Journal of Chromatography B, 905 (2012) 150–154

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Liquid chromatography–tandem mass spectrometric assay for the ALK inhibitor crizotinib in mouse plasma

Rolf W. Sparidans[a,*], Seng Chuan Tang[b], Luan N. Nguyen[b], Alfred H. Schinkel[b], Jan H.M. Schellens[a,c], Jos H. Beijnen[a,c,d]

[a] Utrecht University, Faculty of Science, Department of Pharmaceutical Sciences, Division of Pharmacoepidemiology & Clinical Pharmacology, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands
[b] The Netherlands Cancer Institute, Department of Molecular Oncology, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[c] The Netherlands Cancer Institute, Department of Clinical Pharmacology, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[d] Slotervaart Hospital, Department of Pharmacy & Pharmacology, Louwesweg 6, 1066 EC Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 8 June 2012
Accepted 14 August 2012
Available online 21 August 2012

Keywords:
Crizotinib
ALK inhibitor
LC–MS/MS
Oxidative metabolites
Mouse plasma

ABSTRACT

A quantitative bioanalytical liquid chromatography–tandem mass spectrometric (LC–MS/MS) assay for the ALK inhibitor crizotinib was developed and validated. Plasma samples were pre-treated using protein precipitation with acetonitrile containing crizotinib-$^{13}C_2$-$^{2}H_5$ as internal standard. The extract was directly injected into the chromatographic system after dilution with water. This system consisted of a sub-2 μm particle, trifunctional bonded octadecyl silica column with a gradient using 0.1% (v/v) of ammonium hydroxide in water and methanol. The eluate was transferred into the electrospray interface with positive ionization and the analyte was detected in the selected reaction monitoring mode of a triple quadrupole mass spectrometer. The assay was validated in a 10–10,000 ng/ml calibration range with $r^2 = 0.99980 \pm 0.00014$ for double logarithmic linear regression ($n = 5$). Within day precisions ($n = 6$) were 3.4–4.8%, between day (3 days; $n = 18$) precisions 3.6–4.9%. Accuracies were between 107% and 112% for the whole calibration range. The drug was sufficiently stable under all relevant analytical conditions. Oxidative metabolites of crizotinib were monitored semi-quantitatively. Finally, the assay was successfully used to assess drug pharmacokinetics in mice.

1. Introduction

Crizotinib (PF-02341066; Fig. 1) is an inhibitor of translocated anaplastic lymphoma kinase (ALK) [1,2]. The first discovered ALK-rearrangement was *EML4-ALK* and this oncogenic driver is frequently present (2–7% [3]) in patients suffering from non-small cell lung cancer (NSCLC). Crizotinib showed promising results in the treatment of NSCLC patients with ALK translocations [1,2,4] and therefore received accelerated approval for ALK positive NSCLC in August 2011 by the FDA [4]. Presently, two Phase III clinical studies are ongoing investigating crizotinib as an alternative to current treatments in patients with ALK-rearranged adenocarcinoma of the lung [1,5].

In humans, crizotinib is thought to be extensively metabolized by cytochrome P450 3A4/5 into mainly the active metabolite crizotinib-lactam (PF-06260182; Supplemental Fig. 1); other Phase I metabolites, including their sulfate and glucuronide conjugates can also be observed [6]. In rats and dogs, other oxidative metabolites like crizotinib-N-oxide (Supplemental Fig. 1) and hydroxy-crizotinib can additionally be observed [5,7]. In female rats, however, sulfated crizotinib is the main metabolite [7]. Pharmacokinetic data of this drug have been reported both in humans, the registered 250 mg twice daily oral administration of crizotinib resulted in average trough plasma levels of *ca.* 300 ng/ml and terminal half-lives of 40–50 h [1,6], and in mice [8,9].

So far only concise descriptions of bioanalytical assays for crizotinib have been reported [8,9]. We now report on the development and validation of a bioanalytical LC–MS/MS assay for crizotinib in FVB mouse plasma using protein precipitation as a simple pretreatment procedure and using sub-2 μm LC particles to obtain a high sample throughput. Oxidative metabolites of the drug were assessed semi-quantitatively. The applicability of the assay was demonstrated in a pharmacokinetic mouse study.

Abbreviations: ALK, anaplastic lymphoma kinase; AUC, area-under-the-plasma-time curve; HESI, heated electrospray ionization; k_a, absorption rate; LLOQ, lower limit of quantification; NSCLC, non-small cell lung cancer; SRM, selected reaction monitoring; $T_{1/2}$, elimination half-life; QC, quality control.

* Corresponding author. Tel.: +31 6 20289970; fax: +31 30 2539166.
E-mail address: R.W.Sparidans@uu.nl (R.W. Sparidans).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.08.021

Table 1
Mass spectrometric settings in SRM mode of the protonated molecules ([M+H]$^+$) at 0.7 full width at half height resolutions of both quadrupoles.

Compound	Parent ion (m/z)	Ion tube off set [V]	Daughter ions (m/z)	Collision energy [V] at 1.6 mTorr argon	Skimmer off set [V]	Dwell time [ms]
Crizotinib	450.2	120	260.2	−23	−6	50
			177.1[c]	−35	−12	100
Crizotinib-$^{13}C_2$-$^2H_5$	457.2	120	267.2	−23	−6	50
M+14[a]	464.2	130	177.1	−25	0	50
M+16[b]	466.2	130	276.2	−23	−12	100

[a] Crizotinib-lactam and crizotinib-N-oxide.
[b] Hydroxy-crizotinib.
[c] This transition was not used for quantification.

2. Experimental

2.1. Chemicals

Crizotinib (≥99%) was obtained from Sequoia Research Products (Pangbourne, UK) and crizotinib-$^{13}C_2$-$^2H_5$ (>99%, internal standard (IS), Supplemental Fig. 1) from Alsachim (Strasbourg, France). Water (LC–MS grade), methanol (HPLC grade) and acetonitrile (HPLC-S grade) were from Biosolve (Valkenswaard, The Netherlands). Water, not used as eluent, was home purified by reversed osmosis on a multi-laboratory scale. Ammonium hydroxide was of reagent grade and originated from Sigma–Aldrich Chemie (Steinheim, Germany). Formic acid was of analytical grade originating from Merck (Darmstadt, Germany) and pooled mouse EDTA-disodium plasma was supplied by Seralab Laboratories International (Haywards Heath, UK).

2.2. Equipment

The LC–MS/MS equipment consisted of an Accela quaternary pump and autoinjector and a TSQ Quantum Ultra triple quadrupole mass spectrometer with heated electrospray ionization (HESI; Thermo Fisher Scientific, San Jose, CA, USA). Data were recorded and the system was controlled using the Thermo Fisher Xcalibur software (version 2.07).

2.3. LC–MS/MS conditions

Partial-loop injections (3 μl) were made on an Acquity UPLC® BEH C18 column (30 mm × 2.1 mm, d_p = 1.7 μm, Waters, Milford, USA), protected by the corresponding VanGuard pre-column (Waters, 5 mm × 2.1 mm). The column temperature was maintained at 40 °C and the sample rack compartment of the autosampler at 4 °C. A gradient (0.6 ml/min) using 0.1% (v/v) ammonium hydroxide (A) and methanol (B) was used. After injection, the percentage of solvent B was increased linearly from 55 to 75% (v/v) during 1.5 min. Next, the column was flushed with 90% (v/v) acetonitrile (C) and 10% (v/v) A for 0.3 min and finally, the column was reconditioned at the starting conditions (55% (v/v) B and 45% (v/v) A) for 0.7 min resulting in a total run time of 2.5 min. The whole eluate was transferred into the electrospray probe, starting at 0.6 min after injection by switching the MS divert valve until 2 min after injection. The HESI was tuned in the positive ionization mode by introducing 0.6 ml/min of a mixture of 0.1% (v/v) formic acid in water (50%, v/v) and methanol (50%, v/v) and 5 μl/min of 10 μg/ml of crizotinib. HESI settings of the assay were a 3000 V spray voltage, 304 °C capillary and 391 °C vaporizer temperatures and the nitrogen sheath, ion sweep and auxiliary gasses were set at 50, 8 and 35 arbitrary units, respectively. Mass spectrometric settings of the selected reaction monitoring (SRM) mode are reported in Table 1.

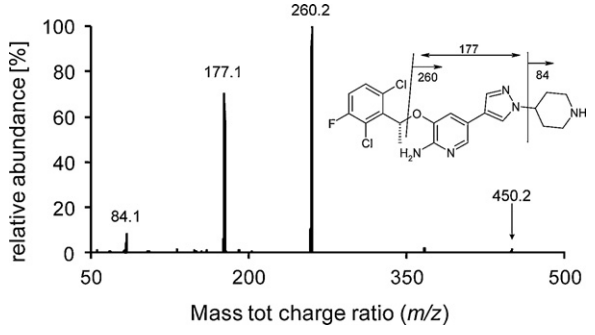

Fig. 1. Chemical structure and product spectrum of crizotinib: m/z 450.2 @ −30 V.

2.4. Sample pre-treatment

To a volume of 20 μl of mouse plasma, pipetted into a polypropylene reaction tube, 30 μl of 100 ng/ml crizotinib-$^{13}C_2$-$^2H_5$ in acetonitrile was added. The tubes were closed and shaken by vortex mixing for 5–10 s. After centrifugation of the sample at 10,000 × g at 20 °C for 1 min, 40 μl of the supernatant was transferred to a 250 μl glass insert placed in an autoinjector vial. Before closing the vial, 100 μl of water was added and finally, 3 μl of the mixture was injected onto the column.

2.5. Validation

A laboratory scheme based on international guidelines was used for the validation procedures [10–12].

2.5.1. Calibration

Stock solutions of crizotinib and crizotinib-$^{13}C_2$-$^2H_5$ at 0.5, 1 or 2 mg/ml were prepared in methanol. A 1 mg/ml stock solution of crizotinib was diluted to a 10,000 ng/ml working solution in mouse EDTA plasma. All stock and working solutions were stored in a 1.5-ml polypropylene tube at −30 °C. Additional calibration samples were prepared daily at 5000, 1000, 500, 100, 50, 20 and 10 ng/ml by dilution with the same blank plasma. The highest and two lowest calibration samples were processed in duplicate for each daily calibration, whereas the levels in between were processed only once. Least-squares double logarithmic linear regression was employed to define the calibration curves using the ratios of the peak area of crizotinib and the labeled IS.

2.5.2. Precision and accuracy

A second stock solution of crizotinib (0.5 mg/ml) was used to obtain validation (quality control, QC) samples in pooled human EDTA-disodium plasma at 8000 (QC-high), 400 (QC-med), 20 (QC-low) and 10 ng/ml (QC-LLOQ). The QC samples were stored in polypropylene tubes at −30 °C. Precisions and accuracies were determined by sextuple analysis of each QC in three analytical runs on three separate days for all QCs (total: n = 18 per QC). Relative standard deviations were calculated for both, the within and between day precisions.

2.5.3. Selectivity

Six individual mouse plasma samples were processed to test the selectivity of the assay. The samples were processed without crizotinib and IS and with crizotinib at the LLOQ level (10 ng/ml), supplemented with the IS. Blank drug response should be below 20% of the LLOQ response [13].

2.5.4. Recovery and matrix effect

The recovery was determined ($n = 4$) by comparing processed samples (QC-high, -med, -low) with reference crizotinib solutions in blank plasma extract at the same levels. The matrix effect was assessed by comparing the reference solutions in blank plasma extracts with the same solutions in acetonitrile–water (1:3, v/v) at the three validation levels.

2.5.5. Stability

The stability of crizotinib was investigated in QC-high and -low plasma samples stored in polypropylene tubes. Quadruplicate analysis of these samples from separate tubes was performed after storage at 20 °C (ambient temperature) for 24 h, three additional freeze–thaw cycles (thawing at 20 °C during *ca.* 2 h and freezing again at −30 °C for at least one day), and storage at −30 °C for 2 months, respectively. Furthermore, QC samples (4 levels, each $n = 6$) and study samples ($n = 10$) were re-injected after additional storage of the extracts at 4 °C for three nights together with freshly prepared calibration samples.

Finally, the responses of crizotinib from the stock solutions in methanol after 6 h at 20 °C ($n = 2$) and after 4 months of storage in the freezer (−30 °C; $n = 3$) were compared to fresh stock solutions with LC–MS/MS after appropriate dilution of the samples and adding IS.

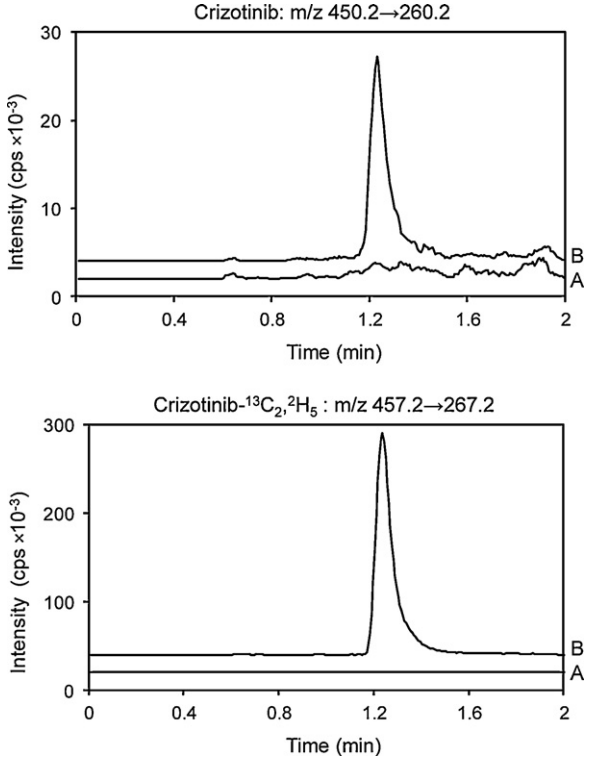

Fig. 2. SRM chromatograms of crizotinib and the labeled IS in plasma extracts: blank mouse plasma (A) and LLOQ (10 ng/ml) spiked plasma (B). An artificial off set was given to the chromatograms.

2.6. Mouse samples

Wild-type (FVB genetic background) male mice ($n = 5$) were housed and handled as reported previously [14] and were treated with 50 mg/kg crizotinib orally. The crizotinib solution was obtained by dissolving the drug in dimethyl sulfoxide (25 mg/ml), followed by five-fold dilution with 50 mM acetate buffer (pH 4.6). Blood samples (~50 μl) were collected in EDTA containing microvettes *via* the tail vein at 0.25, 0.5, 1, 2, 4 and 8 h after administration of the drug. At 24 h, blood was collected in EDTA tubes by cardiac puncture. After centrifugation at 2100 × *g* for 6 min at 4 °C, plasma samples were stored at −30 °C prior to analysis.

Pharmacokinetic parameters (area-under-the-plasma-time curve (AUC), both for 24 h (AUC_{0-24}) and with extrapolation to infinity ($AUC_{0-\infty}$); elimination half-life ($T_{1/2}$); absorption rate (k_a)) of crizotinib were calculated based on a first-order, one-compartment model. For calculation of the half-life and extrapolation the 8 and 24 h time points were used and for calculation of the absorption rate the 0.25–4 h points using curve stripping. Semi-quantitative analysis of crizotinib metabolites was executed using crizotinib calibration samples.

3. Results and discussion

3.1. Method development

Before starting method development the possibility to include one or more oxidative crizotinib metabolites in the assay was considered. No lactam, N-oxide or hydroxy metabolite of crizotinib was, however, commercially available and chemical synthesis was very expensive. Amounts that could be obtained by enzymatic synthesis with liver microsomes were relatively small and very likely insufficient to use for validation of a bioanalytical assay. Therefore, we chose to monitor the oxidative metabolites in samples of treated animals semi-quantitatively without validation with reference compounds. Distinction between the lactam and N-oxide metabolites of crizotinib, both with a nominal mass defect of +14 Da compared to crizotinib, was possible using rat liver microsomes incubation products of crizotinib-$^{13}C_2$-$^{2}H_5$ (Supplemental Fig. 1).

HESI-MS/MS settings were optimized for the crizotinib assay to obtain optimal sensitivity, a product spectrum, formed by collision induced dissociation of the protonated molecule, is shown in Fig. 1. The main product ion at m/z 260 was identical to the main fragment observed in previous investigations [8,9]. The use of protein precipitation as sample pretreatment procedure has been used successfully earlier in our laboratory for several other kinase inhibitors [15]. The choices between formic acid and ammonium hydroxide and between methanol and acetonitrile in the eluent, were made based on the higher crizotinib response using both, ammonium hydroxide and methanol.

3.2. Validation

During a pilot experiment crizotinib plasma levels in the range 500–5000 ng/ml were found. Therefore, we decided to execute the validation in the 10–10,000 ng/ml range. SRM chromatograms are depicted in Fig. 2, showing chromatograms of blank and LLOQ spiked plasma samples.

3.2.1. Calibration

The relative response of crizotinib showed a very small but strongly significant deviation from a linear function ($P = 0.0014$ for a 1-tailed Student's t-distribution of the average double-logarithmic slope ($n = 5$) compared to 1); therefore, the double logarithmic linear function was used for the assay calibration. For 5 calibrations (55 samples) the concentrations were back-calculated

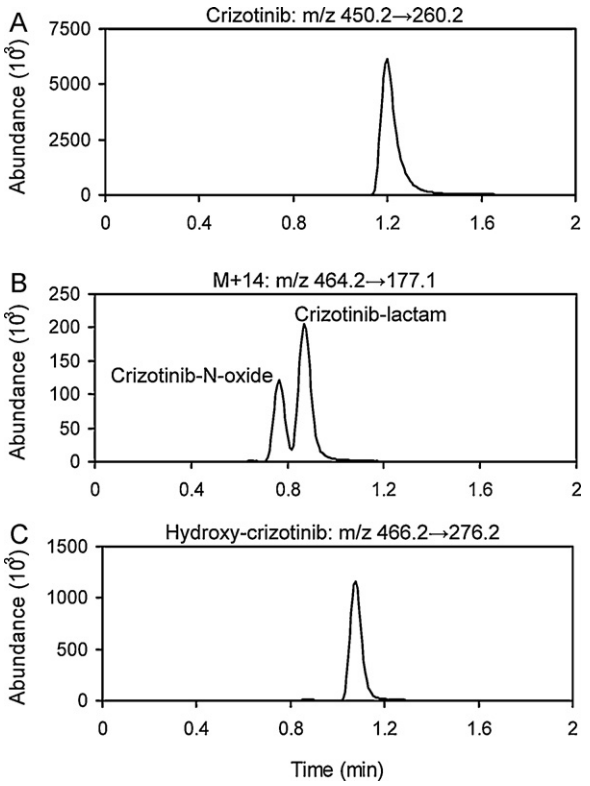

Fig. 3. SRM chromatogram of crizotinib and oxidative metabolites in a wild type male mouse plasma, 15 min after oral administration of 50 mg/kg crizotinib. (A) Crizotinib (2370 ng/ml); (B) lactam and N-oxide metabolites; (C) hydroxy-metabolite.

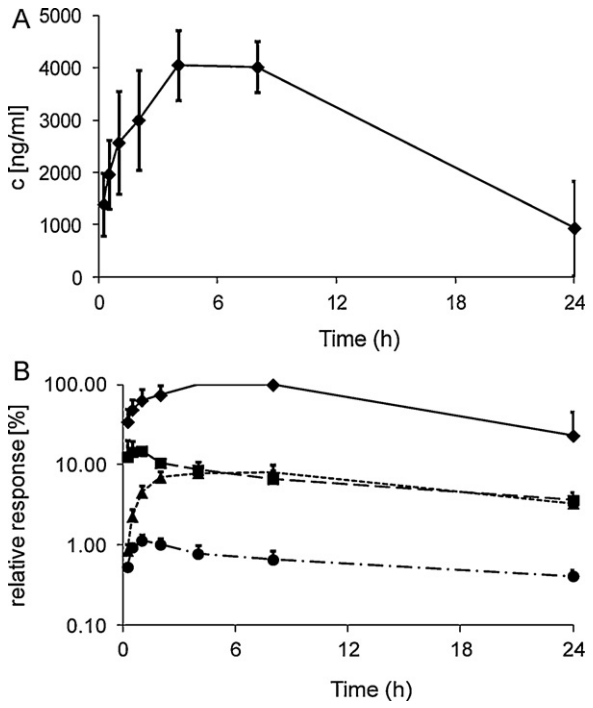

Fig. 4. Pharmacokinetic plots of crizotinib and oxidative metabolites in wild type male mouse plasma after oral administration of 50 mg/kg crizotinib ($n = 5$). (A) Absolute levels of crizotinib; (B) responses, relative to the response of crizotinib at the maximum concentration, of crizotinib and metabolites. Crizotinib (♦: ————); hydroxy-crizotinib (■: – – – –) crizotinib-lactam (▲: ·····); crizotinib-N-oxide (●: – · – · – · –).

from the ratio of the peak areas (analyte and labeled IS) using the calibration curves of the run in which they were included. No deviations of the average of each level higher than 4% were observed (data not shown), indicating the suitability of the logarithmic regression model. The average of the very reproducible regression parameters of the double logarithmic regression functions ($n = 5$) were $\log(y) = -2.024(\pm 0.055) + 0.9824(\pm 0.0027) \log(x)$ with a regression coefficient of 0.99980 ± 0.00014. Here, x is the concentration (ng/ml) and y is the crizotinib response relative to the IS.

3.2.2. Precision and accuracy

Assay performance data from the validation samples at four concentrations are reported in Supplemental Table 1. Within day and between day variations lower than 5% were observed and deviations of the accuracies were lower than 13%. The precision and accuracy therefore met the required ±15% (±20% for the LLOQ) [10–12].

3.2.3. Selectivity

The analysis of six independent blank mouse plasma samples showed no interfering peaks in the SRM traces for crizotinib and its labeled analog. Blank crizotinib responses were all <20% of the LLOQ response [13] and blank IS responses below 0.5% of the normal response. The signals at the LLOQ level (10 ng/ml) were all distinguishable from blank responses; concentrations found at the LLOQ level ($n = 6$) were 9.49 ± 0.41 ng/ml, demonstrating the applicability of the investigated LLOQ level [10–12].

3.2.4. Recovery and matrix effect

The extraction recoveries showed only small losses for crizotinib and ranged from 79% to 92% (data not shown). Small matrix effects were also observed; ionization recoveries ranged from 80% to 94% for crizotinib at the investigated levels. Overall, the small extraction losses and matrix effects contributed to a successful validation of the assay [10–12].

3.2.5. Stability

The stability of crizotinib in mouse EDTA plasma after different storage procedures is presented in Supplemental Table 2. Small losses up to 13.9% were found without any decline of the precision. Re-injection of validation (QC) samples after additional storage at 4 °C for three nights resulted again in successful performances with QC failures being far below a 33% frequency (data not shown) as required [10,13]. For the re-injection of study samples ($n = 10$) the recovery was 85.9 ± 3.3%, the 3 days of storage should therefore not be exceeded. Recoveries of crizotinib in stock solutions were 98.9% (after 4 months at −30 °C; $n = 3$) and 99.0% (after 6 h at 20 °C; $n = 2$), respectively, and were therefore considered satisfactory (>95%) for the validation [10–12].

3.3. Mouse pharmacokinetics

After the successful validation procedure, the new assay was used to investigate the plasma pharmacokinetics of crizotinib in wild type male mice with semi-quantitative monitoring of the oxidative metabolites. Results of the animal experiments ($n = 5$) at the 50 mg/kg oral dosage are depicted in Figs. 3 and 4. Crizotinib levels in the range 218–4629 ng/ml were observed; average (±SD) pharmacokinetic parameters were: $AUC_{0-24} = 67 \pm 11$ μg h ml^{-1}; $AUC_{0-\infty} = 84 \pm 38$ μg h ml^{-1}; $T_{1/2} = 8 \pm 6$ h; $k_a = 0.35 \pm 0.08$ h^{-1}.

4. Conclusions

The first validated assay for crizotinib has now been fully reported for mouse EDTA-disodium plasma samples. The sensitive LC–MS/MS assay uses a fast and simple sample pre-treatment

method. The results show values of accuracy, precision, recovery and stability allowed by international guidelines [10–12]. The new assay was successfully used for a pharmacokinetic mouse study of the drug with simultaneous semi-quantitative analysis of the oxidative metabolites of crizotinib, and can be used for future studies in uptake, metabolism and clearance of crizotinib.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.08.021.

References

[1] S.H. Ou, Drug Des. Dev. Ther. 5 (2011) 471.
[2] G. Scagliotti, R.A. Stahel, R. Rosell, N. Thatcher, J.C. Soria, Eur. J. Cancer 48 (2012) 961.
[3] E.L. Kwak, Y.J. Bang, D.R. Camidge, A.T. Shaw, B. Solomon, R.G. Maki, S.H. Ou, B.J. Dezube, P.A. Janne, D.B. Costa, M. Varella-Garcia, W.H. Kim, T.J. Lynch, P. Fidias, H. Stubbs, J.A. Engelman, L.V. Sequist, W. Tan, L. Gandhi, M. Mino-Kenudson, G.C. Wei, S.M. Shreeve, M.J. Ratain, J. Settleman, J.G. Christensen, D.A. Haber, K. Wilner, R. Salgia, G.I. Shapiro, J.W. Clark, A.J. Iafrate, N. Engl. J. Med. 363 (2010) 1693.
[4] A.T. Shaw, U. Yasothan, P. Kirkpatrick, Nat. Rev. Drug Discov. 10 (2011) 897.
[5] US National Institutes of Health, 2012. http://clinicaltrials.gov/ct2/results?term=crizotinib&pg=2 (accessed 10.05.12).
[6] Center for Drug Evaluation and Research, 2011. http://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/202570Orig1s000ClinPharmR.pdf (accessed 10.05.12).
[7] W.Z. Zhong, J. Zhan, P. Kang, S. Yamazaki, Curr. Drug Metab. 11 (2010) 296.
[8] S. Yamazaki, P. Vicini, Z. Shen, H.Y. Zou, J. Lee, Q. Li, J.G. Christensen, B.J. Smith, B. Shetty, J. Pharmacol. Exp. Ther. 340 (2011) 549.
[9] S. Yamazaki, J. Skaptason, D. Romero, J.H. Lee, H.Y. Zou, J.G. Christensen, J.R. Koup, B.J. Smith, T. Koudriakova, Drug Metab. Dispos. 36 (2008) 1267.
[10] Center for Drug Evaluation and Research of the U.S. Department of Health and Human Services Food and Drug Administration, 2001. http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064964.htm (accessed 26.04.11).
[11] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[12] C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, Pharm. Res. 24 (2007) 1962.
[13] European Medicines Agency, 2009. http://www.ema.europa.eu/pdfs/human/ewp/19221709en.pdf (accessed 26.04.11).
[14] R.W. Sparidans, D. Iusuf, A.H. Schinkel, J.H. Schellens, J.H. Beijnen, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 4090.
[15] R.W. Sparidans, S. Durmus, N. Xu, A.H. Schinkel, J.H. Schellens, J.H. Beijnen, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 895–896 (2012) 174.