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Validation of a liquid chromatography assay for the quantification of the Raf kinase inhibitor BAY 43-9006 in small volumes of mouse serum

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Abstract

BAY 43-9006 is a selective Raf-1 kinase inhibitor with antitumor activity against a variety of human cancers. A highly sensitive HPLC method for determination of BAY 43-9006 in small volumes of serum (30 μl) was developed. Sample preparation involved a liquid–liquid extraction procedure with tolnaftate as internal standard followed by linear gradient elution at a reversed phase C_{18} column and UV detection. The method was selective and the calibration curves were linear over the concentration range of 80–2000 ng/ml. The intra-day accuracy ranged from 99.9 to 107.6% and the inter-day accuracy from 94.6 to 115%. The lower limit of quantitation (LOQ) was 80 ng/ml with an accuracy of 105.8%. Thus, this method has been validated and can be applied for the drug monitoring or pharmacokinetic studies of BAY 43-9006 in small volumes of serum samples.

Keywords: BAY 43-9006; Raf-1 kinase

1. Introduction

BAY 43-9006 is a novel low molecular weight diphenylurea derivative (Fig. 1A) that selectively inhibits Raf-1 kinase [1,2]. This compound represents a new aspect in drug development focusing on protein kinases as targets for cancer treatment. Since protein kinases have been identified as regulators of multiple aspects of cancer initiation and progression, selective inhibitors of various kinases are tested in clinical trials for the treatment of a wide range of cancers [3,4].

BAY 43-9006 displayed potent antitumour and antiproliferative activity in human colon, pancreatic, ovarian and lung cell lines. In vitro inhibition assays with recombinant Raf-1 kinase revealed an IC_{50} value of 12 nM. Currently, BAY 43-9006 is assessed in clinical development phase II trials in patients with various tumour types [2]. The compound is administered orally; doses ranging from 50 to 1600 mg per day have been given to patients in phase I trials. First pharmacokinetic studies suggested substantial interpatient variabilities and will require further investigations [2,5,6].

To our knowledge, no detailed analytical procedures for sensitive and selective quantification of BAY 43-9006 in biological specimen have been published so far. We aimed at the development and validation of a simple method requiring only small volumes of mouse serum.

2. Materials and methods

2.1. Chemicals and reagents

BAY 43-9006 (purity = 95% by HPLC) was obtained from Prof. Rapp or purchased at Calbiochem (Bad Soden, Germany). Tolnaftate (purity = 99% by HPLC) and mouse serum were obtained from Sigma (Taufkirchen, Germany). Methanol HPLC grade was purchased at Merck (Darmstadt, Germany), diethyl ether and olive oil were from Fluka (Taufkirchen, Germany), acetonitrile from Fisher Scientific (Schwerte, Germany), acetic acid was obtained from Grüssing (Filcum, Germany) and gum arabic powered was purchased at Aldrich (Taufkirchen, Germany).

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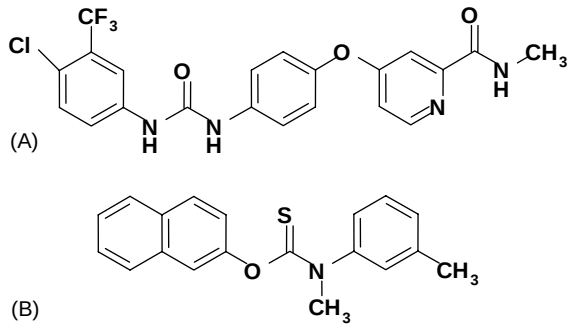

Fig. 1. Chemical structures of BAY43-9006 (A) and tolnaftate (B) as internal standard.

2.2. Sample preparation

Calibration standard (BAY 43-9006) and internal standard (tolnaftate) stock solutions were prepared by exact weighting of the respective compounds, dissolving them in methanol and subsequent dilution with methanol to the indicated concentrations. Calibration standard and internal standard solutions were stored at −20 °C and prepared freshly every month. Five different batches of blank mouse serum were used.

To 30 μl of blank mouse serum for calibration standards or 30 μl unknown samples, 6 μl of the working internal standard solution tolnaftate in methanol was added to obtain a final concentration of 1800 ng/ml of tolnaftate. BAY 43-9006 concentrations in the working standard solutions chosen for the calibration curve were 60, 150, 300, 500, 1000, 1500, 2000 ng/ml by adding 4 μl from a concentrated stock solution in methanol to the mouse serum. After vortex-mixing for 10 s, 30 μl acetonitrile was added to precipitate proteins. Subsequently, all samples were centrifuged at 7833 × *g* for 10 min (Biofuge A, Heraeus Christ, Hanau, Germany). The supernatant was transferred into glass centrifuge tubes and 1.0 ml millipore water was added. The mixture was successively extracted twice with each 3.0 ml diethyl ether. After every addition of ether the centrifuge tubes were shaken for 20 min at room temperature (Rotating wheel, self made by in-house technicians), centrifuged for 2 min (Labofuge II, Heraeus Christ), at 1000 × *g*. The ether layer was transferred into a 5 ml flask and the collected ether layers from both extractions were evaporated to dryness under a stream of nitrogen at 25 °C. The residue was reconstituted in 60 μl methanol.

2.3. Treatment of mice with BAY 43-9006

All of the mice were housed in pathogen-free conditions and handled in accordance with federal animal protection laws, and reviewed by a local ethics committee. Wild type mice (Bl/6 × D2) [7] of 3 months and 20–30 g weight were treated with an emulsion of BAY 43-9006 by oral administration. Bay 43-9006 (2 mg or 20 mg) was dissolved in 200 mg or 300 mg, respectively, olive oil by vortexing for 10 min and subsequent sonication for 15 min. Then 100 mg or 150 mg, respectively, gum arabic was given into the oil phase and mixed well with a pestle. Two hundred microliters of water were added portionwise under vigorous mixing until a homogenous emulsion was formed. The pH was adjusted to pH 7.0 with 0.1N NaOH and water was added to obtain a final volume of 1 ml (the final oil content was 20% or 30%, respectively). The emulsion was sonicated for 15 min with continuous shaking to produce a white homogenous emulsion. The particle size in the emulsion, as determined under the microscope (Eclipse TS 100, Nikon, Germany), ranged between 4.5 and 9 μm. Mice were treated perorally with 100 μl of the emulsion containing 2 mg/ml or 20 mg/ml, respectively. Blood samples were obtained after 2 and 24 h and serum was analyzed by HPLC.

2.4. HPLC conditions

The HPLC system consisted of a Binary HPLC pump (Waters1525), a Waters 717 plus autosampler and a Waters 2487 Dual λ absorbance detector (Waters, Eschborn, Germany). Data acquisition and analysis was performed using the Breeze® software package (Waters). Chromatographic separation was carried out on a reversed phase C_{18} column (Symmetry® C_{18}, 5 μm 4.6 mm × 150 mm HPLC column). Eluent A consisted of 100% acetonitrile and eluent B was water with 0.2% acetic acid (pH 4.0). A 35 min linear gradient started at 40% eluent A and 60% eluent B to 71% eluent A and 29% eluent B. The pump pressure was limited to 4000.0 psi. The temperature of the injector was kept at 10 °C. The temperature of the column was maintained at 25 °C. The detection wavelength was set at 254 nm, the injection volume was 20 μl. The column was equilibrated for at least 30 min with the mobile phase at a flow rate 1 ml/min.

3. Results and discussion

3.1. Selectivity

The selectivity of the method was examined by determining if interfering chromatographic peaks were present in blank mouse serum. Under the chromatographic conditions used for the analysis of BAY 43-9006 (Fig. 1A), the retention times for BAY 43-9006 and internal standard tolnaftate (Fig. 1B) were 18.9 and 29.7 min, respectively. The total chromatography run time was 35 min. Our HPLC assay was found to be selective and free from other possible interferences (Fig. 2).

3.2. Linearity

Calibration standards were set up over the calibration range of 60–2000 ng/ml by adding known amount of BAY 43-9006 to blank mouse serum prior to extraction. For the ratio of peak area of BAY 43-9006 to that of internal

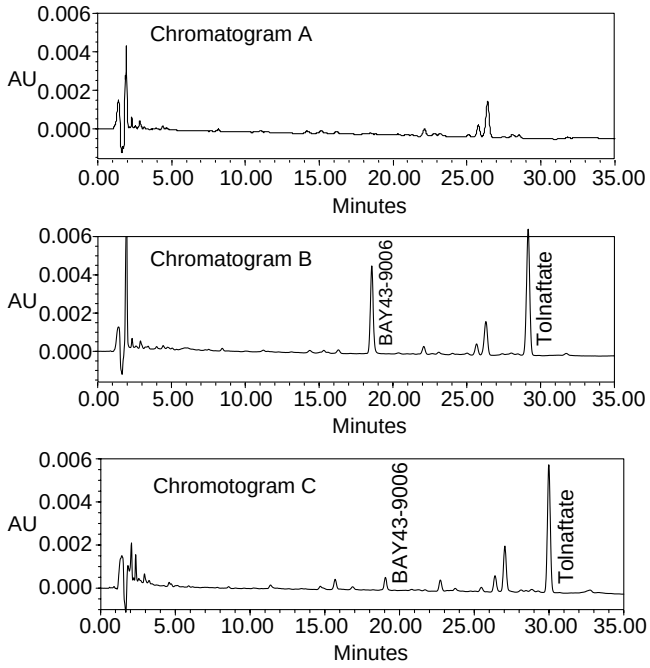

Fig. 2. HPLC chromatograms of blank mouse serum (A) and mouse serum with 1000 ng/ml (B) and 150 ng/ml (C) BAY 43-9006, respectively.

standard linear least-squares regression was conducted to determine the slope, intercept and correlation coefficient [8]. Calibration curves were linear over the concentration range used. Six calibration curves were constructed on six consecutive days in the range of 60–2000 ng/ml for BAY 43-9006 (Table 1). The linearity of the calibration curve was demonstrated by the correlation coefficient (r^2) obtained for the regression line. The slope, the intercept of the regression line, and coefficient of correlation were calculated for the whole data set. Representative results are listed in Table 1.

3.3. Accuracy and precision

Quality control (QC) samples containing BAY 43-9006 were prepared from weightings independent of those used for preparing calibration curves. Final concentrations of the QC samples were 100, 600 and 1800 ng/ml. These samples were prepared on the day of analysis in the same way as calibration standards. The performance of the HPLC method was assessed by analysis of 18 quality control samples (six each of low, medium and high concentrations) on a single assay day to determine intra-day accuracy and precision, and 15 quality control samples (five each for low, medium and high concentration) on each five consecutive days to determine inter-day accuracy and precision [8]. Precision is expressed by relative standard deviation (R.S.D.) in %. Accuracy is expressed as:

$$\text{accuracy (\%)} = \frac{\text{calculated amount}}{\text{actual amount}} \times 100$$

The difference between the calculated and the actual concentration and relative standard deviation were not more than 15% at any QC concentrations [9]. The results of the precision and accuracy for BAY 43-9006 are given in Table 2.

Table 1
Linearity data for BAY 43-9006, calibration standard response values for a calibration curve range of 60–2000 ng/ml

Calibration curve	Slope	Intercept	R^2
1	0.00053	−0.0024	0.9942
2	0.00044	0.0635	0.9926
3	0.0005	0.0161	0.9902
4	0.00067	0.0081	0.9945
5	0.0004	0.0238	0.9933
6	0.0004	0.0231	0.9984
Mean	0.0005	0.0220	0.9938
S.D.	0.0001	0.0225	0.0026

S.D.: standard deviation of the mean.

Table 2
Precision and accuracy of the method for determination of BAY 43-9006 in mouse serum

Concentration (ng/ml)	Mean (ng/ml)	S.D.	Precision R.S.D. (%)	Accuracy (%)
Inter-day ($n = 6$)				
100	94.6	2.79	2.9	94.6
600	692	68.55	9.9	115
1800	1813.8	101.94	5.6	100.7
Intra-day ($n = 5$)				
100	99.9	11.2	11.2	99.9
600	645.8	36.99	5.7	107.6
1800	1810.2	71.29	3.9	100.5

S.D.: standard deviation, R.S.D.: relative standard deviation.

3.4. Sensitivity

The lower limit of quantitation (LOQ) was determined as the minimum concentration which can be accurately and precisely quantified [10]. The LOQ, defined in the presented experiment as the lowest serum concentration in the calibration curve that can be measured routinely with acceptable precision (R.S.D. <20%) and accuracy (80–120%) was 80 ng/ml (Table 3).

3.5. Recovery

The recovery was quantified by the ratio of the slopes of the six calibration curves for extracted to working standard

Table 3
The limit of quantitation of the method for determination of BAY 43-9006 in mouse serum

Concentration (ng/ml)	Mean (ng/ml)	S.D.	Precision R.S.D. (%)	Accuracy (%)	Difference (%)
Inter-day ($n = 6$)					
80	84.7	15.2	17.9	105.8	5.87

S.D.: standard deviation, R.S.D.: relative standard deviation.

Table 4
Recovery study for BAY 43-9006 (by ratio of slopes) the calibration curves were ($n = 6$) for each of the standard curve and extraction curve

	Standard curves	Extraction curves
Intercept	0.0055	0.024
Slope	0.000406	0.000379
Correlation coefficient (r)	0.9994	0.9933

Recovery by ratio of slopes = 0.000379/0.000406 = 93.3%.

solutions [10]. The relative recovery for BAY 43-9006 was 93.3% (Table 4).

3.6. Extraction efficiency

To determine extraction efficiency, mouse serum samples were spiked with BAY 43-9006 to achieve a final concentration of 100 and 1800 ng/ml. Six samples were extracted and analyzed for each concentration. Extraction efficiency was calculated with the following equation [11]:

$$\text{extraction efficiency (\%)} = \frac{\text{peak area extracted sample for} \times \text{ng/ml}}{\text{peak area neat sample for} \times \text{ng/ml}} \times 100.$$

BAY 43-9006 peak areas were proportional over the mouse serum concentrations ranges from 60 to 2000 ng/ml. Mean absolute recoveries of BAY 43-9006 from mouse serum at concentrations of 100 and 1800 ng/ml were 71.8 ± 6.25% and 74.2 ± 8.52%, respectively.

3.7. Serum concentrations of BAY 43-9006 after peroral treatment of wild type mice

To proof that the developed analytical method is applicable to serum samples of mice treated with BAY 43-9006, we prepared an emulsion of BAY 43-9006 and administered it perorally to the animals (Table 5). After 2 and 24 h blood samples were taken and serum (30 μl) was analyzed for BAY43-9006 (Fig. 3). After administration of 0.2 and 2 mg BAY 43-9006 per animal serum concentrations of 999 and 1777 ng/ml, respectively, were determined after 2 h. This indicates that the drug is absorbed from the emulsion. After 24 h the serum levels declined below the lower limit of quantitation in case of the 0.2 mg dose, while 96 ng/ml were still detectable after administration of 2 mg BAY 43-9006. These example results indicate that the analytical method is suitable for further studies.

Table 5
Serum concentrations of BAY 43-9006 after peroral treatment of mice with a single dose of a drug-containing emulsion

Dose of BAY 43-9006 (mg)	Time of sampling after peroral administration (h)	BAY 43-9006 concentration in serum samples (ng/ml)
0.2	2	999
0.2	24	Below LOQ
2	2	1777
2	24	96

LOQ: lower limit of quantitation.

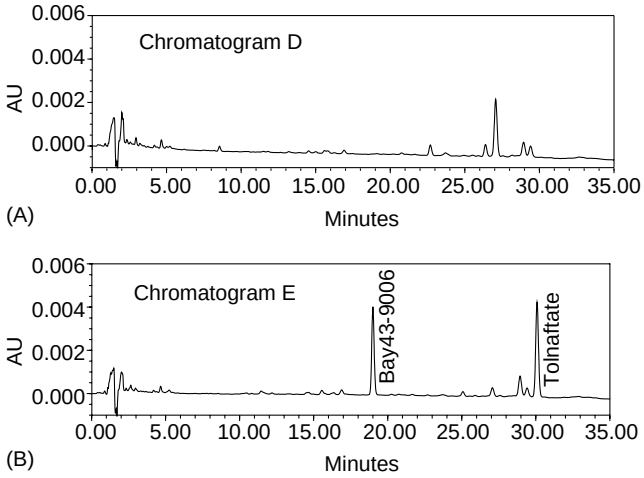

Fig. 3. HPLC chromatograms of blank mouse serum (D) and a serum sample of a mouse treated perorally with 2 mg BAY 43-9006 (E). The sample was obtained two hours after administration of the drug.

4. Conclusion

The proposed liquid chromatographic method is selective for quantification of BAY 43-9006 in serum samples. Serum samples of as little as 30 μl are sufficient for precise determination of the Raf kinase inhibitor at concentrations of 80 ng/ml or above. Thus, this method is applicable for pharmacokinetic monitoring of BAY 43-9006 in small animals. It might as well be applied to extensive human pharmacokinetic studies.

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