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HPLC–DAD protein kinase inhibitor analysis in human serum

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ABSTRACT

We here describe an HPLC–DAD method to analyse different protein kinase inhibitors. Potential applications of this method are pharmacokinetic studies and therapeutic drug monitoring. Optimised chromatography conditions resulted in a very good separation of seven inhibitors (vatalanib, bosutinib, canertinib, tandutinib, pazopanib, dasatinib – internal standard and erlotinib). The good sensitivity makes this method competitive with LC/MS/MS. The separation was performed with a Lichrospher 100-5 RP8, 250 mm × 4 mm column maintained at 30 ± 1 °C, and with a mobile phase of 0.05 M H_3PO_4/KH_2PO_4 (pH = 2.3)–acetonitrile (7:3, v/v) at a flow rate of 0.7 mL/min. A simple and fast sample preparation sequence with liquid–liquid extraction led to good recoveries (73–90%) of all analytes. The recovery hardly reached 50% only for pazopanib. This method can also be used for targeted protein kinase inhibitor quantification. A perfect linearity in the validated range (20–10,000 ng/mL) and an LOQ of 20 ng/mL were achieved. The relative standard deviations and accuracies of all examined drug concentrations gave values much lower than 15% both for between- and within-batch calculations. All analysed PKIs were stable for 6 months in a 1 mg/mL dimethyl sulfoxide stock solution. Vatalanib, bosutinib and erlotinib were also stable in human serum in the whole examined concentration range.

1. Introduction

Protein kinase inhibitors (PKIs) are responsible for the effective blocking of the addition of a phosphate group to other proteins by a protein kinase. Vatalanib and pazopanib are protein kinase inhibitors potent against all vascular endothelial growth factor receptors (VEGFR), the platelet-derived growth factor receptor (PDGFR), the steam cell growth factor receptor (c-Kit) and the colony-stimulating factor 1 receptor [1,2]. Bosutinib is a dual Src/Abl kinase inhibitor with potential activity against chronic myeloid leukaemia (CML) [3]. Canertinib is an irreversible tyrosine-kinase inhibitor, which binds to the whole ErbB family [4]. Tandutinib, an inhibitor of the type III tyrosine kinase receptor, blocks FLT3, PDGFR and c-Kit [5]. Erlotinib inhibits EGFR and EGFRvIII [6].

The analysed PKIs (Fig. 1) are investigated as a potential means for treating several types of cancer. Therefore, a validated analytical method for investigating these relative new active substances could be interesting for scientific research. The PKI blood concentrations depend on the patient, dose and time. According to the reported studies [1,3–7] the analytical method should cover at least the following concentration ranges: vatalanib (370–5500 ng/mL), bosutinib (70–210 ng/mL), canertinib (90–260 ng/mL), tandutinib (30–2300 ng/mL), pazopanib (1500–54,000 ng/mL), erlotinib (90–2400 ng/mL).

A validated assay for the quantitative analysis of vatalanib in human EDTA plasma by liquid chromatography coupled with electrospray ionisation tandem mass spectrometry was proposed by Lankheet et al. [8]. More validated methods were prepared for erlotinib. An LC/MS/MS quantification method for erlotinib and its O-desmethyl metabolite in human plasma was developed by Masters et al. [9]. The samples were prepared by liquid–liquid extraction with hexane:ethyl acetate. Honeywell et al. also used the tandem mass spectrometry for an erlotinib assay [10]. This method, which based on a protein precipitation extraction step, was also used for the quantification of gefitinib, sorafenib and sunitinib. HPLC–UV was also a method of choice for erlotinib analysis in human plasma [11–13]. Our work places emphasis on the possibility of overcoming the need for an LC/MS/MS system to analyse different protein kinase inhibitors. We have already proposed a simple and fast HPLC–DAD method to quantify imatinib in human serum for clinical purposes [14], and developed a nilotinib high-performance liquid chromatography method using a diode array detector with and without internal standard [15]. Both methods are an applicable alternative to other reported, usually more expensive assays. This paper presents an effective separation and selective/sufficient quantification of six PKIs in human serum. For most of them, a validated analytical method was not developed till we started our work.

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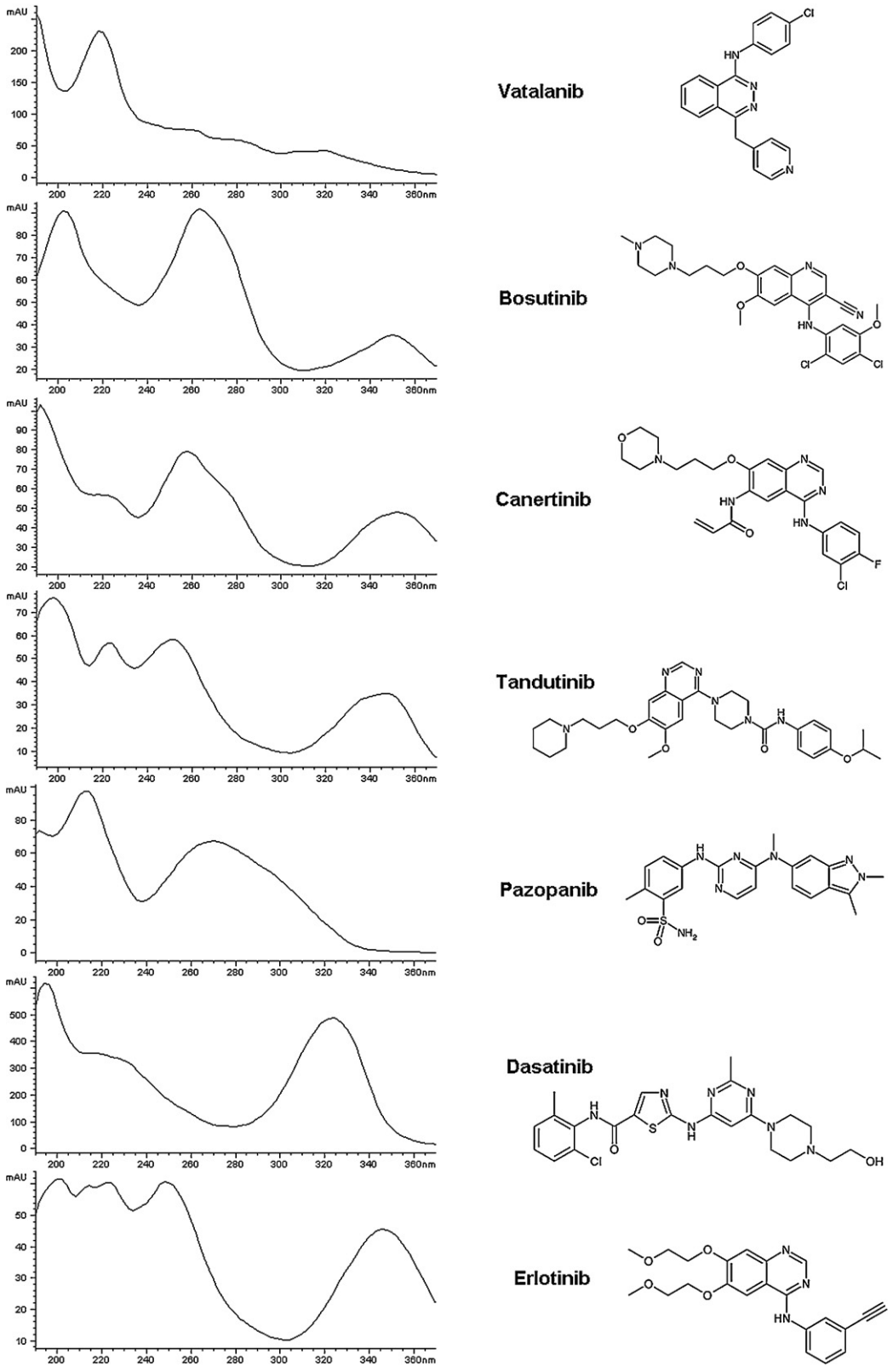

Fig. 1. Analysed protein kinase inhibitors and their UV-spectra.

2. Materials and methods

2.1. Chemicals

Protein kinase inhibitors (LC Laboratories, Woburn, MA 01801, USA) and all other chemicals/solvents (Merck KGaA, Darmstadt, Germany) were of analytical grade. Blank serum (calf serum) was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). New-born calf serum was used as a surrogate matrix, because its biochemical composition is close to that of human serum. It can be obtained easily and is much cheaper than human

serum [16–18]. Different pools of new-born calf serum were used during the validation.

2.2. Equipment

An Agilent HPLC 1200 Series system with a diode array detector was used for all purposes. Data acquisition and integration were performed by ChemStation for LC 3D Systems.

2.3. Chromatography conditions

Protein kinase inhibitors were separated with a Lichrospher 100-5 RP8, 250 mm × 4 mm column (Marcherey-Nagel GmbH & Co. KG, Düren, Germany) maintained at 30 ± 1 °C. The elution was performed with a mobile phase of 0.05 M H_3PO_4/KH_2PO_4 (pH = 2.3)–acetonitrile [19] (7:3, v/v) at a flow rate of 0.7 mL/min. The diode array detector was operating at a wavelength of 265 nm. A volume of 50 μL of a prepared sample was injected into the HPLC, and a single run was completed in 20 min.

2.4. Preparation of calibration standards

A 1 mg/mL PKI stock solution in dimethyl sulfoxide (DMSO) was used. The appropriate dilutions in the range of 20–10,000 ng/mL (each in blank calf serum) were prepared each experimental day.

2.5. PKI extraction from calf serum

A volume of 10 μL 1 mg/mL dasatinib (internal standard – ISTD) was added to a microcentrifuge tube with 300 μL calf serum with PKIs. After the addition of 900 μL of 1-chlorbutane, the standards were mixed for 30 min with a thermomixer (24 °C) and then centrifuged. 800 μL of the supernatant was transferred to a test tube and evaporated in a vacuum centrifuge. The residue was redissolved in 100 μL of the mobile phase and transferred into an HPLC vial.

2.6. Validation parameters

2.6.1. Linearity

The linearity was examined by preparing calf serum dilutions (in the range of 20–10,000 ng/mL) of the PKI 1 mg/mL stock solution and by plotting the analytical signal (area ratio PKI/ISTD) against the PKI concentration. A new calibration curve was prepared each experimental day.

2.6.2. Extraction efficiency

The percentage recovery of three PKI concentrations (20 ng/mL, 2000 ng/mL and 10,000 ng/mL) was calculated by the comparison of the determined PKI peak areas of extracted calf serum samples with the corresponding PKI peak areas of non-extracted control samples, prepared in a defined concentration in the mobile phase. PKIs in the matrix (calf serum) were extracted as described in Section 2.5 and in the mobile phase were directly injected (50 μL) into the HPLC. All of these PKI concentrations (extracted and corresponding non-extracted) were analysed six times.

2.6.3. Determination of LOQ and LOD

The limit of quantification (LOQ) was determined by the ten times replicated analysis of the lowest calibration standard (20 ng/mL) and by the calculation of the relative standard deviation (RSD) and accuracy. The limit of detection (LOD) was examined by the determination of signal-to-noise values of the PKIs at different low (≤LOQ) concentrations.

2.6.4. Accuracy and precision

The accuracy of estimation and between-batch imprecision were examined by the preparation and analysis of quality control samples on different days, and the accuracy of estimation and the within-batch imprecision were examined by the analysis of quality control samples performed during one day. Three concentrations were under examination (20 ng/mL, 2000 ng/mL and 10,000 ng/mL). Every level was analysed three times a day, eight days long.

2.6.5. Stability

The stability of PKIs in calf serum and DMSO was examined by freeze and thaw experiments (three unassisted cycles of at least 20 h freezing and 1 h thawing) and by storing the prepared standards over six months at −20 °C (long-term stability). Six independent analyses per PKI concentration (20 ng/mL, 2000 ng/mL and 10,000 ng/mL in calf serum and 1 mg/mL stock solution in DMSO) were done after these experiments. The results were compared with the analyses of the corresponding standards, performed directly six times per concentration.

2.6.6. Selectivity

The selectivity of this method was demonstrated by the analysis of the blank calf serum (ten times) and by the efficient separation of seven protein kinase inhibitors.

3. Results

Under the applied conditions a very good separation of seven protein kinase inhibitors was achieved. The well resolved peaks were characterised by the subsequent retention times: vatalanib – 4.3 min; bosutinib – 6.5 min; canertinib – 7.4 min; tandutinib – 8.2 min; pazopanib – 9.3 min; dasatinib (ISTD) – 9.9 min; and erlotinib – 17.6 min (Fig. 2). The blank calf serum analyses showed no interferences with the biological matrix. The measured UV spectra of all analysed PKIs were added to the library. This library, together with the reproducible retention times, made the PKI qualification possible. Therefore, the appropriate start conditions for the method validation were given.

All PKI calibration curves were characterised by a perfect linearity in the examined range (20–10,000 ng/mL). The calculated regression coefficient (R^2) exceeded 0.990, and the back-calculated standard concentration differences were lower than 15%. The ten times replicated analysis of the lowest calibration standard set the limit of quantification for the protein kinase inhibitors at 20 ng/mL. The relative standard deviation was lower than 20%, and the accuracy was within ±20% for these analyses. The signal-to-noise (S/N) values of the PKIs determined at different low (≤LOQ) concentrations showed, that 20 ng/mL is also a safe collective limit of detection in this method. For this calibration level an S/N > 3 value was obtained.

As listed in Table 1, the applied extraction method gave recoveries for a low, middle and high calibration standard in the range of 73–90%. The recovery hardly reached the accepted value of 50% only for pazopanib. The relative standard deviations and accuracies of all examined drug concentrations gave values much lower than 15% for both the between- and within-batch calculations. Therefore, the proposed chromatography and extraction method is acceptable for PKI quantification in human serum.

The freeze and thaw and long-term stability experiments performed with DMSO showed, that all analysed PKIs are stable for at least 6 months in a 1 mg/mL dimethyl sulfoxide stock solution. The analysed standard concentrations were in the range of 90–110% of the starting concentrations. Analogue experiments performed with three PKI calf serum concentrations differentiated a little more (Table 2) and showed, that canertinib has a poor serum stability

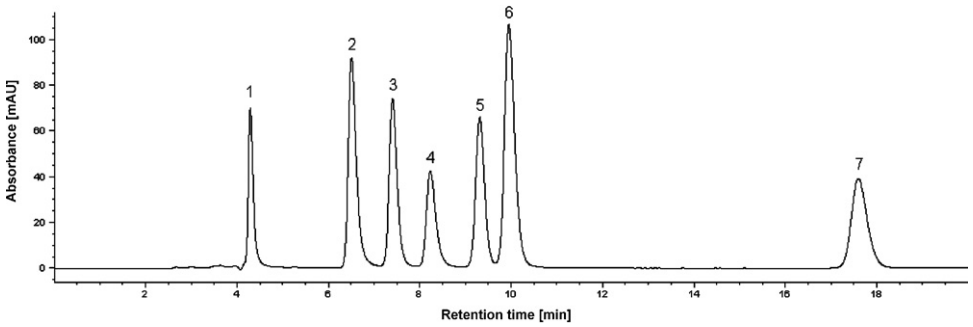

Fig. 2. Chromatogram of an analysed calf serum sample spiced with PKIs – 2000 ng/mL (1 – vatalanib, 2 – bosutinib, 3 – canertinib, 4 – tandutinib, 5 – pazopanib, 6 – dasatinib (ISTD), and 7 – erlotinib).

in the low, middle and high concentrations and tandutinib in the low concentration (20 ng/mL). The long-term stability experiment results of pazopanib were much better but the difference to the starting concentrations for the 20 and 2000 ng/mL standards was still about 15%. Vatalanib, bosutinib and erlotinib were stable in the whole examined concentration range.

4. Discussion

The validated HPLC–DAD method reported here is suitable for the separation/quantification of six protein kinase inhibitors. It can be used for pharmacokinetic studies and therapeutic drug monitoring. Although it is not necessary to quantify more than one PKI in a single run, the possibility of one chromatography method for analysing various PKIs is very advantageous. The sample preparation with the liquid–liquid extraction is a simple and relative fast analytical step which could be adapted by every clinical laboratory with standard equipment. Our results offer an alternative for those institutions that cannot afford an expensive LC/MS/MS to reach low PKI blood concentrations.

It is very important to realise that the method parameters were optimised for six PKIs. The chosen DAD wavelength does not give the highest sensitivity for all analysed drugs (Fig. 1). Therefore, it is possible to reach even lower LOQ/LOD for some inhibitors than the reported 20 ng/mL. Our aim was to present a possibility for analysis of PKIs (in the range of 20–10,000 ng/mL) without method modification for individual analytes. Thus, we left this HPLC–DAD method upgradable for targeted protein kinase inhibitor quantifications. Unfortunately the quantification of pazopanib requires sometimes a calibrated concentration range up to 54,000 ng/mL. In such cases a sample dilution could be applied. We also would like to point out the promising method extension over new protein kinase inhibitors explored in the future.

It is very difficult to compare directly our validated HPLC–DAD method with other validated methods. Only validated vatalanib and erlotinib assays were widely investigated and described in the literature. Two developed HPLC–UV erlotinib quantification methods [11,12] have a similar linearity range 100 (80)–4500 (4000) ng/mL and use also a comparable sample amount 500 (400) μL. Zhang et al. [13] reduced the sample amount to 250 μL and the limit of quantification to 12.5 ng/mL by the use of a complicated and time

Table 1
Accuracy, precision and recovery results of the applied PKI analytical method.

PKI [ng/mL]	Intra-day		Inter-day		Recovery [%]
	RSD [%]	Accuracy [%]	RSD [%]	Accuracy [%]	
Vatalanib					
20	5.0	−5.3	5.3	−3.1	76
2000	4.2	−5.7	5.8	−3.6	84
10,000	4.7	7.0	5.6	0.7	84
Bosutinib					
20	5.1	−5.8	5.8	0.4	73
2000	4.0	−10.5	7.6	−4.5	80
10,000	4.9	−6.7	5.9	0.7	79
Canertinib					
20	4.3	−2.7	4.3	−1.6	73
2000	3.9	4.4	7.8	−1.8	75
10,000	4.9	3.6	5.6	2.4	74
Tandutinib					
20	4.6	−2.3	4.8	−1.6	90
2000	3.4	−6.1	8.0	−5.0	87
10,000	4.7	−4.9	5.8	−2.0	82
Pazopanib					
20	2.4	2.7	3.9	0.2	50
2000	4.0	−5.3	6.5	−4.2	50
10,000	4.9	2.5	5.0	0.1	49
Erlotinib					
20	5.8	−7.2	6.8	−4.2	82
2000	4.0	−3.6	5.0	−1.7	85
10,000	4.6	3.3	4.8	−0.2	83

Table 2
Results of the freeze and thaw/long-term stability experiments.

PKI [ng/mL]	Freeze and thaw [%]	Long-term stability [%]
Vatalanib		
20	93	96
2000	92	97
10,000	95	104
Bosutinib		
20	112	94
2000	93	98
10,000	100	104
Canertinib		
20	81	70
2000	79	59
10,000	79	62
Tandutinib		
20	72	61
2000	91	101
10,000	104	97
Pazopanib		
20	98	85
2000	92	84
10,000	97	103
Erlotinib		
20	112	93
2000	98	97
10,000	103	106

consuming extraction method. In comparison our method has a wider linearity range (20–10,000 ng/mL) and is based on a simple and fast extraction, which is validated for six PKIs. The small difference in the LOQ in comparison to the method of Zhang et al. [13] is not relevant. The LC/MS/MS methods developed for the erlotinib estimation by Masters et al. [9] and by Honeywell et al. [10] have lower limits of quantification than our method (10 ng/mL and 1 ng/mL, respectively). In general the LC/MS/MS technique leads to lower quantification limits than the HPLC–UV methods. However such small concentrations are important only for the inhibitor quantification in tumour cells but not in human serum [12]. For a safe substance qualification the use of at least one qualifier is recommended in the clinical toxicology. Unfortunately, some published tandem mass spectrometry methods are validated with only one mass-transition, so that an error-free substance qualification is not warranted [8,10].

In addition, in the liquid chromatography–tandem mass spectrometry targeted substance tune and method preparation are very time-consuming and require highly specialised scientific staff. It is also worth mentioning that the linearity range of a diode array detector is a lot wider. In the LC/MS/MS methods, the highest standard did not exceed 4000 ng/mL. At higher concentrations, lower peak areas were observed as theoretically expected [10]. A different important issue for the toxicology is the fact that LC/MS/MS enables targeted analysis. Thus, potential intoxications with other drugs are not detectable without the help of other methods. These facts, together with the enormous price difference, make an alternative HPLC method for all clinical laboratories working in this field very attractive.

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