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RAZVOJ BIOANALITIČKE METODE ZA ODREĐIVANJE INHIBITORA O CIKLINU D OVISNIH KINAZA 4 I 6 I DRUGIH LIJEKOVA U TERAPIJSKIM KOMBINACIJAMA ZA LIJEČENJE HR+/HER2− RAKA DOJKE

DOKTORSKI RAD

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DEVELOPMENT OF A BIOANALYTICAL METHOD FOR THE DETERMINATION OF CYCLIN D DEPENDENT KINASE 4 AND 6 INHIBITORS AND OTHER DRUGS IN THERAPEUTIC COMBINATIONS FOR HR+/HER2− BREAST CANCER TREATMENT

DOCTORAL DISSERTATION

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SAŽETAK

Rak dojke, kao jedan od najčešće dijagnosticiranih tumora u svijetu, velik je javnozdravstveni problem. Liječenje se nastoji unaprijediti razvojem novih lijekova, ali i personaliziranim pristupom postojećoj terapiji s obzirom na specifične karakteristike pacijentica kroz postupak terapijskog praćenja lijekova. Temeljni preduvjeti terapijskom praćenju lijekova su provedena istraživanja farmakokinetičkih parametara u ciljanoj populaciji te povezanosti doze lijeka, njegove koncentracije u plazmi ili drugom tkivu i učinka ili toksičnosti. Za sve navedeno potrebna je prikladna, selektivna i osjetljiva bioanalitička metoda. U ovom doktorskom radu proveden je razvoj i optimizacija uvjeta pripreme uzorka ljudske plazme, kromatografskog razdvajanja i detekcije šest lijekova-kandidata za terapijsko praćenje – inhibitora o ciklinu D ovisnih kinaza 4 i 6 (CDK4/6) palbocikliba, ribocikliba, abemacikliba, inhibitora aromataze anastrozola i letrozola te selektivnog degradatora estrogenskih receptora fulvestranta. Ispitana su i optimizirana četiri različita pristupa pripremi složenog biološkog uzorka za analizu: taloženje proteina, disperzivna tekućinska mikroekstrakcija, ekstrakcija na čvrstoj fazi i pročišćavanje uklanjanjem fosfolipida. Razvijene su i validirane četiri nove kromatografske metode za razdvajanje lijekova od interesa primjenom fenilne i bifenilne stacionarne faze na različitim kromatografskim sustavima. Evaluacija pročišćavanja složenog uzorka plazme od UV-apsorbirajućih interferencija provedena je robusnim detektorom niza dioda i detektorom fluorescencije, dok su klinički relevantni linearni rasponi i praćenje zaostalih plazmatskih fosfolipida postignuti osjetljivim spektrometrima masa s analizatorom kvadrupola i vremena leta ili trostrukog kvadrupola. Provedena je validacija optimiziranih metoda u skladu s međunarodno prihvaćenim smjernicama, kao i unakrsna validacija naprednijih metoda pročišćavanja na istom kromatografskom sustavu i setu realnih uzoraka pacijentica te podrobna evaluacija ekoloških i ekonomskih svojstava primjenom *Analytical Greenness Assessment* (AGREE, AGREEprep), *Blue Analytical Grade Index* (BAGI) i *Red-Green-Blue-12* (RGB-12) alata. Naposlijetku, sve validirane metode su uspješno primijenjene za određivanje koncentracija te procjenu farmakokinetičkih parametara lijekova od interesa u stvarnim uzorcima pacijentica. Time je kao rezultat ovog doktorskog istraživanja stvorena potrebna bioanalitička podloga, kao i demonstrirana njena praktična valjanost za implementaciju u kliničkoj praksi u svrhu provođenja daljnjih ispitivanja terapijskog praćenja šest lijekova od interesa radi poboljšanja skrbi i terapijskih ishoda za pacijentice s rakom dojke.

Ključne riječi: terapijsko praćenje lijekova, LC-MS/MS, bioanalitička metoda, palbociklib, ribociklib, abemaciklib, antihormonska terapija, rak dojke, priprema uzorka

SUMMARY

Introduction: Breast cancer poses an on-going public health issue, with incidence and mortality in the rise despite the advances in diagnostics and the development of new medicines. The most prevalent type of breast cancer is hormone receptor-positive, and it is typically treated with antihormonal drugs such as aromatase inhibitors anastrozole and letrozole or selective oestrogen receptor degrader fulvestrant. Cancer resistance to these drugs may develop over time, hindering their efficacy and leading to disease progression. Inhibitors of cyclin D dependent kinases 4 and 6 (CDK4/6), palbociclib, ribociclib and abemaciclib, represent a targeted approach in breast cancer treatment and are used in combination with the aforementioned drugs. They enable postponing or even preventing cancer resistance to antihormonal therapy but are characterised with a range of toxic effects which may lead to dose reductions and reduced adherence. Therapeutic drug monitoring (TDM) and precision medicine may help improve treatment outcomes of previously ineffective or toxic drugs based on individual patient characteristics, as well as enable continuous treatment adjustments according to the patient needs. Development of TDM relies on the existence of adequately sensitive, selective and robust bioanalytical methods for the determination of drug concentrations in patient samples such as plasma. Apart from their analytical merits, the importance of such methods' ecological properties and economical practicality is increasingly recognised in order to provide sustainable solutions applicable in routine clinical practice. The six breast cancer drugs of interest may be good candidates for TDM due to observed inter-patient variabilities in efficacy and toxicity, indicating that a personalised approach may be beneficial. The aim of this doctoral thesis is to develop and optimise different procedures for the bioanalysis of the drugs of interest in patient plasma samples. This encompasses the development of a selective preanalytical plasma sample preparation method, optimisation of chromatographic separation conditions, achieving sensitive detection in clinically relevant linear ranges, validation of the developed procedure according to internationally acclaimed guidelines and application of the validated procedure on real patient samples for the quantitation of the drugs of interest. Furthermore, detailed evaluation from an ecological and economical perspective illuminates the potential merits and weaknesses of the proposed methodology in a routine clinical setting. As a result, the necessary analytical support for future testing and development of TDM with additional applicability in pharmacokinetic studies is provided, ultimately aiming to enhance breast cancer patient care and treatment outcomes.

Methods: Various sample preparation methods with liquid chromatography (LC) coupled to diode array (DAD), fluorescence (FLD) and mass spectrometry (MS) detectors were optimised. Protein precipitation (PPT) with organic solvents, concentrated acid or salt solutions were first tested as the simplest plasma sample preparation technique. More advanced dispersive liquid-liquid microextraction (DLLME), solid-phase extraction (SPE) and phospholipid removal (PLR) methods were further developed to achieve improved sample cleanup. The choice of disperser and extractant solvent types, volumes and volume ratios as well as pH and addition of salt for three distinct modes of DLLME were optimised in detail. Six different sorbents, including silica-based and polymeric, reversed-phase and cation-exchange, were assessed during the optimisation of SPE. Protic and aprotic solvents of acidic, alkaline and neutral pH were tested for analyte elution. Phospholipid removal was achieved using a cyclodextrin-based sorbent, where a simple flow-through protocol was enhanced with an additional elution step. Cleanup capabilities and matrix effects of the sample preparation methods were assessed with DAD-FLD for UV-absorbing interferences and MS detection for phospholipids. Detection conditions for achieving clinically relevant linear ranges of all the analytes were explored for three different MS systems. Chromatographic conditions for successful separation of the analytes of interest mutually as well as from matrix interferences were optimised using octadecyl-silyl, phenyl and biphenyl stationary phases with water, acetonitrile and/or methanol containing 0.1 % formic acid as the mobile phase. The optimised methods were validated according to recent international bioanalytical method validation guidelines (ICH M10) and applied on real patient samples for quantitation of the drugs of interest in order to confirm their applicability and identify potential drawbacks.

Results: Four sample preparation methods for extraction and cleanup of six breast cancer drugs from human plasma samples were developed, based on PPT, DLLME, SPE, and PLR. Chromatographic separation was optimised on four stationary phases and three different LC instruments. Four bioanalytical methods employing different MS detectors or DAD-FLD were finally validated, and each was applied on real patient samples. The methods' critical features were thoroughly ecologically and economically evaluated, while the quantitated concentrations of the drugs of interest were used for the estimation of their pharmacokinetic parameters: minimal (C_{min}) and maximal plasma concentrations (C_{max}), time to achieve maximal concentration (T_{max}), constant of elimination (k_e) and the elimination half-life ($T_{1/2}$), and area under the 24 h concentration-time curve (AUC_{0-24}). The obtained results demonstrate the applicability of the developed methodology for routine clinical use.

PPT is performed by adding a precipitation agent and collecting the resulting supernatant. Excellent efficacy with high analyte recoveries (>91 %) was obtained using acetonitrile, in the volume ratio to plasma sample 4:1. This method was extremely simple and fast, albeit fully manual, however it achieved only partial sample cleanup, which may limit the long-term applicability. Nevertheless, it was successfully validated, using an LC-MS system with a quadrupole-time-of-flight analyser, in terms of selectivity and carry-over (peak areas of any interferences smaller than 20 % of the peak areas in the lower limit of quantitation, LLOQ, sample), matrix effects (soft to moderate ionisation changes relatively consistent over different plasma lots and analyte concentrations), linearity (adequate in the clinically relevant ranges for all analytes), accuracy (maximal between-day bias in the range −14.8–15.0 %), precision (maximal between-day RSD of non-LLOQ samples ≤15.0 % and LLOQ samples ≤19.9 %), stability (bias within ± 15 % and RSD ≤15 % at all tested conditions).

In DLLME, the extraction is carried out using a ternary solvent mixture containing a polar aqueous phase, a nonpolar organic extractant solvent and a polar organic disperser solvent which increases the contact area between the phases. In this work, three modes were developed: aqueous-sample (AqS-DLLME), organic-sample (OrS-DLLME) and air-assisted DLLME (AA-DLLME). The optimised AqS-DLLME procedure was performed by injecting a mixture of i-propanol and chloroform into an aqueous solution of the sample residue obtained after PPT and evaporation, in the volume ratio water:i-propanol:chloroform = 100:50:100 µL. The extraction mixture in OrS-DLLME was composed of water, the acetonitrile supernatant after PPT, and chloroform, in the volume ratio 100:200:100 µL. AA-DLLME encompassed extraction with 100 µL of chloroform added to 100 µL of the aqueous phase containing 10 % *w/V* NaCl, without the use of a disperser. Extraction recoveries >82 % were achieved with AqS- and AA-DLLME modes, while the recoveries obtained with OrS-DLLME were >75 %. A representative AqS-DLLME method coupled to LC-DAD-FLD was validated and acceptable selectivity (absence of co-eluting interferences), robustness (bias <15% for variations in the volumes of all three phases, the time of vortex mixing and the number of pipette mixing cycles), linearity (in clinically relevant ranges for the CDK4/6 inhibitors and letrozole), accuracy (maximal between-day bias on all concentration levels −13.1–13.1 %), and precision (maximal between-day RSD on all concentration levels ≤13.8 %) were confirmed. This method was subsequently transferred to an LC-MS system for cross-validation, comparison with the other developed procedures and application to patient samples for pharmacokinetic parameter estimation. Adequate linearity in appropriate ranges for all six analytes, accuracy (bias −13.6–

7.7 %) and precision (RSD ≤5.0 %) on this system were confirmed, while the matrix effects were corrected using stable isotope-labelled internal standards (IS).

In SPE, a favourable combination of the extraction sorbent and the elution solvents is necessary to achieve a selective and efficient extraction. The highest extraction recoveries of all six analytes (>75 %), together with the best purification efficacy were achieved using octyl-silyl (C8) sorbent with methanol as the elution solvent. Among the other tested sorbents, hydrophilic-lipophilic balance and octadecyl-silyl sorbents also showed acceptable recoveries of CDK4/6 and aromatase inhibitors, while high amounts of co-extracted interferences were observed with the tested cation-exchange sorbents. The optimised method was validated on a sensitive LC-MS system with a triple quadrupole analyser in terms of linearity in clinically relevant ranges for all analytes, accuracy (maximal between-day bias on all concentration levels −12.7–13.5 %), precision (maximal between-day RSD on all concentration levels ≤14.3 %), and matrix effects (soft to moderate ion suppression was mostly observed). Thereafter, it was transferred to another LC-MS system with stable isotope-labelled IS-correction for the purposes of cross-validation with DLLME and PLR, where appropriate linearity, accuracy (bias −11.2–8.1 %) and precision (RSD ≤4.5 %) were also confirmed.

In PLR, analyte purification is achieved through a selective retention of plasma phospholipids on the sorbent. In accordance with the obtained results, an additional elution step with 5 % ammonia in methanol after the initial sample loading was found to increase the extraction recoveries of all the analytes (to >71 %), especially the CDK4/6 inhibitors, as they appeared to have been partially retained in the structure of the sorbent. The linearity in clinically relevant ranges for all six analytes, accuracy (bias −10.9–11.8 %) and precision (RSD ≤6.9 %) of this method on an LC-MS system with stable isotope-labelled IS-correction were successfully validated.

Cross-validation of the developed DLLME, SPE and PLR methods using the same LC-MS system with a triple quadrupole mass analyser and the same set of 38 real patient samples revealed excellent agreement between the methods, as demonstrated by differences between paired quantified analyte concentrations (within ±20 % of the mean for ≥92 % of the samples), RSD between the concentrations obtained with all three methods (≤13.1 %), Bland-Altman method comparison (mean difference between quantitated concentrations 1.020–6.295 %), two variants of folded empirical cumulative distribution plots (centred near zero and with most differences within the guideline-defined ranges for accuracy), Deming regression (95 %

confidence intervals of the regression curves' slopes for different method pairs encompassing zero; a slight bias of the intercept observed for PLR in relation to SPE and DLLME), and Lin's concordance correlation coefficient (lower limit of the 95 % confidence intervals of the coefficient for each method pair >0.99). The analytical, ecological and economical properties of the three sample preparation procedures were compared using red-green-blue index (RGB-12), where PLR was revealed as the most cost-effective, DLLME most eco-friendly and SPE most analytically favourable. Overall, DLLME was the highest-scoring sample preparation method with a total score of 90.8, closely followed by PLR (score 90.0) and SPE in the third place (score 85.0).

All the developed analytical and sample preparation methods were further assessed from ecological and economic standpoint using the recently reported analytical greenness metric (AGREE) or analytical greenness metric for sample preparation (AGREEprep) and blue applicability grade index (BAGI). All chromatographic methods paired with different detection systems achieved AGREE scores ≥0.52, indicating acceptable greenness, as well as BAGI scores ≥72.5, demonstrating adequate cost-effectiveness. The AGREEprep scores of the sample preparation approaches ranged from 0.31 (SPE) to 0.51 (PPT). Smaller volumes of sample and solvents as well as a smaller number of steps are favourable for increased analyst safety, making PPT and DLLME advantageous. All sample preparation methods were economically feasible (BAGI score ≥67.5), with PPT and PLR being most cost-effective due to a favourable balance of simplicity and speed or necessary investment and automatability, respectively.

Finally, the application of all methods on real patient samples proved their suitability for the designated purpose in TDM development: the determined concentrations of the analytes in the samples collected in various timepoints after dose administration including the trough values were within the methods' linear ranges and the estimated pharmacokinetic parameters (C_{max}, C_{min}, T_{max}, $T_{1/2}$, k_e, AUC_{0-24}) were in accordance with those previously reported.

Conclusions: This doctoral thesis provides a detailed overview of bioanalytical method development and validation for the analysis of six breast cancer drugs in patient plasma samples. Due to extensively validated and clinically appropriate linear ranges, selectivity, accuracy and precision, matrix effects, extraction recoveries, robustness, and stability, the developed methodology can be readily applied in a clinical setting for further studies of TDM of the drugs of interest, as well as pharmacokinetic studies and adherence monitoring. The applicability of the methods is assessed not only on spiked validation samples, but also on a

larger number of real patient samples in order to provide insight into possible drawbacks and pitfalls. Furthermore, ecological and economic assessment results of the developed methods elucidate the long-term suitability depending on the available equipment and funds as well as the desired ecological footprint. Finally, the described methodology can be applied as a guideline and a starting point in future method development for the bioanalysis of other similar drugs. Thus, the obtained results may prove beneficial for improving the patient care and treatment outcomes in breast cancer.

Keywords: therapeutic drug monitoring, LC-MS/MS, bioanalytical method, palbociclib, ribociclib, abemaciclib, antihormonal therapy, breast cancer, sample preparation