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Short communication

Liquid chromatography–tandem mass spectrometric assay for the JAK2 inhibitor CYT387 in plasma

Rolf W. Sparidans [a,*], Selvi Durmus [b], Ning Xu [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, Departments of Medical Oncology and Molecular Biology, 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

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ABSTRACT

A quantitative bioanalytical liquid chromatography–tandem mass spectrometric (LC–MS/MS) assay for the JAK2 inhibitor CYT387 was developed and validated. Plasma samples were pre-treated using protein precipitation with acetonitrile containing cediranib 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 0.005% (v/v) of formic acid in a mixture of 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 0.25–1000 ng/ml calibration range. Within day precisions were 3.0–13.5%, between day precisions 5.7% and 14.5%. Accuracies were between 96% and 113% for the whole calibration range. The drug was stable under all relevant analytical conditions. Finally, the assay was successfully used to assess drug levels in mice.

1. Introduction

CYT387 (Fig. 1) is a novel inhibitor of Janus kinase (JAK) 2, designed to suppress the over-activity of the mutant $JAK2^{V617F}$ [1,2]. This mutation plays an essential role in myeloproliferative disorders and is highly present in patients suffering from myelofibrosis, post-polycythemia Vera and essential thrombocythemia [2,3]. Clinical Phase I and II studies in these populations are ongoing [4]. The first Phase I study for CYT387 has been completed and showed the drug to be well tolerated up to 300 mg once daily [5].

To support the future clinical investigations with CYT387 such assay will be indispensable, however, as far as we know a validated bioanalytical assay for CYT387 has not been reported hitherto. Only the use of LC–MS/MS and protein precipitation with an unknown internal standard (IS) [2] has been reported previously. On the basis of our good experiences with other kinase inhibitors (axitinib [6], vemurafenib [7] and cediranib [8]) we decided to develop and validate an LC–MS/MS assay, also with protein precipitation, using sub-2 μm LC particles to obtain a high sample throughput.

2. Experimental

2.1. Chemicals

CYT387·H_2SO_4 (≥99%) and cediranib (≥99%, internal standard (IS)) were obtained from Sequoia Research Products (Pangbourne, UK). 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. Formic acid was of analytical grade and originated from Merck (Darmstadt, Germany) and analytical grade dimethyl sulfoxide (DMSO) from Acros Organics (Geel, Belgium). Pooled human EDTA-disodium plasma was supplied by Seralab Laboratories International (Haywards Heath, UK), EDTA-disodium human plasma from individual human donors by Innovative Research (Southfield, MI, USA).

2.2. Equipment

The LC–MS/MS equipment consisted of an Accela quaternary pump and autoinjector and a TSQ Quantum Ultra triple quadrupole

Abbreviations: HESI, heated electrospray ionization; JAK, Janus kinase; LLOQ, lower limit of quantification; SRM, selected reaction monitoring; QC, quality control.

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

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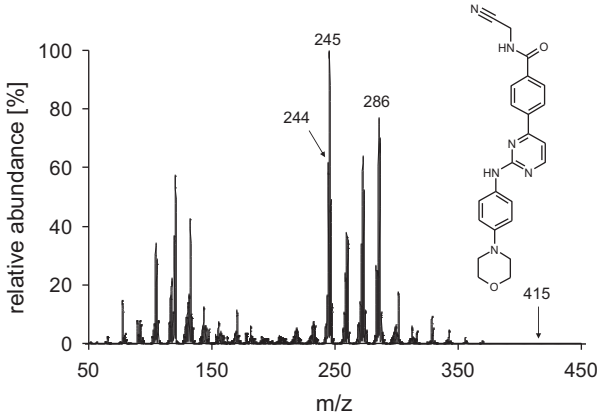

Fig. 1. Chemical structure and product spectrum of CYT387: *m/z* 415.15 at −60 V.

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 (10 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 was obtained by linearly increasing methanol from 45% to 50% (v/v) during the first minute after injection while water was reduced from 50% to 45% (v/v). Next, the column was flushed with 95% (v/v) acetonitrile for 0.5 min and reconditioned at the starting conditions (45% (v/v) methanol and 50% (v/v) water) for 0.8 min until starting the next injection, resulting in a total run time of 2.3 min. During the whole run at a 0.6 ml/min flow rate, the solvents were supplemented with 5% of formic acid (0.1% (v/v)) in water. The eluate was transferred into the electrospray probe, starting at 0.5 min after injection by switching the MS divert valve until 1.6 min after injection. The HESI was tuned in the positive ionization mode by introducing 0.5 ml/min of a mixture of 0.1% formic acid in water (40%, v/v) and methanol (60%, v/v) and 5 μl/min of 100 μg/ml of CYT387. HESI settings of the assay were a 4000 V spray voltage, 350 °C capillary and vaporizer temperatures and the nitrogen sheath, ion sweep and auxiliary gasses were set at 50, 8 and 50 arbitrary units, respectively; the skimmer voltage was set at −10 V. The SRM mode was used with argon as the collision gas at 2 mTorr. CYT387 was monitored at *m/z* 415.15 → 244.1; 245.1; 286.1 at −77, −59 and −40 V collision energies, respectively, and cediranib at *m/z* 451.2 → 84.1; 112.1 at −44 V and −31 V, respectively. The tube lens off set was 104 V for both compounds and the dwell time was 100 ms for each transition. Mass resolutions were set at 0.7 full width at half height (unit resolution) for both separating quadrupoles. The electromultiplier was set at 5×10^6.

2.4. Sample pre-treatment

To a volume of 50 μl of human plasma, pipetted into a 1.5 ml polypropylene reaction tube, 75 μl of 20 ng/ml cediranib 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, 100 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, 10 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 [9–11].

2.5.1. Calibration

Stock solutions of CYT387·H_2SO_4 and cediranib (IS) at 0.5 mg/ml were prepared in methanol. All stock solutions were stored at −30 °C and all calculations were based on the sulfuric acid salt. One stock solution of CYT387 was diluted to a 25 μg/ml working solution in 50% (v/v) methanol in water and diluted further to a 1000 ng/ml calibration sample in pooled human EDTA-disodium plasma, stored in a polypropylene tube at −30 °C. Additional calibration samples were prepared daily at 100, 10, 1, 0.5 and 0.25 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 linear regression with the reversed square of the concentration ($1/x^2$) as the weighting factor was employed to define the calibration curves using the ratios of the peaks of CYT387 and the IS.

2.5.2. Precision and accuracy

A second stock solution of CYT387 and a corresponding 25 μg/ml working solution were used to obtain validation (quality control; QC) samples in pooled human EDTA-disodium plasma at 750 (QC-high), 30 (QC-med), 0.75 (QC-low) and 0.25 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). Relative standard deviations were calculated for both, the within day precisions (repeatability) and the between day precisions (reproducibility).

Additionally, mouse QC samples (QC-med and -high level; n = 6) were processed after dilution with the human matrix as a partial validation for this species. Mouse EDTA-dipotassium plasma samples (10 μl) were diluted with 190 μl of the human plasma before processing 50 μl of the diluted samples.

2.5.3. Selectivity

Six individual human EDTA-disodium plasma samples were processed to test the selectivity of the assay. The samples were processed without CYT387 and IS and with CYT387 at the LLOQ level (0.25 ng/ml), supplemented with the IS. Additionally, individual blank diluted mouse EDTA-disodium plasma (20%; n = 4) and EDTA-dipotassium (5%; n = 4) were tested.

2.5.4. Recovery and matrix effect

The recovery was determined in triplicate by comparing processed samples (QC-high, -med, -low) with reference 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 water–methanol–acetonitrile (63/10/27; v/v/v) at the three validation levels (QC-high, -med, -low). Recovery and matrix effect of the IS were assessed using an identical procedure (n = 4) at the cediranib concentration used in the assay.

2.5.5. Stability

The stability of CYT387 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

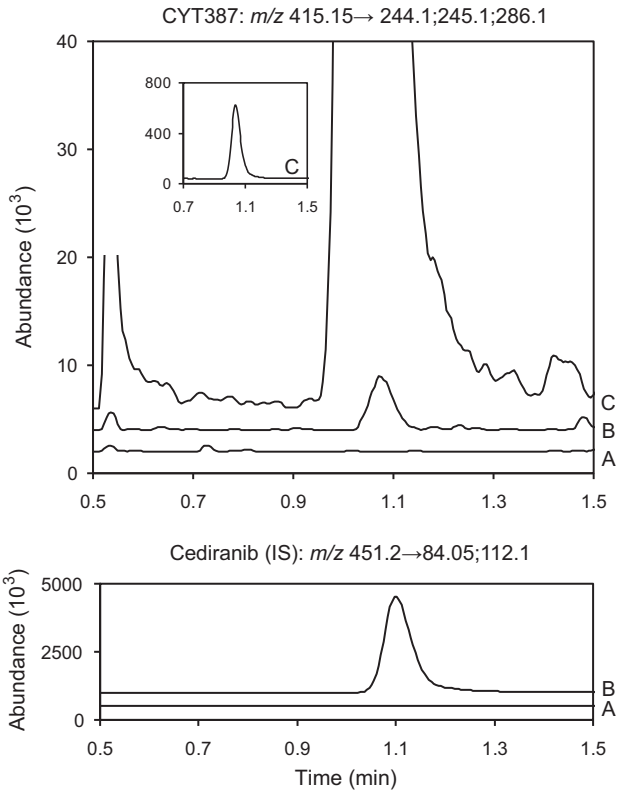

Fig. 2. SRM chromatograms of CYT387 and the IS in plasma extracts: blank EDTA-disodium plasma (A), LLOQ (0.25 ng/ml) spiked plasma (B) and wild type mouse EDTA-dipotassium plasma (C) taken 0.25 h after administration of 33 mg/kg CYT387, diluted 1:20 with human EDTA-disodium plasma and containing 673 ng/ml CYT387. An artificial off set was given to the chromatograms and an inlay of CYT387 in mouse plasma (C) with a less magnified scale was added.

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 2.5 months, respectively. Furthermore, analytical runs were re-injected after additional storage of the extracts at 4 °C for one night.

Finally, the responses of CYT387 from the stock solutions in methanol and working solutions in 50% (v/v) methanol after 6 h at 20 °C and after 2.5 months of storage in the freezer (−30 °C) were compared in duplicate to fresh stock and working solutions.

2.6. Mouse samples

Wild-type (FVB genetic background) mice ($n = 4$) were housed and handled as reported previously [6] and were treated with 33 mg/kg CYT387 orally. The CYT387 solution was obtained by dissolving the drug (sulfuric acid salt) in DMSO (500 mg/ml), followed by dilution (1:100) with Tween 80–ethanol–water (20:13:67, v/v/v). Blood samples were collected in tubes containing EDTA-dipotassium *via* the tail vein at 0.25, 0.5, 1 and 2 h after administration of the drug. After centrifugation at 2100 × *g* for 6 min at 4 °C, plasma samples were stored at −30 °C. Mouse plasma samples were diluted 20-fold with human EDTA-disodium plasma as reported in Section 2.5.2.

3. Results and discussion

3.1. Method development

MS/MS settings were optimized for the CYT387 assay to obtain optimal sensitivity. To obtain optimal sensitivity, responses of 3 main product ions were added up, a product spectrum of CYT387 is shown in Fig. 1. The use of simply using protein precipitation as pretreatment procedure may result in insufficiently cleaned-up samples. However, exceptional instrument maintenance costs or poor life time of the analytical columns was never experienced in our laboratory for over 5 years now using this approach [6,7,12–17]. The use of the divert valve to discard the eluate during and shortly after the chromatographic dead time may play in prominent role in this matter. Because a stable isotopically labeled compound was not available, an alternative compound with a retention time close to CYT387 and the ability to correct response variations of the method was selected.

Table 1
Assay performance data of CYT387 resulting from 18 validation (QC) samples (human EDTA-disodium) plasma in 3 analytical runs with additional data of diluted mouse plasma ($n = 6$).

Nominal concentration [ng/ml]	Within day precision [%]	Between day precision [%]	Accuracy [%]
750	3.0	5.7	99.7
30	6.1	6.1	96.3
0.75	8.8	9.1	105.5
0.25	13.5	14.1	100.9
750[a]	7.9		97.3
30[a]	7.3		112.7

[a] 5% mouse EDTA-dipotassium plasma in human plasma.

3.2. Validation

SRM chromatograms are depicted in Fig. 2, showing chromatograms of blank and LLOQ spiked plasma samples.

3.2.1. Calibration

Clinical studies using 300 mg CYT387 once daily are ongoing [5]. Expected drug levels in patients are not yet known but levels up to *ca.* 5000 ng/ml were observed in a mouse model after treatment with a 25 mg/kg oral dosage of CYT387 [2]. Therefore a range with optimized sensitivity (0.25 ng/ml) up to 1000 ng/ml was chosen. For 6 calibrations (54 samples) the concentrations were back calculated from the ratio of the peak areas (analyte and IS) using the calibration curves of the run in which they were included. No deviations of the average of each level higher than 8% were observed (data not shown), indicating the suitability of the linear regression model with quadratic weighting. The average regression parameters of the linear regression function ($n = 6$) was $y = -0.0002(\pm 0.0005) + 0.065(\pm 0.014) \times x$ with regression coefficients (r^2) being 0.994 ± 0.003.

3.2.2. Precision and accuracy

Assay performance data from the validation samples at four concentrations are reported in Table 1. Between day variations and deviations of the accuracy lower than 9.1% (14.1% at the LLOQ) were observed. The precision and accuracy therefore met the required ±15% (±20% for the LLOQ) [9–11]. Results of diluted mouse plasma did also meet these criteria (Table 1), these data show the applicability of the new assay for mouse EDTA-dipotassium plasma after dilution with the human matrix [7,8,18].

3.2.3. Selectivity

The analysis of six independent blank human EDTA-disodium plasma samples, as well as all ten diluted mouse samples, showed no interfering peaks in the SRM traces for CYT387. Blank responses were all <10% of the LLOQ response, meeting the required 20% [19]. The signals at the LLOQ level (0.25 ng/ml) were all distinguishable from blank responses; concentrations found at the LLOQ level

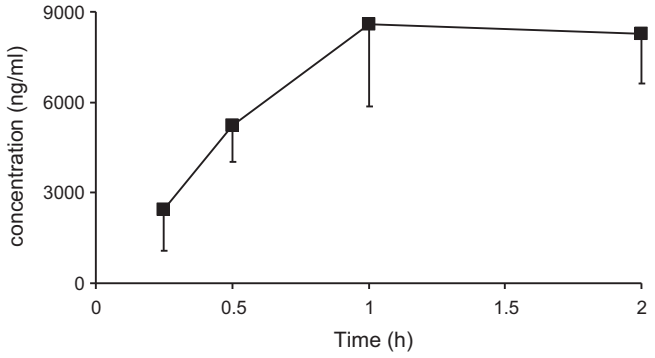

Fig. 3. Concentration–time profile of the average concentrations with standard deviations of CYT387 in 4 wild type mice after oral administration of 33 mg/kg of the drug.

Table 2
Stability data (recovery [%]; ±SD; $n = 4$) of CYT387 in human EDTA plasma, reporting the percentage of the initial concentration.

Condition	QC-high	QC-low
24 h at ambient temperature	108.6 ± 5.1	98.8 ± 8.1
3 freeze–thaw cycles	110.8 ± 2.3	88.9 ± 4.1
2.5 months at −30 °C	104.1 ± 2.0	92.1 ± 6.6

($n = 6$) were 0.255 ± 0.037 ng/ml, demonstrating the applicability of the investigated LLOQ level [9–11].

3.2.4. Recovery and matrix effect

The extraction recoveries showed no losses for both target compound and IS and ranged from 99% to 114% (data not shown). Small matrix effects were observed; ionization recoveries ranged from 114% to 137% for CYT387 at the investigated levels while 83.3 ± 9.2% was found for the IS. Overall, the absence of extraction losses and only relatively small matrix effects contributed to a successful validation of the assay [9–11].

3.2.5. Stability

The stability of CYT387 in human EDTA-disodium plasma after different storage procedures is presented in Table 2. No losses higher than 11.1% were found without any decline of the precision. Re-injection of validation (QC) samples after additional storage at 4 °C for 1 night resulted again in successful performances with QC failures being below a 33% frequency (data not shown) as required [9,19]. Recoveries of CYT387 in the stock and working solutions (after 2.5 months at −30 °C and after 6 h at 20 °C) all exceeded 95% and could therefore be considered satisfactory for the validation [9–11].

3.3. Mouse samples

To show the applicability of the new assay after the successful validation procedure, the concentration–time profile of CYT387 was investigated in mice. Plasma samples from human pharmacokinetic studies were not available. Results of the animal experiments at the 33 mg/kg oral dosage are depicted in Figs. 2 and 3 and CYT387 levels in the range 673–11,140 ng/ml were observed, after 20-fold dilution this range was within the 30 and 600 ng/ml QC samples of this matrix. The highest levels (*ca.* 10,000 ng/ml ≈ 20 μM) correspond to the previously reported levels at 25 mg/kg (*ca.* 8 μM) and 50 mg/kg (*ca.* 32 μM) in Balb/c mice [2].

4. Conclusions

The first validated assay for CYT387 has now been fully reported for human EDTA-disodium plasma samples. The sensitive LC–MS/MS assay uses a fast and simple pre-treatment method. The results show values of accuracy, precision, recovery and stability allowed by international guidelines [9–11]. The new assay can be applied to clinical CYT387 studies and can also be used for mouse studies of the drug.

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