6
	2.50	2.54	1.82	101.4
	3.00	3.03	1.01	101.0
M2	0.10	0.11	4.76	105.0
	0.50	0.49	0.76	98.3
	1.00	0.98	3.06	97.5
	1.50	1.50	0.83	99.8
	2.00	1.97	3.42	98.7
	2.50	2.53	1.89	101.3
	3.00	3.01	0.68	100.2
M3	0.10	0.10	4.56	103.3
	0.50	0.49	1.67	98.0
	1.00	0.98	3.03	98.3
	1.50	1.48	1.28	98.4
	2.00	1.99	3.50	99.3
	2.50	2.54	2.35	101.5
	3.00	3.00	1.45	99.8

[a] Six replicates at each level.

Table 3

Inter-day Precision and Accuracy Observed with Plasma Quality Control Samples Assayed Concurrently with Clinical Samples During 15 Months

Analyte	Theoretical Conc. (μg/mL)	N	Mean Observed[a] Conc. (μg/mL)	Precision, Relative Standard Deviation (%)	Accuracy, Mean Observed/ Theoretical (%)
BAY 129566	0.14	85	0.14	6.08	99.4
	1.00	46	1.02	1.46	102.0
	10.00	136	10.20	2.49	102.0
	20.00	136	20.21	2.32	101.1
	40.00	82	40.00	6.76	100.0
M1	0.15	79	0.14	7.31	96.1
	0.80	78	0.80	3.36	99.8
	1.80	78	1.79	3.14	99.6
	2.80	76	2.69	4.06	96.1
M2	0.15	81	0.15	4.60	99.8
	0.80	80	0.81	3.64	100.7
	1.80	80	1.80	3.05	100.0
	2.80	78	2.68	5.55	95.7
M3	0.15	83	0.16	4.18	103.4
	0.80	82	0.84	3.63	104.8
	1.80	82	1.87	3.21	103.7
	2.80	80	2.78	4.06	99.4

CONCLUSIONS

The procedure described in this report is sufficiently accurate, precise, and sensitive for the analysis of BAY 12-9566 within a 0.1 μg/mL to 50 μg/mL concentration range, and for the analysis of the three metabolites in plasma within a concentration range of 0.1 μg/mL to 3 μg/mL. The stability data on BAY 12-9566 and its metabolites indicate that all four compounds are stable during storage in plasma at -20°C for over one year. Also, there is no evidence of instability in plasma after four freeze-thaw cycles, and no evidence of instability in precipitated plasma supernatant at room temperature after 48 hours on the autosampler.

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