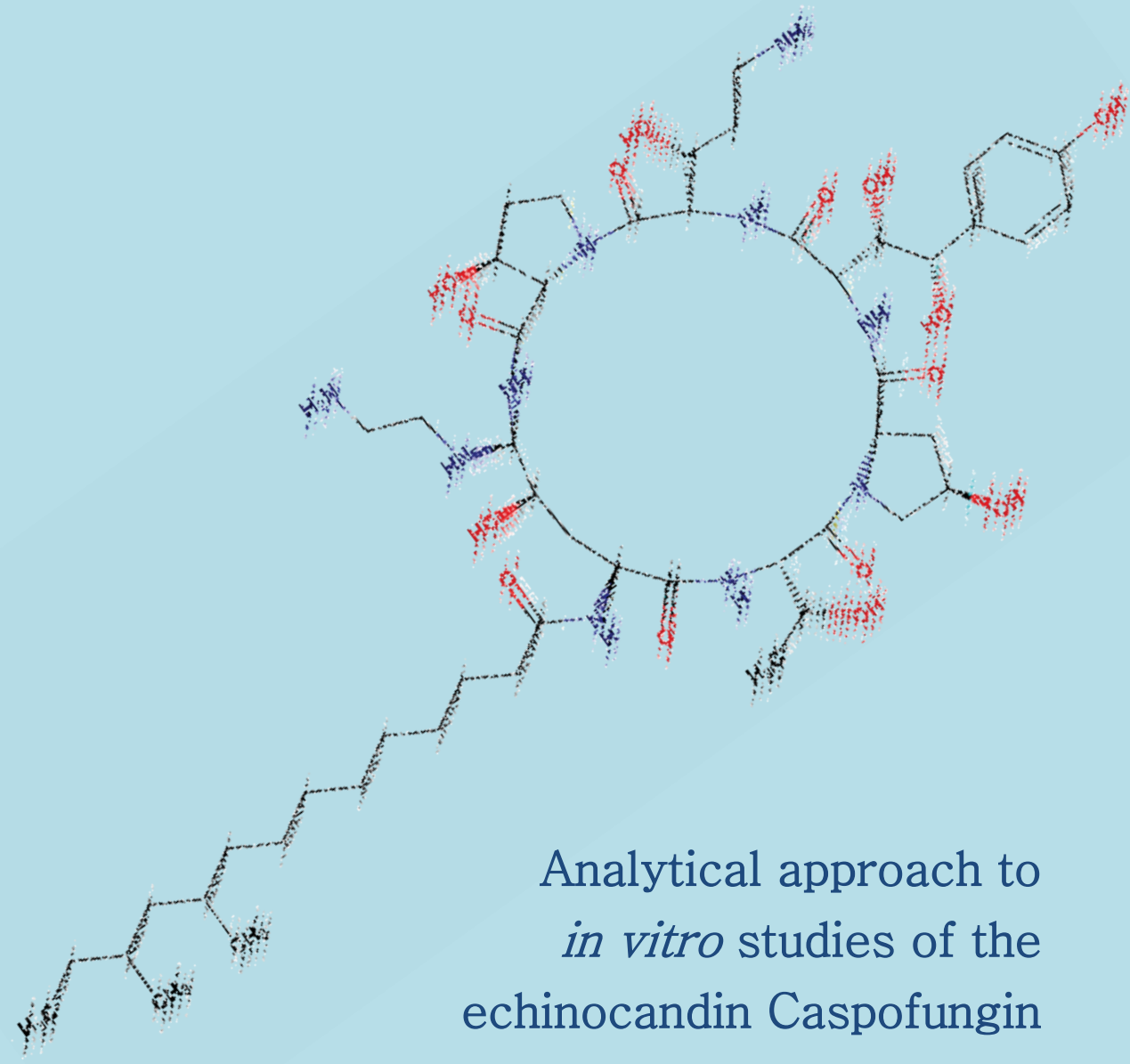

Analytical approach to *in vitro* studies of the echinocandin Caspofungin

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Kozuzendaritzapean aurkeztutako tesia

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Auzitegiko analisiko doktorego eskola
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Beatriz URIBE-ren eskutik

Kaspofungina ekinokandinaren *in vitro* azterlanen ikuspegi analitikoa

Approche analytique pour des études in vitro d'une échinochandine, la caspofungine

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Kaspofungina ekinokandinaren in vitro azterlanen ikuspegi analitikoa

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka!" but "That's funny…"

Isaac Asimov

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Laburpena

Infekzio fungiko inbaditzaileek, mundu mailan, garrantzia hartu dute erakusten duten erikortasun- eta hilkortasun-tasa altuak direla eta. Infekzio hauen erantzule nagusiak *Aspergillus, Cryptococcus* eta *Candida* onddo generoak dira. Horien artean, kandidak espezialisten arreta lortu du populazio talde batzuetan erakusten duen hilkortasun maila altuagatik, non, batzuetan % 40-ra heldu daitekeen.

Egun, kandidiasi inbaditzailearen tratamendurako lau farmako familia erabili ohi dira: azolak, polienoak, pirimidinaren analogoak eta ekinokandinak. Urte askoz, kandidiasiaren tratamendua azolen familiako farmakoekin aurrera eraman da batez ere. Baina onddoek farmakoekiko garatzen ari diren erresistentzia mekanismoak direla eta, terapien aldaketa bultzatuta ikusi da. Testuinguru horretan, berriki garatutako ekinokandinen familia bilakatu da espezialisten lehen aukera kandidiasiaren tratamendurako hainbat populazio multzotan.

Ekinokandinen akzio mekanismoa onddoen horma zelularraren osagai nagusi baten inhibizioan oinarritzen da, β-(1,3)-D-glukanoarena hain zuzen ere. Ikuspuntu kimiko batetik, ekinokandina familia osatzen duten molekulak hainbat onddoen hartziduratik lortutako lipopeptido semisintetikoak dira. Gaur egun, familia horretan hiru molekula onartuta daude giza terapian erabiltzeko: kaspofungina, mikafungina eta anidulafungina. Momentu honetan, laugarren bat garapen klinikoaren azken faseetan aurkitzen da: rezafungina.

Ekinokandina molekulekiko onddo erresistentzia baxu mantentzen da kandida espezie gehienetan. Zoritxarrez, azken hamarkadan *C. glabrata* espeziearen erresistentziak ekinokandinekiko gora egin du, batez ere kaspofunginarekiko (CSFrekiko). Horrela izanik, nahitaezkoa da in vitro ikerketak garatzea onddoek farmakoekiko duten erresistentzia aztertu ahal izateko. Guzti hau kontuan hartuta, lan honek *C. glabrataren* erresistentziaren azterlanak aurrera eramateko beharrezkoak diren ikuspuntu analitikoak aurkezten ditu.

2. Kapituluan, ekinokandina familiako molekulen kuantifikazioa matrize biologikoetan egiteko argitaratutako metodo analitikoen berrikuspen bibliografikoa egin zen. Literaturan aurkitutakoaren arabera, gehien erabilitako metodo analitikoa alderantzizko faseko likido kromatografia (LC) da. Farmako horien detekzioa egiteko, masa espektrometria (MS) eta fluoreszentzia (FLD) detektagailuak erabili dira, eta kasu batzuetan, ultramore ikuskorreko absortzio espektroskopia detektagailua. Ikertzaile batzuek aurkeztu dutenen arabera, ekinokandinekin lan egitean, lagin tratamendu batzuk aurrera eramatean gerta daitekeen analito galera da aurkitutako zailtasun handiena, adibidez, fase solidoko erauzketa (SPE) egitean.

Deskribatutako informazioa kontuan harturik, bereizmen handiko likido kromatografia metodo bat garatu egin zen fluoreszentziako detekzioa erabiliz (HPLC-FLD). Metodo honetan, column-switching prozedura bat aurrera eraman zen lagina zutabe kromatografikora heldu baino lehen (**3. Kapitulua**). *In vitro* ikerketetan erabiltzen diren kultibo zelularrak konposatu askotariko matrize konplexuak dira eta prozedura horrek laginaren garbiketa egitea ahalbidetzen du analisi kromatografikoa egin baino lehen. Garatutako metodologia analitikoa CSF farmakoaren kuantifikazioa RPMI-1640 matrizean egiteko metodo eraginkorra, azkarra eta sentikorra dela frogatu egin zen. Alde batetik, column-switching teknikak, lagina zuzenean injektatzeko aukera ematen du laginaren aurretratamendurik egin gabe, literaturan deskribatutako analito galera murriztuz. Bestetik, bolumen txikiekin lan egitea ahalbidetzen du, *in vitro* ikerketetan aplikatu ahal izateko beharrezkoa dena. Metodoa balidatu egin zen elikagai eta medikamentuen administrazioaren (FDA) irizpideak jarraituz, hurrengo parametroekiko: selektibitatea, carryover, kontzentrazio tarte lineala, zehaztasuna eta doitasuna. Metodoa egoki aplikatu egin zen CSF kantitatearen determinazioa egiteko *C. glabrataren* 24 h-ko hazkundearen ikerketa zinetikoan.

4. kapituluan *C. glabrataren* erresistentziaren ikerketa aurrera eramaten saiatu egin zen. Hori helburua izanik, bi konpartimentutako in vitro eredu farmakozinetiko/farmakodinamiko (PK/PD) bat (SIMULI-PHARM 1 deitua) erabili egin zen plasman aurkitzen den CSFren profil zinetikoa simulatzeko. Esperimentazio hori burutzean CSF kantitatearen galera nabarmena ikusi zen. Hori horrela izanik, beste farmako batzuen ikerketetan aurretiaz ikertzaile batzuen eskutik aurrera eramandako prozedura bat saiatu egin zen, analitoaren adsortzioa sisteman zehar ekiditzeko. Bertan, esperimentua burutu baino lehen, PK/PD sistema analitoarekin asetzen da adsorbatu daitekeen kantitate guztia adsorbatu daiten. Modu horretan, ondoren esperimentua burutzean adsortzioa eragozten da. Asetze prozedura burutu arren analito galera ekiditzea ezinezkoa izan zen. Hori dela eta, C. glabrataren CSFrekiko erresistentziaren ikerketa, SIMULI-PHARM 1 *in vitro* PK/PD ereduaren laguntzaz, aurrera eramatea ez zen posiblea izan.

4. kapituluan lortutako emaitzetan oinarrituz eta aurretiaz bibliografian aurkitutako peptidoen materialekiko adsortzioa kontuan hartuta, ikusitako analito galeraren azalpenaren inguruan ikertzea erabaki zen, ondoren PK/PD metodoa modu egokian garatu ahal izateko. Horretarako, eman ahal ziren bi prozesuak plazaratu ziren: degradazio eta adsortzio fenomenoak hain zuzen ere.

Lehenengo eta behin molekularen degradazioaren ikerketa egin zen, **5. Kapituluan** deskribatuta dagoena. Bertan erresonantzia magnetiko nuklearra (RMN), HPLC-FLD eta HPLC-MS teknikak erabili egin ziren CSF molekularen degradazio produktuak identifikatzeko helburuarekin. Analitoaren degradazioa bultzatzeko baldintza azido, basiko eta oxidatzaile bortitzak erabili ziren. RMN eta HPLC-FLD teknikekin degradazio produktu bat ikusi egin zen baldintza azidoetan aurrera eramandako degradazioan. RMN eta HPLC-(Q)-TOF-MS (ESI+ ionizazioarekin) teknikak erabili arren, konposatuaren egitura kimikoa guztiz argitzea ezinezkoa izan zen. Hala ere, kapitulu honetan ondorio nagusi bat ateratzea lortu zen: lortutako emaitzei erreparatuz, degradazioa analito galeraren erantzule nagusia ez zen.

Hori horrela zelarik, CSFren adsortzio fenomenoaren ikerketa aurrera eraman zen (**6. Kapitulua**). CSFren adsortzioa zenbait laborategiko materialetan aztertu egin zen. Gainera, disoluzioaren pH balioa eta disolbatzaile organikoaren adizioak eduki ahal zuen eragina ikertu egin zen. Horretarako pH balio azido eta basikoa ikertu egin ziren. Disolbatzaile organikoen artean, azetonitriloa eta metanolaren eragina aztertzea erabaki zen. Baldintza horiekin jokatuz adsortzioa ekiditu edo gutxienez minimizatzeko aukerarik zegoen ebaluatu egin zen. Laginak berriki prestatuta eta 24 h ondoren (ingurune tenperaturan) analizatu egin ziren. Ikertutako laborategiko materialen artean ezberdintasun esanguratsuak aurkitu egin ziren, baita, plastiko mota berdinen artean ere. Disoluzio urtsuetan adsortzioa ekiditzeko baldintza egokiena pH azidoa zela ondorioztatu egin zen. Disolbatzaile organikoen gehipena, ere, adsortzioa saihesteko eraginkorra zela ikusi zen.

Azkenengoz, adsortzioa ekiditzeko gai zen materialaren tratamendu bat proposatu eta ebaluatu egin zen. Tratamendu hori aurretiaz bibliografian deskribatutako prozedura batean oinarritzen da, farmako batzuen adsortzio ez-espezifikoa saihesteko erabilia izan dena. Hiru erreaktibo ezberdin frogatu egin ziren bial kromatografikoetan eta 96-mikroputzuetako plaketan ematen den adsortzioa ekiditzeko erakusten zuten gaitasuna ebaluatzeko. Ikertutakoen artean N-(2-aminoetil)-3-aminopropiltrimetoxisilanoa ikusi zen eraginkorrena zela. Erreaktibo horrek adsortzioaren zinetika moteltzeko gai zela aurkitu zen, CSFren determinazio kuantitatiboa ahalbidetuz. Dena den, denbora pasa ahala CSF materialean adsorbatzen zela ikusi zen, beraz, tratamendua guztiz eraginkorra ez zela ikusi zen.

Laburbiltzeko, tesi honek CSFrekin *in vitro* baldintzetan (giza gorputzaren baldintzetan: pH 7 eta disoluzio urtsuan) lan egitearen zailtasunak aurkezten ditu, aurkitutako adsortzioko fenomenoa dela eta. Ikerketa honek mikrobiologiako eta terapia klinikoetan lortutako emaitzak ebaluatzeko ikuspegi berria eskaintzen du. Ez hori bakarrik, erresistentziak zehazteko eta drogak administratzeko erabiltzen diren metodologien fidagarritasuna ebaluatzeko ikuspuntu berri bat aurkezten du.

Resumé

Les infections fongiques invasives sont devenues une préoccupation mondiale en raison de la morbidité et de la mortalité observées. Les principaux responsables de ces infections sont les genres *Aspergillus, Cryptococcus et Candida*. Cependant, *Candida* a attiré l'attention des spécialistes en raison de la mortalité observée pour certains groupes de population comme les personnes immunodéprimées, pouvant atteindre 40%.

Les traitements pharmacologiques de la candidose invasive reposent sur quatre familles de médicaments antifongiques : les azolés, les polyènes, les analogues de pyrimidine et les échinocandines (EC). L'augmentation de la résistance aux antifongiques, tels que la famille des azolés, a donné lieu au développement de nouveaux traitements. Dans ce contexte, les EC sont apparues comme une alternative prometteuse pour les thérapies antifongiques et sont devenues la thérapie de première intention pour la candidose invasive dans différentes populations de patients.

Le mécanisme d'action des EC est basé sur l'inhibition de la synthèse du β- (1,3) -D-glucane, une molécule essentielle de la paroi cellulaire mycocètes. Du point de vue chimique, les molécules composant la famille des EC sont des lipopeptides semi-synthétiques issus de la fermentation de différentes levures. Trois molécules de cette famille ont été approuvées pour leur usage thérapeutique : la caspofungine, la micafungine et l'anidulafungine, et une quatrième est en cours de développement, la rezafungine.

La résistance aux échinocandines reste faible dans la plupart des espèces de *Candida*. Malheureusement, au cours de la dernière décennie, la résistance des espèces de *C. glabrata* aux EC a augmenté, et en particulier à la caspofungine (CSF). Dans ce contexte, ce travail présente de nouvelles approches analytiques pour étudier la résistance des souches de *C. glabrata* à la CSF.

Premièrement, une revue de la littérature sur les méthodes analytiques développées pour la quantification des EC dans des matrices biologiques, a été soigneusement effectuée et constitue le **chapitre 2**. Elles utilisent la chromatographie liquide haute performance en phase inverse (RP-HPLC). Pour la détection, en général la spectrométrie de masse (MS) et la fluorescence (FLD) sont utilisées, dans certains cas, un détection UV-Vis. La littérature mentionne qu'une attention toute particulière doit être suivie lors de l'analyse des EC en raison d'une perte de concentration de l'analyte pendant les étapes de traitement d'échantillons comme l'extraction en phase solide (SPE).

Grâce à cette revue, une méthode par commutation de colonnes utilisant la RP-HPLC couplée à une détection par fluorescence, a été développée (**chapitre 3**). Elle a été validée en termes de sélectivité, d'effet mémoire, de linéarité, d'exactitude et de précision selon le guide de validation de la Food and Drug Administration (FDA) pour la bioanalyse. Cette méthodologie analytique s'est avérée être efficace, rapide et sensible pour la quantification de la CSF dans des milieux de culture cellulaire RPMI-1640 pour des études *in vitro*. Cette approche offre la possibilité d'une injection directe de l'échantillon sans traitement préalable (évitant toute perte d'analyte due à des manipulations), et permet de travailler avec des volumes réduits d'échantillon, un avantage pour son utilisation lors d'études *in vitro*. Elle a été appliquée avec succès pour la détermination de la CSF lors d'une cinétique de 24h en présence de *C. glabrata*.

Dans le **chapitre 4**, une tentative d'étude de la résistance de *C. glabrata* à la CSF a été effectuée. Dans ce but, un modèle *in vitro* pharmacocinétique / pharmacodynamique (PK/PD) à deux compartiments, appelé SIMULI-PHARM 1, a été utilisé pour simuler le profil cinétique de la CSF dans le plasma. Au cours de cette expérimentation, une perte importante de l'analyte a été observée. Même avec un essai de saturation du système par la CSF (une procédure déjà testée avec succès par certains auteurs vis à vis d'autres molécules), la perte d'analyte s'est toujours

produite. Par conséquent, l'étude de la résistance de *C. glabrata* à l'aide du modèle *in vitro* PK/PD n'a pas pu être réalisée.

Sur la base des résultats obtenus et la connaissance du comportement des peptides décrit dans la littérature, une étude tenant compte à la fois de la dégradation et de l'adsorption de la CSF a permis d'expliquer ce phénomène.

L'étude de dégradation de la molécule a été réalisée au **chapitre 5** grâce à la Résonance magnétique nucléaire (RMN), et l'HPLC-FLD ou HPLC-MS. Des conditions de stress, acides, alcalines, oxydantes et de température, ont été utilisées pour forcer l'analyte à se dégrader. Un produit de dégradation a été observé en RMN, HPLC-FLD ou HPLC- (Q) -TOF-MS par mode d'ionisation positive. Cependant son élucidation structurale n'a pas été obtenue. En revanche, cette étude a montré que la CSF n'est pas une molécule qui se dégrade rapidement et donc la dégradation ne peut pas être l'unique responsable de la diminution de la concentration de CSF.

Par conséquent, le phénomène d'adsorption de la CSF a été étudié au **chapitre 6**. Divers matériaux présents au laboratoire ont été testés. Puis l'effet du pH ou l'utilisation de solvants organiques a été étudié dans l'optique d'éviter ou du moins minimiser l'adsorption. Des échantillons analysés dès leur préparation à température ambiante puis étudiés sur 24, ont montré d'énormes différences, notamment pour certains matériaux plastiques. La mise en solution à pH acide s'est avérée la meilleure condition pour éviter l'adsorption de la CSF en solution aqueuse. L'addition de solvants organiques est aussi une approche qui s'est révélée efficace.

En dernier lieu, une procédure déjà établie de modification de surface par un traitement chimique, a été testée sur différents matériaux (verre ou plaque 96 puits). L'efficacité de trois réactifs a été évaluée. Le N- (2-aminoéthyl) -3-aminopropyltriméthoxysilane, a donné des résultats positifs comme le ralentissement de la cinétique d'adsorption de la CSF permettant ainsi d'améliorer

sa quantification. Cependant, le phénomène d'adsorption est ralenti mais pas complètement éliminé.

Pour résumer, les résultats obtenus ont montré les difficultés de travailler avec la CSF lors d'études *in vitro* comme des conditions à pH physiologique en solution aqueuse, en raison principalement du phénomène d'adsorption. Ce travail de recherche offre une nouvelle perspective pour aborder les résultats obtenus dans les domaines microbiologique et thérapeutique, des moyens d'améliorer la fiabilité des méthodologies utilisées pour la détermination de la résistance et l'administration de la CSF.

Summary

Invasive fungal infections have become a concern around the world due to the morbidity and mortality shown. The main responsible of these infections are *Aspergillus, Cryptococcus* and *Candida* genus. However, Candida has gained the attention of specialists due to the mortality shown in some population groups such as immunocompromised, where it can arrive to 40 %.

Pharmacological treatment of Invasive candidiasis includes four antifungal drug families: azoles, polyenes, pyrimidine analogues and echinocandins (ECs). The increase fungal resistance to drugs, such as azole family, gave rise to the development of new antifungals. In this context, ECs emerged as a promising alternative for antifungal therapies and become the first-line therapy for invasive candidiasis in different patient population.

ECs mechanism of action is based on the inhibition of the β-(1,3)-D-glucan synthesis, an essential molecule of the fungi cell wall. From the chemical point of view, the molecules comprising ECs family are semisynthetic lipopeptides derived from the fermentation of different fungi. Three molecules of this family have been approved for their therapeutic use: caspofungin, micafungin and anidulafungin, and a fourth one is under development, rezafungin.

Echinocandin resistance remains low in most candida species. Unfortunately, in the last decade resistance of *C. glabrata* species to ECs has risen, especially to caspofungin (CSF). In this context, this work presents new analytical approaches to study the resistance of *C. glabrata* strains to the antifungal agent CSF.

Firstly, a literature review of the analytical methods developed for ECs quantification in biological matrices was carefully done and it is included in **Chapter 2**. Mainly, the analytical methods developed for the analysis of ECs used reversed phase liquid chromatography (LC). For detection, mass spectrometry (MS) and fluorescence (FLD)

were chosen, and in some cases, ultraviolet-visible absorption spectroscopy. As seen in literature, special care must be taken when analysing ECs due to an analyte concentration loss during sample treatment procedures as solid phase extraction (SPE).

With the information previously described, a column-switching high performance liquid chromatography coupled to fluorescence detection method was developed (**Chapter 3**). Due to the complexity of the matrix used for in vitro studies, column-switching technique allowed sample cleaning before its analytical determination. The method developed was validated in terms of selectivity, carryover, linear concentration range, accuracy and precision according to the criteria established by the Food and Drug Administration (FDA) for validation in bioanalysis. This analytical methodology demonstrated to be an efficient, rapid and sensible technique for the quantification of CSF echinocandin drug in RPMI-1640 cell culture media for *in vitro* studies. Column-switching technique gives the chance of direct injection of the sample with no prior sample treatment (avoiding any analyte loss due to sample manipulation) and permits to work with small sample volumes, which is adapted for *in vitro* studies. The method was successfully applied for the determination of CSF amount during 24h kinetic study of *C. glabrata* cell proliferation.

In **Chapter 4,** a study of the resistance of *C. glabrata* to CSF was tried to perform. For this aim, a two compartment pharmacokinetic/pharmacodynamic (PK/PD) *in vitro* model, called SIMULI-PHARM 1, was used to simulate the kinetic profile of CSF in plasma. During this experimentation, a significant analyte loss was observed. Even though, an attempt of system saturation with CSF was carried out (a procedure already performed by some authors with other drugs), the analyte loss was not possible to avoid. Therefore, the resistance study of *C. glabrata* using the SIMULI-PHARM 1 PK/PD *in vitro* model was not possible to carry out.

Based on the results obtained and the previously reported behaviour of peptides in literature, explanation of this phenomenon was studied taking in consideration both degradation and adsorption processes.

Degradation study of the molecule was carried out in **Chapter 5** by nuclear magnetic resonance (NMR), HPLC-FLD and HPLC-MS techniques. Stress acidic, alkaline and oxidative conditions and temperature were used to force analyte degradation. A degradation product was observed in NMR and HPLC-FLD or MS analysis, but its structural elucidation was not obtained by means of NMR or HPLC-(Q)-TOF-MS in ESI+ ionization mode. Additionally, degradation was found to be slightly responsible for the decrease in CSF concentration compared to the adsorption on the materials.

Consequently, the adsorption phenomenon of CSF was studied in **Chapter 6**. Various laboratory materials were tested to evaluate CSF adsorption in each material. Furthermore, the impact of the pH value of the solution (acidic or alkaline) and the addition of organic modifiers (acetonitrile and methanol) were studied to clarify in which conditions adsorption could be avoided or at least, minimized. Samples were analysed freshly prepared and after 24 h at room temperature. Huge differences were found when using different laboratory materials and dissimilarities within plastic types were noticed. Acidic pH was established as the best condition to avoid adsorption in aqueous solution. The addition of organic solvents was also positively evaluated for adsorption avoidance.

Finally, using a procedure already reported in bibliography for reduction of non-specific adsorption of drugs to plastic containers, different coating agents were tested for material (glass vials and plastic 96-well plates) treatment. The efficacy of the three agents for adsorption avoidance assayed was evaluated. One of the agents, the N-(2-Aminoethyl)-3-aminopropyltrimethoxysilane (AATMS), shown positive results. AATMS use underwent a decrease of the adsorption kinetic of CSF allowing

its reliable quantification. However, the treatment was not sufficient to avoid adsorption completely and the effect of time was perceptible.

To summarize, results obtained shown the difficulties of working with CSF for *in vitro* studies (human body conditions: pH around 7 and in aqueous solution) due to the adsorption phenomenon. This research provides a new perspective to assess the results obtained previously in microbiological and therapeutic fields and, supports researchers to go deeper in the evaluation of the reliability of the methodologies used for resistance determination and drug administration.

Irudien zerrenda / List of figures

CHAPTER 6:

Taulen zerrenda / List of tables

1go Kapitulua

Sarrera

Gizakiaren immunitate sistema ahulduta dagoenean agertzen diren infekzioei, infekzio oportunistak deritze. Urteak pasa ahala, infekzio oportunistetatik infekzio fungikoak nabarmenki hasi dira mundu mailan. Infekzio fungikoak bi taldetan banatu ahal dira: superfizialak eta inbaditzaileak. Superfizialen artean azalekoa, azazkalekoa, ilekoa edo mukosetakoak aurkitzen dira. Infekzio horien kasu kopurua altua da mundu mailan, azaleko, azazkaletako edo ileko infekzio fungikoak bilio bat pertsonak sufritzen dituzte, adibidez. Infekzio fungiko inbaditzaileak (IFIak) sufritzen dituzten pertsonen kopurua txikiagoa izan arren (millioi bat), infekzio hauek garrantzitsuenetarikoak bihurtu egin dira azken urteetan [1-5].

Garatutako edo garatzen ari diren herrialdeetan IFIei bidea errazten dioten zenbait gaixotasun identifikatu egin dira, hots, IHESA, minbizia, neutropenia, tuberkulosia edo asma aurkitu ahal dira horien artean [1,6,7]. Horrela izanik, IFI ezberdinak paziente larri eta immunitate arazoak dituztenen artean ematen dira batez ere [6] eta horiei erlazionatutako erikortasuna eta hilkortasuna dela eta, batzuetan % 50era heltzen dena, mundu osoko espezialistak arduratuta daude [2,4,7].

IFIen erantzule nagusiak *Aspergillus, Cryptococcus* eta *Candida* onddo generoak dira [2,4,5]. *Aspergillus* espezieen hilkortasuna mundu mailan aurretiaz % 30 - 80 zen, eta azken urteetan jaitsi izan arren, oraindik % 20koa da. Era berean, *Cryptococcus* espezieek % 20 - 30ko hilkortasuna erakusten dute osasun sistema on bat duten herrialdetan, baliabide gutxikoetan ordea, hilkortasuna % 50 baino gehiagora heldu daiteke. Azkenengoz, *Candida* spp.-ek % 30 – 40eko hilkortasuna erakusten dute [1,5,7,8]. Izan ere, erakusten duen hilkortasun altua dela eta, kandidiasiak espezialisten arreta lortu du.

1. Kandidiasia

Kandida onddoaren edozein espeziek sortutako infekzio fungikoari kandidiasia deritzo. Oso ezaguna den infekzioa izan arren, azken hamarkadetan eman den kandidiasi nosokomialaren intzidentziaren gorakadak espezialisten aditasuna lortu du. Kandidiasi mota hori ospitalizatuko pazienteetan ematen den kandidiasi inbaditzailea (KIa) da, eta batez ere zainketa intentsiboen unitatean (ZIUn) dauden pazienteen artean ikusi ohi da [1,9-11]. Ospitalizatutako populazioan infekzio mota ohikoenak infekzio intra-abdominalak, kateterren erabilerarekin erlazionatutakoak eta odol-korronteko infekzioak dira. Paziente kritikoetan ordea, barreiatutako kandidiasia da erikortasun eta hilkortasun altueneko kausa, bai immunitate arazoak dutenen eta ez dutenen artean [12].

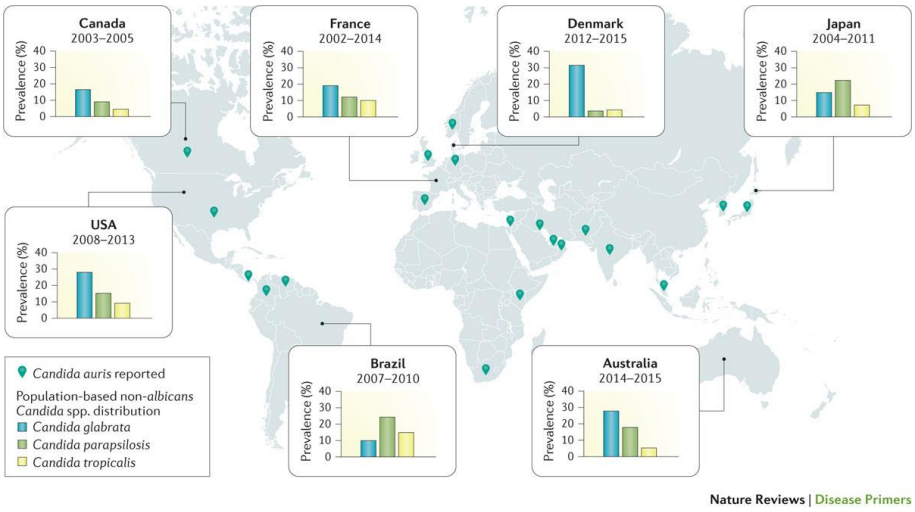
Kandidiasi inbaditzailearen hilkortasun-tasa orokorrean % 30 – 40koa dela aipatu da aurreko paragrafo batean. Hori orokorrean izanik, ospitalizatutako pertsonetan % 27 - 55 tarteko balioak lortzen dira [13]. Egia esan, 2018. urtean Pappas *et al*.-ek [8] zehaztu egin zuten nola hilkortasun-tasa orokorra txikiagoa izan daitekeen, % 10 - 20 tartekoa. Horrela izanik, hainbat faktorek hilkortasunarekin zuzenki erlazionatuta egon daitezkela aurkeztu zuten. Hala nola, adina (zenbat eta nagusiago, hilkortasun-tasa altuagoa), kandida espeziea, immunoezabatzaileen erabilpena …

Esan beharra dago kandida onddo endogeno bat dela. Beraz, gizakian berez kantitate txikietan bizi den onddoa da eta orokorrean ostalariarentzako ez kaltegarria da. Hala ere, batzuetan, ostalariaren eta patogenoaren arteko elkarrekintzan eman daitekeen aldaketa baten ondorioz, bizitza arriskuan jartzeko gai da onddo populazioaren hazkunde bortitz bat dela medio [4,13]. Gorputzean kantitate txikietan eduki ez ezik, iturri exogenoa ere eduki ahal du kandida infekzioak. Hala nola, aurretiaz aipatutako kateterren erabilerarekin erlazionatutako kasuak adibidez [4,14].

2. Kandida espezie nagusiak

Kandida generoan 60 espezie baino gehiago identifikatu egin dira historian zehar eta multzo horretatik 20 espezie dira gehien isolatzen direnak. Dena den, kandida infekzioen erantzule nagusiak bost dira: *albicans, glabrata, tropicalis, papapsilosis* eta *krusei* espezieak [1,4,15-20].

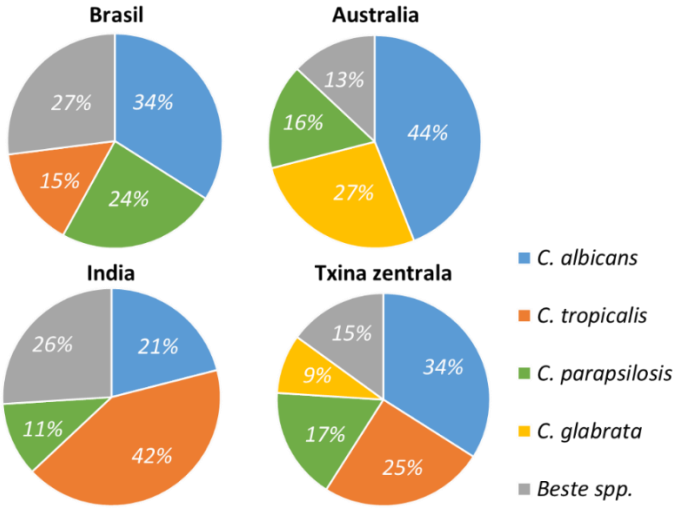
Munduan zehar kandida espezie nagusien distribuzioa ezberdina da. Herrialde guztietan isolatutako espezie ohikoena *C. albicans* dela aurkitu da, baina beste espezieen maiztasuna aldatzen da herrialde ezberdinetako epidemiologia aztertzean. Ez-*albicans* espezie nagusien (*C. glabrata, C. parapsilosis* eta *C. tropicalis*) distribuzioa azaldu egin zuten Pappas *et al*.-ek [8] 2018. urtean. Aurkeztutako informazioan oinarrituz (**1. Irudia**), orokorrean *C. glabrata* espezie ohikoena dela ikus daiteke baina Brasil eta Japonian *C. parapsilosis* espeziea da nagusiki isolatu egin dena.



1. Irudia: Kandida ez-albicans espezieen distribuzioa (Pappas *et al*.-en irudia [8])

Mundu mailan kandida espezieen distribuzio orokorra desberdina izanik, logikoa da KIaren erantzule diren espezieen distribuzioa ere aldatzea herrialdeetan zehar, **2. Irudian** ikus daitekeen bezala.

Horrela izanik, AEBetan, Australian eta Europan *C. glabrata*-ren kasu kopuruen hazkunde nabarmen bat ikusi da KIen bigarren erantzule nagusia suertatuz [21]. Afrika eta Latinoamerikan espezie distribuzioa apur bat aldatzen da, *C. parapsilosis* izanik gehien isolatutako espeziea *C. albicans*-aren ostean. Asian ordea, hainbat joera identifikatu dira Txina zentrala, Taiwan eta India konparatzean. Alde batetik, Taiwan-en Europan bezala, *C. glabrata-ren* hazkundea nabarmendu egin da [22]. Txinan ostera, *C. tropicalis* aurkitu da KIren *ez-albicans* espezieen arteko ohikoena [23]. Indian aurkitzen da distribuzio ezberdinena, non, *C. tropicalis* gehien isolatutako espeziea den, *C. albicans*-aren gainetik ere [22].



2. Irudia: Kandidiasi inbaditzailearen erantzule diren espezie nagusien distribuzioa hainbat herrialdetan [21-23]

C. *albicans, C. glabrata,* eta *C. parapsilosis* espezieak espezien konplexu kriptikoak direla aurkitu da. Hau da, espezie bakarra izan ordez, multzoak direla. Kriptiko hitzak grekotik datorren *kruptós* hitzean oinarritzen da, zeinek "ezkutatuta" esan nahi du, hain zuzen ere [24]. Konplexu hauek beste espezieetatik ugalketa prozesuari dagokionez isolatuta dauden espezie multzoak dira. Espezie bakar bat bezala hartzen dira askotan haien artean morfologikoki ezberdintzea ezinezkoa delako. Hori lortzeko DNA ikerketa bat erabili beharko litzateke, adibidez [17,24,25].

Orokorrean, infekzioen % 50 *C. albicans* konplexu kriptikoaren ondorio izaten jarraitzen dute [26,27]. Multzo horrek *C. albicans*, *C. dublimiensis* eta *C. africana* espezie kriptikoek osatzen dute. Ikerketa epidemiologiko batean ikusi zen, nola *C. dublimiensis*-ak isolatutako espezieen % 1,5 errepresentatzen zuen eta *C. africana* bulbobaginitisaren erantzule zela [28-33]. *C. albicans* konplexu kriptikoak sistema gastrointestinalean eta genitourinarioan aurkitzen den patogeno ez kaltegarri ohia da, populazioaren % 70ean aurkitu daitekelakoan [19]. Espezie ezberdinen artean multzo hau da infekzio sistemiko eta azalekoen erantzule nagusia.

C. albicans kultibo klinikoetan aurkitzen den espezie nagusia izan arren, *ez-albicans* espezieen kasuek gora egin dute. Modu horretan, *C. glabrata* gehien isolatutako espezieen bigarren postuan aurkitzen da [27,34]. Espezie hori, adin altuko pazienteen hemokultiboetan isolatzen da orokorrean, 65 urte baino gehiagokoetan, *C. tropicalis*-a eta *C. krusei*-arekin batera [33,35]. *C. glabrata* konplexuak, *C. glabrata, C. bracarensis* eta *C. nivarensis* espezie kriptikoz osotuta dago [17,34,36,37]. Konplexu kriptiko hau izango da lan honen ikerketaren helburu, Europan ematen ari den kasuen gorakada dela eta.

3. Kandidiasi inbaditzailearen diagnosia

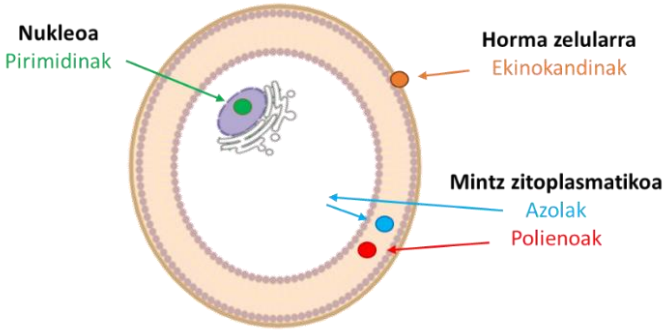
KIak ez du sintoma karakteristikorik, bere adierazpen klinikoa inflamazio sistemikoa baita. Horrela izanik, sukar txiki batetik shock septiko batera heldu daiteke. Hori dela eta, beste infekzioetatik ezberdintzea zaila da. Kandida gorputzean zehar barreiatu daiteke odola garraiobide erabilita eta infekzioaren larritasuna handitu ahal du (endorkarditisa edo endopalmitisa sortuz, edo, nerbio sistema zentrala kaltetuz) [16].

Gaixotasun honen laborategi diagnosia lagin esteril baten kultiboan oinarritzen da. Lagina odola, likido peritoneala edo likido pleurala izan daiteke. Orokorrean, 2 edo 3 egunez kultibatuz diagnosia egiteko hazkunde egokia lortzen da, hala ere, kultiboaren hazkundea 8 egunetaraino luzatu ahal da. Ondoren, espezieen identifikazioa komertzializatutako sistema ezberdinez egin daiteke [16]. Hala ere, eta urte askoz sail honetan ikertu arren, diagnosi ikerketa gutxi dira eskuragarri eta fidagarriak [27].

Laborategiko ikerketen bidez diagnosia aurrera eramatea zaila dela ikusi denez, klinikoki modu azkar batean lortzea garrantzitsua da. Kontuan hartu behar da, terapiaren eraginkortasuna diagnosiaren abiaduraren menpekoa dela [27]. Espezialistek KIaren diagnosia hurrengoetan oinarritzen dute: alde batetik, sintomak eta laborategiko odol analisiak infekzio batekin egokitzea, eta bestetik, arrisku-faktoreak edukitzea eta espektro zabaleko antibiotikoen eraginik ez ikustea. Baldintza horiek betetzen dituen paziente baten aurrean egotean, nahiz eta onddo espeziaren identifikazioa ez eduki, KIaren diagnosia egin daiteke farmako egokia eman ostean erantzun positiboa ikusten bada [27].

4. Kandidiasiaren tratamendurako farmakoak

Hainbat farmako familia erabili dira kandidiasiaren tratamendurako historian zehar. Farmako familiak onddoen zelularen zein punturi (**3. Irudia**) eta nola erasotzen diotenen arabera sailkatu ahal dira. Horrela izanik, lau talde nagusi daude kandidiasi inbaditzailearen tratamendurako: azolak, polienoak, pirimidinaren analogoak eta ekinokandinak [38,39].



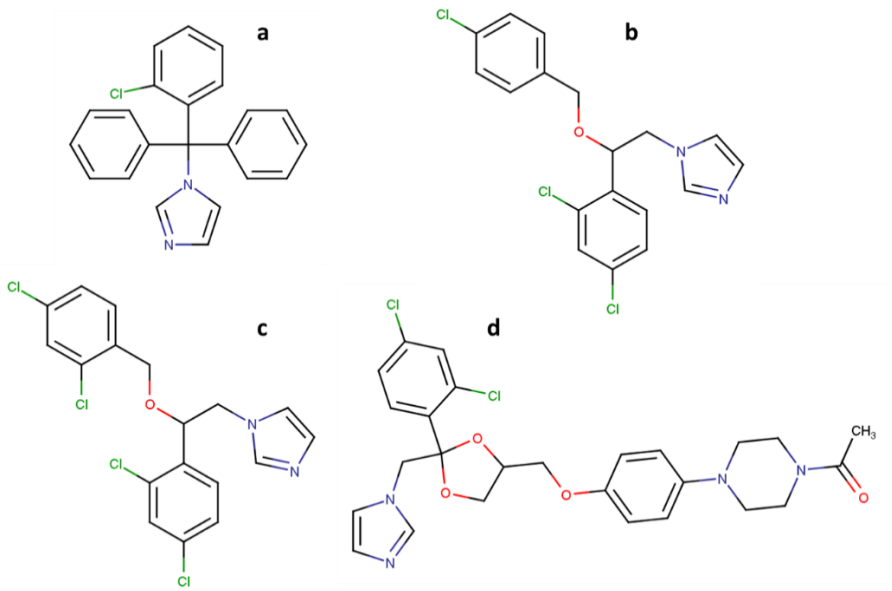
3. Irudia: Antifungiko familia garrantzitsuenen akzio puntuak zelulan

4.1 Azolak

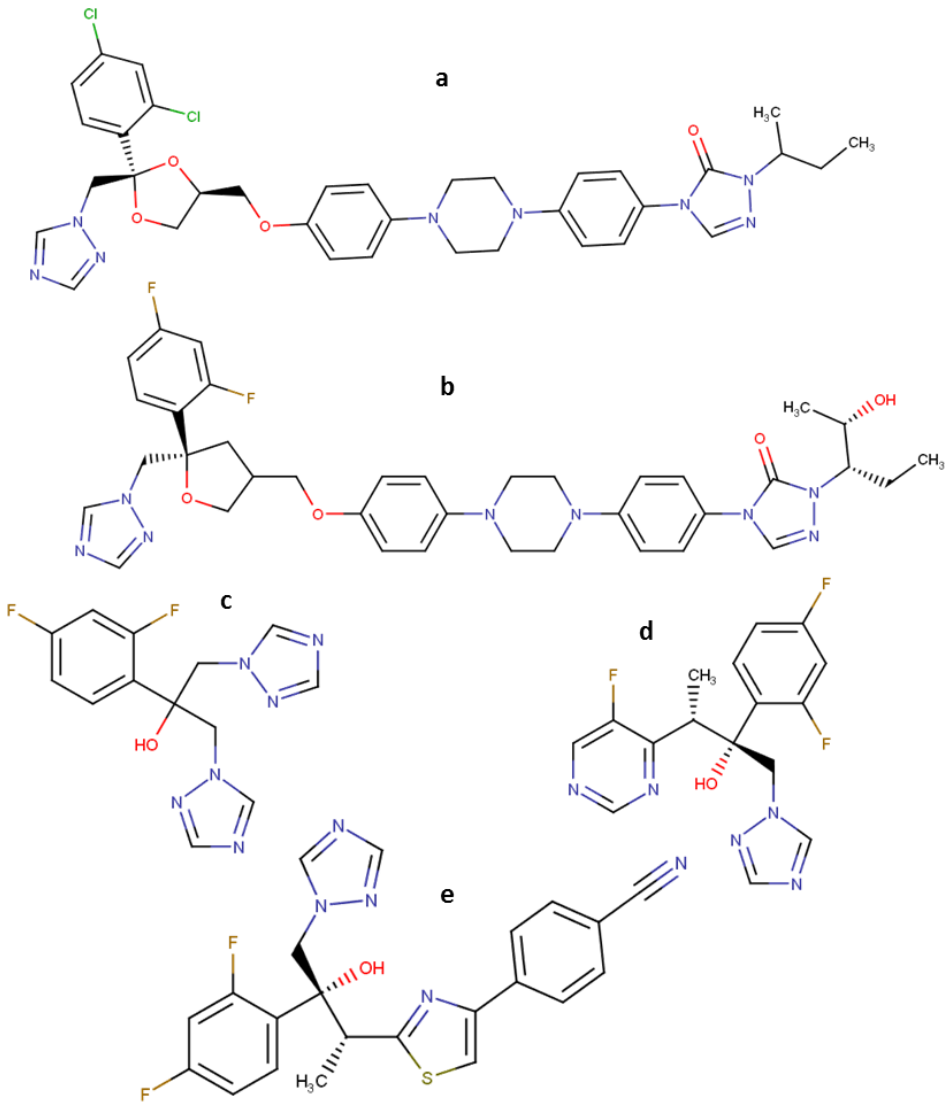
Droga familia honen akzio mekanismoa ez dago argi. Orain arte, zitokromo P450-ren menpe dagoen lanosterol 14α-dimetilasa inhibitzen zutela pentsatzen zen, lotura ez-konpetitibo baten bidez. Zehazki, azolak entzimaren zati ferrikora lotzen zirela uste zen entzimaren substratuaren lotura oztopatuz [40]. Lanosterol 14α-dimetilasak, lanosterolaren 14α-metil taldearen kanporaketa ahalbidetzen du. Pausu hau, ergosterolaren (onddoen mintz zitoplasmatikoaren konposatu nagusia) biosintesirako beharrezkoa da [41-43]. Azken urteetan ordea, farmako hauen mekanismoa inhibizio konpetitibo batean oinarritu ahal dela ikusi da [43]. Hala izanda ere, ondorioa parekoa da. Biak ergosterolaren sintesian eragiten dute eta horrela, zelulen hazkundea eta ugaritzea oztopatzen dute.

Ikuspuntu kimiko batetik farmako hauek bost atomoko eraztun azolikoa dute oinarritzat eta, bertara, albo-kate karbonatu konplexu bat dute lotuta [44]. Azolak bi taldetan banatu ahal dira eraztun azolikoan dauden nitrogeno kopuruaren arabera. Alde batetik, imidazolak aurkitzen dira, eraztunean bi nitrogeno dituztenak. Giza tratamendurako onartutako imidazolak klotrimazola, ekonazola, mikonazola eta ketokonazola dira (**4. Irudia**). Bestetik, eraztunean hiru nitrogeno atomo dituztenak aurkitzen dira, triazolak [41,42]. Talde horretan itrakonazola, posakonazola, flukonazola, borikonazola eta rabukonazola aurkitzen dira (**5. Irudia**).

Iraganean imidazol batzuk tratamendu sistemikorako erabili izan arren, gaur egun erabilera topikoa dute erakutsitako toxizitatea dela eta [42,45]. Horrela izanik, infekzio sistemikoen tratamendurako triazolak erabili ohi dira [41,42]. .



4. Irudia: Imidazol nagusien egitura kimikoak; a) klotrimazola, b) ekonazola, c) mikonazola eta d) ketokonazola



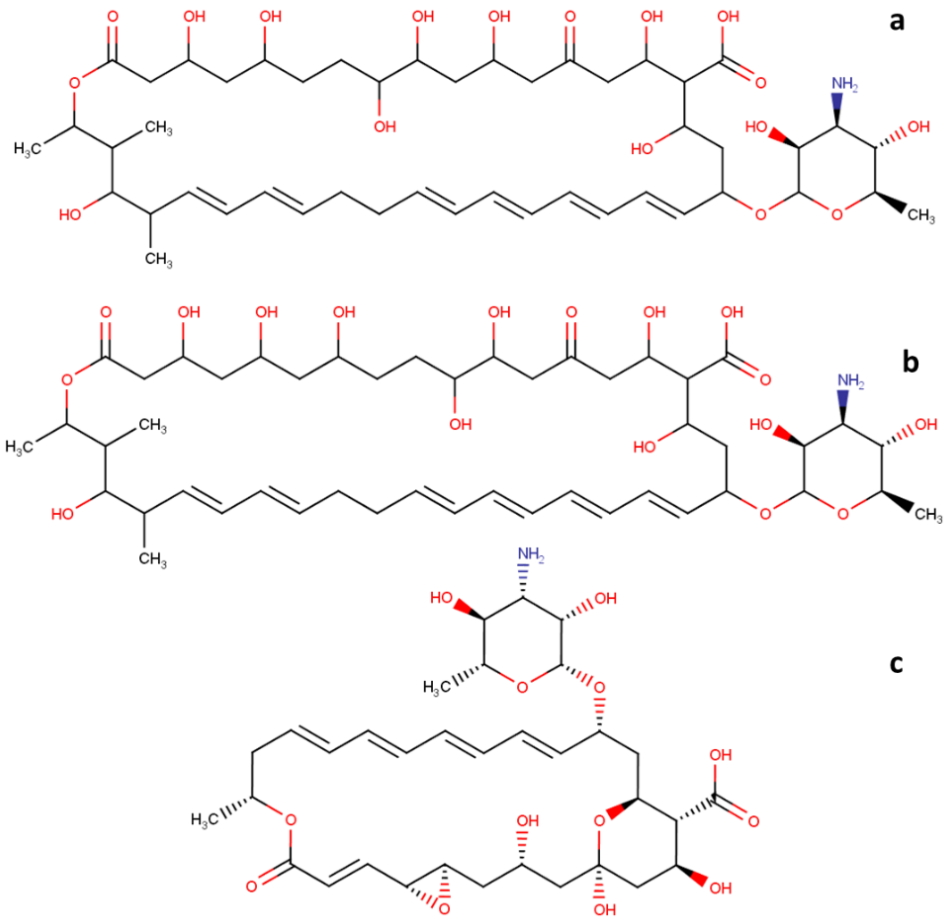
5. Irudia: triazol nagusien egitura kimikoak; a) itrakonazola, b) posakonazola, c) flukonazola, d) borikonazola eta e) rabukonazola

4.2 Polienoak

Farmako hauen akzio mekanismoa ere ergosterolarekin erlazionatuta dago. Kasu honetan, polienoak mintz zelularretan dauden ergosterol molekulei (**2. Irudia**) lotzen dira mintzean poroak sortuz [38,42,46]. Poro horiek oreka ionikoaren galera sortarazten dute mintzaren osotasuna kaltetuz eta, azkenengoz, zelularen heriotza eragiten dute [42].

Polienoak molekula natural organikoak dira eta orokorrean *Streptomyces* bakteriak jariatzen ditu [42]. Droga hauek makrolido izena ere hartzen dute laktona eraztun makrozikliko bat dutelako. Familia honetan aktibitate antifungikoa duten 200 molekulen artean, hiruk bakarrik erabili ahal dira klinikoki besteen toxizitatea altuegia delako [38,42]. Hiru farmako horiek anfoterizina B, nistatina eta natamizina dira (**6. Irudia**).

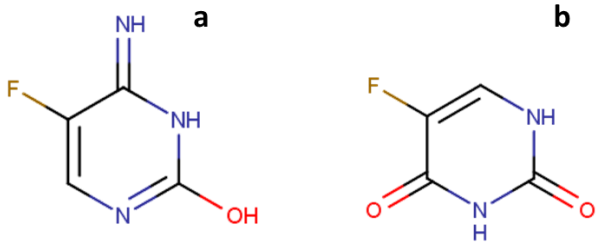
Infekzio sistemikoak tratatzeko anfoterizina B erabili ohi da, nistatina eta natamizina erabilera topikoa duten bitartean. Konposatu hauek berez aktibitate antifungikoa izan arren, albo-efektu asko dute. Horregatik, anfoterizina B-n oinarritzen diren farmako berriak (lipido formulazioak) garatzen ari dira [42].



6. Irudia: Polieno familiako farmakoen egiturak: a) anfoterizina B, b) nistatina eta c) natamizina

4.3 Pirimidinaren analogoak

Talde honetan 5-fluorozitosina, edo 5-fluzitosina, (5FCa) eta 5-fluorouraziloa (5FUa) aurkitzen dira (**7. Irudia**). Farmako horiek zitosinaren analogoak dira eta haien akzio mekanismoa inhibizio konpetitiboan oinarritzen da, zehazki, zitosinarekin konpetitzen dute RNAren sintesian (zelularen nukleoan, **3. Irudia**). 5FCak onddoen zelula horma pasatzen du, non zitosina deaminasak, farmakologikoki aktiboa den 5FU molekulan bihurtzen duen [38,41]. Ondoren, 5FUa zelularen nukleoan sartzen da eta bertan proteinen sintesia blokeatuz edo DNAren erreplikazioa inhibituz zelularen funtzionamendua oztopatzen du [46].



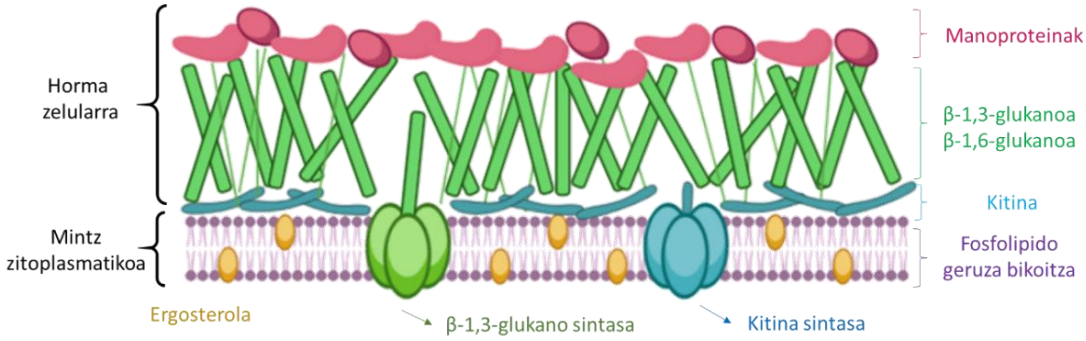
7. Irudia*:* Pirimidinaren analogoen farmakoen egiturak: a) 5-fluorozitosina eta b) 5-fluorouraziloa

5FCak bioerabilgarritasun handia izan arren, gorputzean azkar absorbatzen da, albo-efektu asko ditu. Orokorrean albo-efektuak arinak dira, baina, noiz behinka, larriak ere eman dira, hepatotoxizitatea garatuz, adibidez [38]. Gainera, onddo zelulak askotan tolerantzia garatzen dute. Horregatik farmako hau, orokorrean, beste antifungiko batzuekin batera erabiltzen da terapia konbinatuetan, adibidez, lehen aipaturiko anfoterizina B polienoarekin. [38,42,44].

4.4 Ekinokandinak

Onddoen infekzioen borrokan garatutako azkenengo farmako familia, ekinokandina familia da. Momentu honetan KIaren aurrean gomendatutako tratamendua ekinokandinen erabilpenean oinarritzen da, paziente neutropeniko eta ez-neutropenikoetan [15,47-49]. Paziente neutropenikoek globulu zurien mota baten (neutrofiloak) kontzentrazio eskasa duten pertsonak dira, horren ondorioz, infekzio larriak garatzeko erraztasuna daukate. Farmako familia honen garrantzia dela eta, hau izango da lan honen helburu.

Ekinokandinen akzio mekanismoaren itua onddoen horma zelularra da, polisakaridoz konposatutako egitura zurruna dena [50]. (1➔3)-β-D-glukanoa, (1➔4)-β-D-glukanoa, (1➔6)-β-D-glukanoa, kitina eta galaktomanana (manoproteina) osatzen dute nagusiki horma zelularra (**8. Irudia**). Ekinokandinak (1➔3)-β-D-glukan sintasara lotzen dira (1➔3)-β-D-glukanoaren sintesia inhibituz. Honen ondorioz, horma zelular anormala sortzen da zeinek, ezinezkoa duen presio osmotikoa jasatea eta, azkenean, zelularen lisia sortarazten duen [44,47,51].

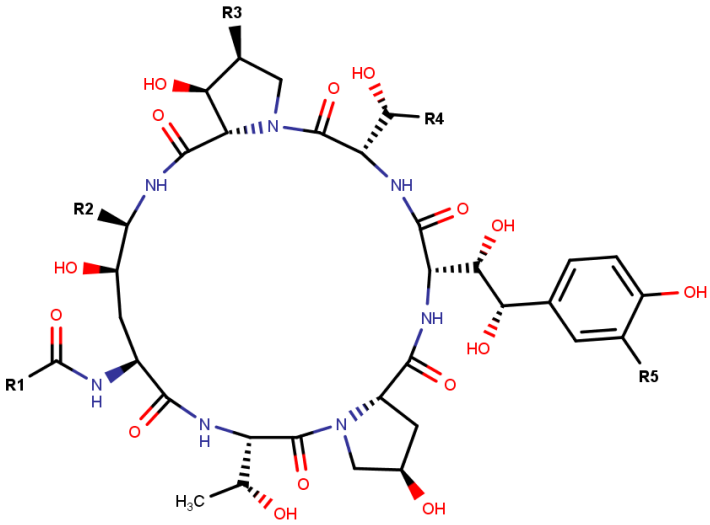


8. Irudia: Horma zelularraren eta mintz zitoplasmatikoaren egiturak

Farmako hauen abantaila bat da efektua aurresateko aukera ematen dutela, terapiaren arrakasta onddoek duten glukano proportzioaren menpekoa izango baita. Horrela, glukano proportzio altua duten onddoetan, *C. albicans* eta *Aspergillus fumigatus* onddoetan adibidez, efektu eraginkorragoa izango dute [47]. Ez hori

bakarrik, kontuan hartuta gizakion zeluletan ez dagoela horma-zelularrik, ekinokandinek, beste antifungikoekin konparatuz, albo-efektuen agerpenaren arrisku gutxiago erakusten dute [42,44,50,51]. Haien toxizitate baxua dela eta, infekzio inbaditzaile eta sistemikoak tratatzeko erabiltzen dira [50].

Droga horiek hainbat onddoen hartziduratik lortzen diren lipopeptido semisintetiko luzeak dira [48]. Familia honetako molekulen egiturak 6 aminoazidoz konposatutako ziklo baten oinarritzen dira (**9. Irudia**). Ziklo horretara lotuta azilo albo-kate karbonatu bat dute, N-lotura baten bidez [50]. Farmakoen arteko ezberdintasun nagusia albo-kate horretan aurkitzen da (**9. Irudia, R1**).



9.Irudia: Ekinokandina farmakoen egituraren oinarria. Molekulen arteko ezberdintasunak R1, R2, R3, R4 eta R5 puntuetan lotutako taldeetan aurkitzen da

1974. urtean ekinokandinen molekula aitzindaria identifikatua izan zen, ekinokandina B [47]. Konposatu horretan oinarriturik garatutako lehenengo konposatuak neumokandina A_0, azuleazina, kriptokandina eta mulundokandina izan ziren eta kandidaren aurrean eraginkorrak ziren. Hala ere, erakusten zuten propietate hemolitikoak direla eta (globulu gorrien desintegrazioa sortaraztea), ez ziren giza tratamendurako proposatu [52]. Ekinokandina B eta neumokandina A_0-ren

deribatizazioa eginez haien ezaugarri hemolitikoak jaistea lortu zen bioaktibitatea mantenduz [52].

Horrela izanik, giza tratamendurako garatzen hasi zen lehenengo ekinokandina zilofungina (LY121019) izan zen [53,54]. Hau *Aspergillus* onddoaren hartziduratik lortutako molekula semi sintetikoa da. Baina, erabiltzen zen farmakoaren disolbatzaileak (polietilenglikolak) sortzen zituen nefrotoxizitate eta azidosi metabolikoak direla eta, garapen klinikoa bigarren fasean bertan behera utzi zuten [53].

Giza tratamendurako onartutako ekinokandina farmako familia, egun, hiru farmakok osatzen dute; kaspofunginak, mikafunginak eta anidulafunginak. Laugarren bat garapen klinikoan aurkitzen da, rezafungina, hain zuzen ere [48].

4.4.1 Kaspofungina (CSF)

L-743 827 edo MK-0991 kodeekin ere ezagutzen den farmakoa, onartutako lehen ekinokandina izan zen, 2001. urtean Elikagaien eta Sendagaien Administrazioagaitik (FDA) [48,55,56]. Farmako hau, *Glarea lozoyensis* onddoak produzitzen duen neumokandina B_0-tik lortzen den konposatu semisintetikoa da [53,57].

Giza tratamenduan 70 mg CSF hartzen dira karga dosi lez ordu batean zehar zaineko administrazioaz baliatuz. Ondoren, 50 mg/egun-eko dosi erregimena jarraitzen da [48].

4.4.2 Mikafungina (MCF)

Mikafungina FK-463 kodearekin ere ezagutzen den farmakoa da [58,59]. 2005. urtean onartutako farmakoa, *Coleoma empetri* onddotik eratorriko neumokandina A_0 bat da [47,60].

CSFn ez bezala, MCFaren erabileran giza helduentzako eta umeentzako dosi erregimenak ezberdinak dira. Umeen kasuan pisua kontuan hartzen da. Hori horrela izanik, karga dosia 1,5 mg/Kg-koa da eta ondoren 1 – 4 mg/kg/egunero hartzen dira. Helduentzako, ordea, 100 g/egunero hartzen dira karga dosirik gabe [48].

4.4.3 Anidulafungina (ADF)

FDAk anidulafungina, LY-303366 ere deitua, 2006. urtean onartu zuen eta *Aspergillus nidulans* onddoaren hartziduratik eratorriko lipoproteina da [47,60,61].

Giza tratamendua ADFrekin denean, umeek 1,5 mg/kg-ko dosia hartu behar dute egunero, 3 mg/kg-ko karga dosia hartu ostean. Helduentzako, 200 mg-ko karga dosia gomendatzen da, 100 mg/eguneroko dosiak jarraituz [48].

4.4.4 Rezafungina (RZF)

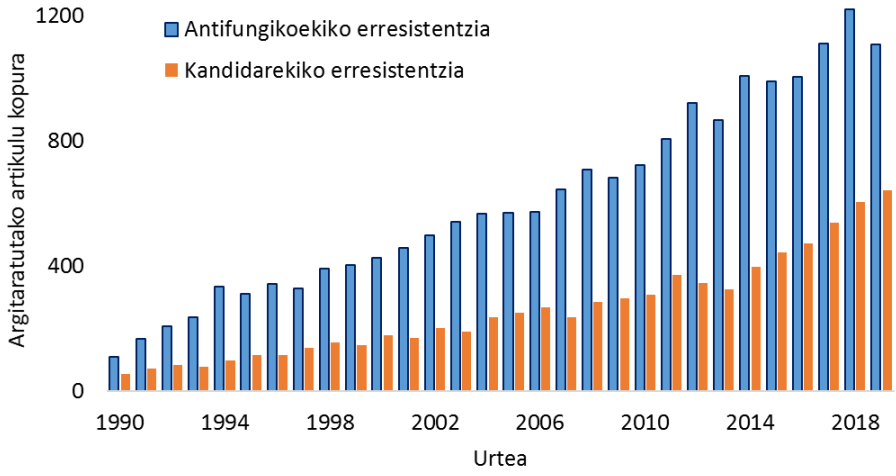
CIDARA Therapeutics enpresak garatzen ari du eta momentu honetan ikerketa klinikoaren hirugarren fasean aurkitzen da [62]. CD-101 edo biafungina izenekin ere ezagutua da RZF [48].

Farmako hau ADFren analogoa da baina ziklo peptidikoari lotuta (**9.Irudia**) kolina talde bat dauka, alkoholaren ordez (**9.Irudia**, R2). Aldaketa horrek, kimikoki eta metabolikoki egonkorragoa izatea eragiten du [48,63-65].

Familiako beste drogekin konparatuta, zain administrazioko dosiak astero hartzeko diseinatuta daude eta azaleko administrazioa posible egingo duela dirudi [65,66]. 400 mg-ko karga dosi bat eta ondoren, 200 mg/astero da momentuz proposaturiko dosi erregimena [62].

5. Antifungikoekiko erresistentzia

Kandidiasia tratatzeko hainbat farmako egon arren, haien gehiegizko erabilpena dela eta, urteak pasa ahala terapien eraginkortasuna txikitzen joan da. Kandida onddoak farmakoei galdutako sentikortasuna da honen erantzule. Antibiotiko baten aurrean patogenoak eduki ahal duen erantzunen arabera, hiru zepa mota ezberdindu ahal dira: zepa sentikorrak, erdi-mailako sentikortasuna duten zepak eta zepa erresistenteak [67]. Azken horiei, farmako baten kantitate terapeutiko baten aurrean, ugaritu daitezkeen onddo zepei deritze [40]. Antifungikoekiko erresistentziaren garapenaren larritasuna dela eta, ikertzaile askok hori haien ikerkuntzen helburu bihurtu dute. **10. Irudian** ikus daiteke nola antifungikoekiko erresistentziari buruzko argitaratutako artikulu kopurua nabarmenki igo da urteak pasa ahala. Argitalpen horien erdia, gutxi gora-behera, kandidaren erresistentziari buruzkoak diren.



10. Irudia: Antifungikoen erresistentziari buruzko eta kandidaren erresistentziari buruzko argitalpen kopuruaren aldaketa urteetan zehar (Datu-basea: PubMed. Hitz-gakoak: “antifungal resistance” eta “candida resistance”. Bilaketa data: 20/04/2020)

Erresistentziaren garapena prozesu konplexua da eta faktore askoren menpekoa da, hala nola, ostalariaren sistema immunologikoa eta kateterren edo beste tresna kirurgikoen erabilpenak (tresnerian onddo populazioak sortu ahal dira, biofilmak deritzenak), beste batzuen artean [40].

Gutxienez, bi erresistentzia mota ezberdindu ahal dira onddoetan [38,68-71]. Alde batetik, lehen mailakoa erresistentzia, berez patogenoek antifungiko batzuei dioten erresistentzia. Bestetik, bigarren mailako edo eskuratutako erresistentzia aurkitzen da [40], genoman eman ahal diren aldaketei edo mutazioei esker garatzen den erresistentzia.

Onddoek erresistentzia garatzean, orokorrean, hiru estrategia jarraitzen dituzte [38]:

1. **Motako estrategia:** Zelulan ematen den farmakoaren akumulazioaren murrizpena
2. **Motako estrategia**: Farmakoaren helburuarekiko afinitatearen murrizpena
3. **Motako estrategia:** Metabolismoaren aldaketa drogaren efektua orekatzeko

Hau guztia kontuan hartuta, aurretiaz aipatutako antifungiko familietan ematen diren erresistentzia mekanismoak azalduko dira hurrengo paragrafoetan, estrategia mota ere aipatuz.

5.1 Azolak

Azolekiko erresistentziaren garapenak gorakada esanguratsua eman zuen orain dela 30 urte onddo ezberdinetan, eta sakonki ikertutako gaia da. Zorionez, farmako berrien garapen eta erabilerak erresistentzia horren garapena moteldu egin du [38].

Tesi honetan esan den arabera, azolek ergosterolaren biosintesia dute haien akzio mekanismoaren helburu. Aditu gehienen ustean lanosterol 14α-dimetilasaren (Erg11 geneagatik kodifikatuta legamietan eta Cyp51A/Cyp51B lizunetan) inhibizio ez-konpetitiboa dela medio ematen da mekanismo hori [40,68].

Aipatutako hiru estrategien molekularren arabera, azolekiko erresistentzia mekanismoak hurrengoak dira:

1. Motako estrategia: Droga garraiatzaileen erregulazioaren gorakada. Kandida espezieen erresistentzia mekanismo nagusia kontsideratzen da [44], CDR1 eta CDR2 deituriko geneen gainespresioa dela medio [38,44,68,72]. Modu honetan, droga zelula barnetik kanporatzeko gaitasunak gora egiten du eta farmakoaren efektua murrizten du [46].

2. Motako estrategia: Erg11 genearen mutazioa. Gene horretan arginina bat lisina batengatik aldatzean azolek entzimarekiko erakusten duten afinitatea murrizten duela ikusi da, eman ahal den egitura edo funtzio aldaketagatik [38,46,68,72].

3. Motako estrategia: Erg11 edo Cyp51A/Cyp51B geneen gainespresioa. Mekanismo honen erabilgarritasuna kandidaren erresistentzian, kolokan jarri zen lehenengo erresistentzia ikerketetan. Adituek ikusi zuten, nola, orokorrean mekanismo hau beste batekin batera garatzen zen [46,71]. Horrela izan arren, urteak pasa ahala, ikusi da nola gainespresio hori zepa erresistente gehienetan garatzen da [68,72].

5.2 Pirimidinaren analogoak

Farmako familia honetan erresistentzia oso ohikoa da, lehen mailakoa *C.tropicalis*-ean, adibidez, edo bigarren mailakoa beste kandida espezie batzuetan gertatzen den bezala [38,41,44]. Esan beharra dago erresistentziarik ez dela momentuz deskribatu *C. albicans* espeziearentzako [44].

Pirimidinaren berreskurapen-bidezidorrean parte hartzen duten entzima askok 5FC-aren akzio mekanismoan ere erlazionatuta daude. Hori dela eta, erresistentzia garatzeko mekanismo molekular ugari eman ahal dira entzimen mutazio edo aldaketak direla medio [38].

Pirimidinaren analogoetan deskribatutako erresistentzia mekanismoak hurrengoak dira:

3. Motako estrategia: Mutazioa FUR1 genean. Gene hori, 5FU eta 5FC farmakoekiko erresistentziaren erantzulea da, 5FU-a zitosinaren metabolismoan sartzeko gai diren metabolitoetan eraldatzen baitu [38,41].

Mutazioa FCy1 genean. Zitosina deaminasa kodifikatzen duen genea, 5FC-a 5FU-an eraldatzearen erantzulea da [38,41].

Mutazioa edo aktibitate galera FCy2 genean. Mekanismo hau lehen mailako erresistentziaren erantzule da [44]. Zitosina permeasa entzima kodifikatzen duen gene horren eraldaketak, drogaren absortzioaren murrizpena sortarazten du [41].

5.3 Polienoak

Familia honetan erresistentzia baxu mantentzen da zenbait kasu argitaratu izan arren. Orokorrean, onddo espezie gehienak sentikorrak dira farmako hauekiko. Dena den, aipatu beharrekoa da, *C. glabrata* espezieak lehen mailako erresistentzia erakusten duela polienoen aurrean [38,46,71].

Polienoen bigarren mailako erresistentzia mekanismoak zelulen esterol kantitatearen aldaketa kualitatibo zein kuantitatiboan oinarritzen dira. Horrela izanik:

3. Motako estrategia: Ergosterol kantitatearen aldaketa. Gehien deskribaturiko mekanismoa ergosterolaren sintesiaren murrizketan oinarritzen da. Horren kausaz, beste esterol batzuen sintesia bultzatzen da. Mintz plasmatikoan aurkitu ahal den ergosterol kantitatea gutxitzean edo guztiz ezabatzean datza. Horretarako, oinarrizkoak ez diren geneen aldaketak ematen dira. Modu horretan, ergosterola ezabatuz polienoen akzio mekanismoaren helburua ezabatzen da [38,46,71].

5.4 Ekinokandinak

Ekinokandinekiko erresistentzia ez-ohikoa da kandida espezie gehienetan [38,71,73]. Salbuespena *C. glabrata* da, non, ekinokandinekiko erresistentzia kasuak gora egin duten, terapia klinikoen hutsegiteak sorraraziz. Batez ere, CSF tratamenduekin gertatu da [41]. Hori dela eta, tesi hau, *C. glabrata*-k CSFrekiko duen erresistentzian ardazten da.

Ekinokandinen kasuan ez da farmakoaren akumulazioaren kontrako mekanismorik deskribatu (1. Motako estrategia) [41]. Beraz, aurretiaz zaldutako hiru mekanismoetatik, bi bakarrik garatzen dituzte kandida zepek ekinokandinekiko:

2. Motako estrategia: : FKSan emandako polimorfismoak. FKS geneak (FKS1 eta FKS2) glukanoaren sintesiaren erantzule den konplexu entzimatikoa kodifikatzen dute. Literaturan, FKS genetan naturalki gertatzen diren polimorfismoak deskribatu dira. Hauen ondorioz, akzio mekanismoarekiko afinitatea eta onddoen sentikortasuna ekinokandinekiko jaisten da [41,73].

3. Motako estrategia: Estrategia honetan bi mekanismo deskribatu dira ekinokandinentzako. Alde batetik, amino azido aldaketa FKS azpiunitateetan. Ekinokandinen kasuan, glukan sintasa entzimaren FKS1 eta FKS2 genetan ematen diren mutazio puntualak dira erresistentziaren arrazoi nagusia [68,71,74]. Mutazio hauek entzimaren zinetika aldatzen dute. Horren ondorioz, inhibizioa lortzeko beharrezkoa den farmako kontzentrazioak %50eko gorakada jasaten du [73]. FKS1ean mutazioak *C. albicans, C. tropicalis, C. krusei* eta *C. glabrata* espezieetan deskribatu dira, FKS2an ordea, soilik *C. glabrata*-n [75].

Bestetik, Kitinaren gainespresioa ere deskribatu da. Kitina eta glukanoak horma zelularraren egiturazko osagai nagusiak direnez (8. Irudia), kitinaren proportzioa handitzean, ekinokandinen efektua horma zelularraren egituran gutxitzen da [44,73,76,77].

Deskribaturiko erresistentzia mekanismo hauek guztiak direla eta, kandidiasia tratatzea gero eta zailagoa da, zepa erresistenteen sorrerak terapien aldaketak eskatzen baitituzte. Horri erantzuna emateko zenbait alorretan lanean ari da komunitate zientifikoa.

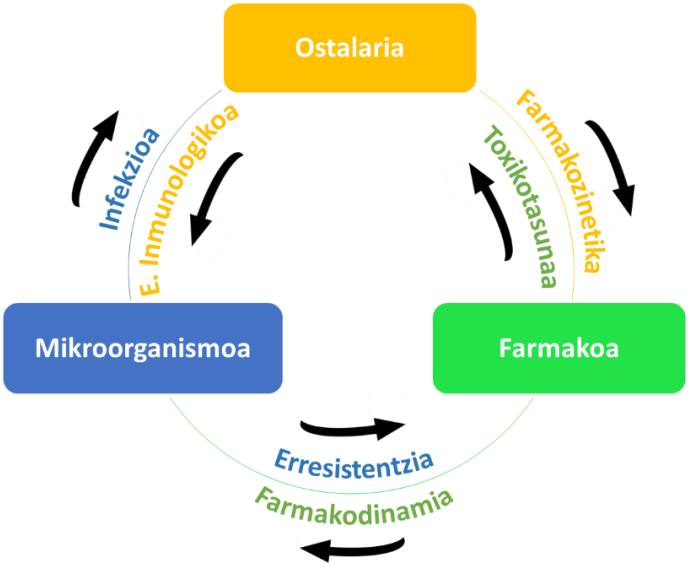
Alde batetik, farmako berrien garapena etengabe aurrera egiten ari du, adibidez, azolen familiako otesekonazola. VT-1161 kodearekin ere ezagutzen den farmakoa mycovia Pharmaceuticals-ek garatzen ari du, momentu honetan. Farmako horrek azolen familian talde berri baten sorpena ekarriko du, tetrazolak (bost atomoko eraztunean 4 nitrogeno dituzten farmakoen taldea). Momentu honetan, garapen klinikoko hirugarren fasean aurkitzen da kandidiasi bulbobaginalaren tratamendua tratatzeko helburuarekin [78-81]. Baita, aurretiaz aipaturiko rezafungina (CD101), garatzen ari diren farmakoen artean aurkitzen da. Hau, Cidara Therapeutics garatzen ari du. Garapen klinikoko hirugarren fasean egonik kandidiasi inbaditzailearen tratamendurako frogatzen ari da, beste erabilera batzuen artean [62,82].

Bestetik, drogen administrazio bide berriak garatzen ari dira. Estrategia honen helburu nagusia dosien kantitatea eta maiztasuna txikitzea da. Talde honetan aurkitu ahal dira nano-partikuletan edo mintz lipidikoetan oinarritutako farmakoen garraio sistemak [83,84].

Hala eta guztiz ere, ikerkuntzek aurrera doazen bitartean, espezialistek egun agertzen diren kandidiasiak tratatu behar dituzte. Data esperimental eta klinikoek ziurtatzen dute dosi erregimenek erresistentziaren garapenean eragina dutela [85]. Horrela izanik, adituek dosi erregimen berrien ebaluaketa proposatzen dute terapia eraginkorragoak lortu ahal izateko eta, era berean, erresistentzia garapena gelditzen saiatzeko.

6. Dosi erregimen berrien ikerkuntza

Infekzio bati aurre egiteko terapiaren arrakastan hainbat elkarrekintzek hartzen dute parte. Izan ere, mikroorganismoaren, drogaren eta ostalarien arteko elkarrekintzak kontuan izan behar dira [86]. Elkarrekintza horien ondorioz sei interakzio bereizi ahal dira (**10. Irudia**). Horien artean farmakozinetika eta farmakodinamia aurkitzen dira, oinarrizko kontzeptuak dosi erregimenen ikerkuntza ulertu ahal izateko.



10. Irudia: Ostalari, mikroorganismo eta farmakoen artean ematen diren elkarrekintzak

6.1 Farmakozinetika (PK)

PK "gorputzak droga nola maneiatzen duen" moduan definitu ohi da, hau da, absortzio, distribuzio eta eliminazio prozesuek gorputzean dagoen farmako kantitateari nola eragiten dioten [85-87]. PKk drogaren dosifikazioa eta drogaren kontzentrazio-denbora profilaren arteko erlazioa deskribatzen du [85].

Farmako baten PK profila ondo simulatu ahal izateko (gorputzean gertatzen dena ahalik eta hoberen mimetizatzeko), hainbat parametro farmakozinetiko kontuan hartu behar dira.

PK parametro nagusiak hurrengoak dira [33,88-90]:

- **Banaketa bolumena (Vd)** (unitatea: bolumena)
  Gorputzean dagoen farmako kantitatea eta plasman dagoen kontzentrazioa erlazionatzen dituen parametroa da. Farmakoa disolbatzeko behar den bolumen teorikoa da, nahi den kontzentrazio plasmatikoa lortu ahal izateko.

- **Argitzapena (CL)** (unitatea: bolumena/denbora)
  Gorputzak, farmako bat eliminatzeko duen gaitasuna da. Askotan, plasma argitzapena (CL_p) edo odol argitzapena (CL_b) moduan ere definitzen da.

- **Kontzentrazio maximoa (C_{max})** (unitatea: kantitatea/bolumena)
  Farmakoak lortzen duen kontzentrazio plasmatiko maximoa da.

- **Erdi-bizitza denbora ($t_{½}$)** (unitatea: denbora)
  Plasman edo gorputzean dagoen farmako kantitateak %50-era gutxitzeko behar duen denbora da.

- **Bioerabilgarritasuna:**
  Akzio mekanismoaren helburura heltzen den aldaketarik gabeko farmako frakzioari deritzo bioerabilgarritasuna.

- **Kontzentrazio minimo inhibitzailea (MIC):**

  Patogeno baten hazkundea inhibitzeko behar den kontzentrazio minimoa da, antibiotiko edo antifungiko baten efektua ebaluatzeko erabiltzen den kontzeptua.

Farmako kontzentrazioa (C) plasman determinatzen da, orokorrean. Ekuazio esponentzial baten bitartez, denboran zehar ematen den C aldaketaren hurbilketa egin daiteke:

$$C = C_0 \, x \, e^{-k_{el} \, x \, t} \qquad (1)$$

Non, C_0 farmakoaren administrazioa amaitzean dagoen droga kontzentrazioa den, *t* denbora eta k_{el} eliminazio abiadura konstantea.

Berehalako distribuzioa eta CL balioa farmakoaren kontzentrazioarekiko menpekotasunik ez duten drogen kasuan, hurrengoa betetzen da:

$$K_{el} = \frac{CL}{Vd} \qquad (2)$$

6.2 Farmakodinamia (PD)

PD "droga batek gorputzari zer egiten dion" moduan definitu ohi da, hau da, gorputzak farmako baten aurrean ematen duen erantzuna [85,90,91]. Aldaketa fisiologikoek, mutazio genetikoek, adinak edota beste farmakoen interakzioak PDn eragina eduki ahal dute [91].

6.3 In vitro ereduak

Antibiotikoen PK/PD parametroen karakterizazioan *in vivo* (organismo bizidunean) egindako ikerketen emaitzak osatzeko eta indartzeko, hainbat *in vitro* eredu garatu dira [85]. Azken horiek, ingurune kontrolatu batean egiten diren esperimentuak dira, organismo bizidunetik kanpo, hain zuzen ere. *In vitro* PK/PD ereduak, PK eta PD kontzeptuak erlazionatzen dituztenak, dosi erregimenak eta efektu klinikoak konektatzeko erabilgarritasun handiko tresnak dira. Eredu horietan, farmakoa, kultibo zelularra eta onddoa kontaktuan jartzen dira ontzi batean eta denboran zehar onddoaren hazkundea aztertzen da. *In vitro* ikerketek aurrera eramateko erraztasun gehiago dute, *in vivo*-koekin konparatuz. Gainera, *in vitro* ereduak

erabiltzean animaliekin edo gizakiekin egiten diren ikerketek aurkezten dituzten etika arazoak ekiditen dira [85,92,93].

In vitro PK/PD ereduetan lortutako emaitzak, animalia eta gizakietan lortutako emaitzekin ondo bateratzen dira. Are gehiago, ostalari baten erantzun inmunologikoaren gabeziak (**9. Irudia**), orokorrean kontrako alde bat izango litzatekeena, primerakoa da paziente neutropenikoetan gertatzen dena simulatu ahal izateko [92].

in vitro sistemen klasifikazio orokor bat existitzen ez denez, Nielsen *et al.*-ek [85] erabilitakoa jarraituko da lan honetan. Horrela izanik, ontzian dagoen farmako kontzentrazioa konstantea bada "sistema estatiko" lez definituko da eta farmako kontzentrazioa aldatzen bada, "sistema dinamikoa" dela adieraziko da [94].

6.3.1 Sistema estatikoak

Sistema estatikoetan onddoa farmako kantitate batekin kontaktuan jartzen da eta ez da farmako kontzentrazio hori konstante mantentzen da denboran zehar. Horrek ez du esan nahi farmako kontzentrazioaren aldaketarik ez dela ematen, izan ere, farmakoa degradatu edo metabolizatu daiteke esperimentua aurrera joan ahala [85].

PK/PD sistema errazenak talde horretan kokatzen dira. Adibidez, denbora-heriotza ikerketak, non, erlenmeyer batetan kultibo zelularra, onddoa eta farmakoa jartzen diren eta 35 edo 37 ^{o}C-tan inkubazioa aurrera eramaten da. Inkubazio denboran zehar nahastea homogeneo mantendu ahal izateko, Erlenmeyerra irabiatze sistema batean kokatzen da. Kultiboaren kontaminazioa ekiditeko helburuarekin, aluminiozko papera dela medio, Erlenmeyerra estaltzen da. Denboran zehar zenbait laginketa egitea ahalbidetzen dute sistema horiek eta orokorrean 24 h baino gutxiagoko esperimentuak izaten dira.

6.3.2 Sistema dinamikoak

In vitro PK/PD eredu dinamikoetan mikroorganismoa drogarekin kontaktuan jartzen da eta kultibo zelular garbiaren gehikuntzaz baliatuz, gizakian ematen den farmakoaren PK simulatzen da, denboran zehar, mikroorganismoaren erantzuna aztertzeko helburuarekin [85,93]. Orokorrean, dinamikoek estatikoek baino denbora eta lan gehiago eskatzen dute baina giza PK hobeto simulatzea ahalbidetzen dute, gorputzean ematen den farmako kontzentrazioa momentu oro aldatzen doalako [85]. Sistema dinamikoetan hurrengoak ezberdindu daitezke: urratsez urratseko diluzio metodoak, konpartimentu bateko metodoak eta bi konpartimentuko metodoak.

Metodo sinpleenak urratsez urratseko diluziokoak dira. Bertan, onddoak denbora batez inkubatzen dira farmako kontzentrazio konstante batean. Ondoren, kultibo zelular garbia gehitzen da (farmakoaren erdi bizitza kontuan hartuz) eta berriz inkubatzen uzten da. Prozedura hau zenbait aldiz errepikatzen da [93]. Kultibo zelular garbia eskuz zein ordenagailu baten laguntzaz gehitzen da. Sinpleena izanik ez du giza gorputzean gertatzen dena ondo simulatzen, gorputzean diluzio konstante bat ematen baita [93].

Konpartimentu bakarreko eta bikoitzeko ereduen oinarria aurrekoaren bera da, baina kultibo zelular garbiaren gehikuntza ponpa peristaltiko baten bidez egingo da fluxu jarrai batean. 4. Kapituluan era sakonean azalduko dira metodo horien ezaugarriak eta haien garapena urteetan zehar.

7. Farmakoen determinazio analitikoa

Azaldutako *in vitro* ikerketen oinarria giza PK/PD profila simulatzea da. Helburu hori lortu ahal izateko, lehenengo eta behin gizakietan ematen den farmako kontzentrazio aldaketa denboran zehar neurtu behar da eta ondoren, kontzentrazio horiek *in vitro* ereduan simulatu. Hori dela eta, farmakoen edo haien metabolitoen determinazioa lagin biologikoetan egitea nahitaezkoa bihurtu da (odolean, plasman,…). Hau da, farmakoen bioanalisia [95].

Orokorrean, farmakoen analisian likido kromatografia teknika erabiltzen da matrizean egon daitezkeen konposatuen banaketa aurrera eramateko [96-99]. Banaketa kromatografikoa aldera batera utziz, determinazio analitikoak burutzeko metodoek bi atal garrantzitsu dituzte: laginaren prestaketa eta konposatuaren detekzioa.

Konposatuaren detekzioa burutzeko zenbait detektagailu daude eskura; diodoseko detektagailua (PDA), fluoreszentzia detektagailua (FLD) eta masa espektrometria (MS) detektagailua, beste batzuen artean. Egun, MS bidezko detekzioak dauzkan sentikortasuna ta espezifizitate altuen ondorioz, bioanalisiak egiteko lehen aukerako detektagailua bihurtu da.

Laginaren prestaketari dagokionez nabarmendu behar da lagin biologikoak konposatu anitzeko matrizeak direla. Horren ondorioz, orokorrean laginak garbitu behar dira, bai detekzioa hobetzeko bai aparatuen eta zutabe kromatografikoen erdi-bizitza luzatzeko ere. Bioanalisian laginen aurretratamendu ohikoenak fase solidoko erauzketa (SPE) eta proteinen hauspeatzea dira [95]. Horrela izan arren, CSFren determinazioan SPE prozedurak erabiltzea ez da gomendagarria berreskurapen arazoak deskribatu direlako [48]. Proteinen hauspeatzea burutzean, orokorrean, lagin bolumen jakin bati disolbatzaile organiko (azetonitriloa edo metanola) gehitzen zaio, 1:3 eta 1:6 proportzio tartean [99]. Prozedura horren proportzioak direla eta, sentikortasun arazoak eduki ahal dira detekzioan.

Farmakoaren kontzentrazioa baxua bada, eta kontuan hartuta hauspeatzean gehiago diluitzen dela, posible da kontzentrazioa kuantifikazio mugatik behera egotea eta kontzentrazioa determinatzea ezinezkoa izatea. Hori guztia kontuan hartuta, laginaren injekzio zuzena erabiltzen dituzten metodoak garatzen ari dira [99]. Prozedura horri ingelesez column-switching deritzo. Sistema kromatografikoan, SPE prozedura bat aurrera eramaten da lagina zutabe analitikora heldu baino lehen. Laginaren garbiketa sisteman bertan egiten da aurretiaz manipulaziorik egin gabe. Horrela izanik, behar den bolumena oso txikia izan daiteke, nahikoa injekzioa modu egokian egin ahal izateko.

Esan bezala, *in vitro* ikerketek farmakoen determinazioaren beharra dute. Ikerketa horiek kultibo zelularreko matrizeetan aurrera eramaten dira, matrize biologiko konplexuak ere direnak. Hori dela eta, erabilitako teknika analitikoak berdintsuak dira (HPLC analisia batez ere) [100-104]. Garbiketa prozesu bat behar da sistema kromatografikoan kalterik ekiditeko bertan aurkitzen diren gatz, amino azido eta bitaminengatik. *In vitro* ikerketetan erabiltzen diren lagin bolumen txikiak ere kontuan hartuta (laginketa maiz egin behar da), column-switching-a erabiltzen duten metodologiak oso erabilgarriak dira baita ere.

Hori horrela delarik, tesi honetan teknika hori erabiltzen duen metodo analitiko bat garatu egin da, zehazki, column-switching-HPLC-FLD metodo bat. Honek, kultibo zelularreko matrizean (RPMI-1640n) CSFren determinazioa ahalbidetzen du *in vitro* ikerketetan lagundu ahal izateko.

8. Farmako peptidikoen determinaziorako zailtasunak

Kandidiasi inbaditzailea tratatzeko farmako berrien garapena etengabekoa da. Horrela izanik, aipatu beharra dago azken hamarkadetan produktu farmazeutikoen garapena proteina eta peptidoetan ardaztu egin dela. Maes *et al*.-ek [105] esan bezala, biomolekula horiek intereskoak dira giza gorputzean dituzten funtzio ugariengatik eta funtsezko prozesu fisiologiko gehienetan parte hartzen dutelako.

Beste batzuen artean, ekinokandina antifungikoen familia testuinguru horretan kokatzen da, molekula lipopeptidikoak direlako.

Zoritxarrez, peptido eta proteinen erabilerak eragozpen garrantzitsu bat dute, jakina baita peptidoek adsortzio ez-espezifikoa erakusten dutela materialekiko [105-114]. Urteetan zehar adsortzio hori ulertzeko hainbat ikerketa aurrera eraman dira, baina, momentuz, ez da joera orokor bat ikusi peptido eta proteinen konposizioen aldakortasuna dela eta [115,116]. Goebel-Stengel *et al*.-ek [110] peptidoen ikerketetan material egokia erabiltzearen garrantzian oinarritu zuten haien ikerketa. Zortzi peptidoen ikerketa aurrera eraman zuten eta lortutako emaitzetan oinarrituz, peptido bakoitzak materialetara lotzeko/adsorbatzeko propietate ezberdinak dituela ondorioztatu zuten. Aurkitu zuten adsortzioa aurresateko indikatzaileek, hala nola, karga netoa, kate luzera, edo hidrofobizitatea, ez zutela material egoki bat aurkitzeko informazio nahikoa ematen. Horrela izanik, molekula bakoitzak erakusten duen adsortzioa ikertu beharko litzateke, konposatu horrekin lan egiteko material aproposa zein den jakin ahal izateko.

Materialen egokitasuna aurresatearen ezintasuna ikusita eta analito bakoitzarentzat edukiontziaren material egokia ikertzeko lan-ordu asko beharrezkoak direnez, adsortzioa minimizatzeko estrategiak bilatu dira. Ikertutako estrategien artean, disolbatzaile organikoen edo surfaktanteen gehipena eta pH balioaren aldaketa aurkitu daitezke [105,108]. Ikusi da nola, laginari disolbatzaile organikoen edo surfaktanteen bolumen bat gehitzeak adsortzio prozesua ekiditen duen. Baita, pH azidoek orokorrean adsortzioa murrizten dutela. Lan honetan zehar ikusiko den bezala, adsortzio ez-espezifiko horrek garrantzi handia du CSF determinatzerako orduan.

Tesiaren helburuak

Azken urteotan IFIen kasuen gorakada nabarmendu da, batez ere, ospitalizaturik dauden eta inmunitate arazoak duten pertsonen artean. Kandidiasia IFIrik ohikoena izan ez ezik, giza erikortasun eta hilkortasun arrazoi garrantzitsu bat da. Egun, infekzio fungikoei aurre egiteko lau farmako familia erabili ohi dira: azolak, polienoak, pirimidinaren analogoak eta ekinokandinak. Dena den, onddo askok farmakoei garatutako erresistentziaren ondorioz, ekinokandinak kandidiasiaren tratamendurako lehen aukera bihurtu dira.

Urteak pasa ahala onddoen erresistentzia ekinokandinekiko baxu mantendu arren, kandidaren lehen kasua antzeman zenetik 2005. urtean, kasu kopurua nabarmenki handitu da, batez ere *C. glabrata*-n. Kandidiasi inbaditzaileak medikuntza arloan hartzen ari duen garrantzia, ematen ari diren akats terapeutikoekin batera (batez ere CSFrekin), farmakoen erresistentzia ikerketen premia bistaratu egin dute. *In vitro* erresistentzia ikerketa egokiak behar dira antifungikoen eraginkortasuna ebaluatu eta konparatu ahal izateko.

Hau guztia dela eta, tesi honen helburu nagusia da:

> *C. glabratak* CSFrekiko duen erresistentziaren ikerketa bi konpartimentutako *in vitro* eredu farmakozinetiko/farmakodinamikoaz baliatuz

Helburu nagusi hau lortu ahal izateko, hurrengo helburu partzialak proposatu dira:

- Kaspofunginaren analisia RPMI-1640 kultibo zelularrean ahalbidetzen duen metodo analitikoaren garapena eta balidazioa
- Kandida glabrataren hainbat zepen kaspofunginarekiko erresistentziaren ikerketa, bi konpartimentuko in vitro ereduaren laguntzaz
- Esperimentazioan ikusitako CSFren galeraren ikerketa. Degradazioaren azterketa HPLC-FLD, RMN eta HPLC-MS teknikak erabiliz. Adsortzioaren ikerketa hainbat baldintzetan eta adsortzioa ekiditeko materialaren tratamendu baten proposamena eta ebaluazioa

Objectives of the Thesis

In the last years invasive fungal infection (IFI) cases have risen, specially, among hospitalized and immunocompromised patients. In this context, candidiasis is one of the most frequent IFI in humans and it is an important reason of human morbidity and mortality. Nowadays, there are four drug families for fungal infection treatment: azoles, polyenes, pyrimidine analogues and echinocandins. However, due to the resistance development of fungi to different drugs, echinocandins are the first-line therapy for candidiasis.

Even though fungal resistance to echinocandins remains low since the first case was reported in 2005, resistance has increased significantly particularly in *C. glabrata spp*. The increasing importance of invasive candidiasis in medical care, in addition with the therapeutic failures reported, have shown the importance of the study of fungal resistance to drugs. To be able to evaluate and compare the effectivity of antifungal drugs, *in vitro* resistance studies are needed.

Taking this in consideration, the main objective of this thesis is;

> Study of *C. glabrata* resistance to caspofungin antifungal drug by means of two-compartmental *in vitro* pharmacokinetic/pharmacodynamics model

To achieve this main objective the next secondary objectives have been proposed:

- Development and validation of an analytical methodology which allows the determination of CSF in RPMI-1640 cell culture matrix
- Resistance study of *C. glabrata* strains to CSF using the two compartmental *in vitro* model
- Study of the CSF loss during experimentation: Degradation study of CSF by HPLC-FLD, NMR and HPLC-MS techniques. Adsorption study of CSF in different condition and the evaluation of a material treatment for adsorption avoidance

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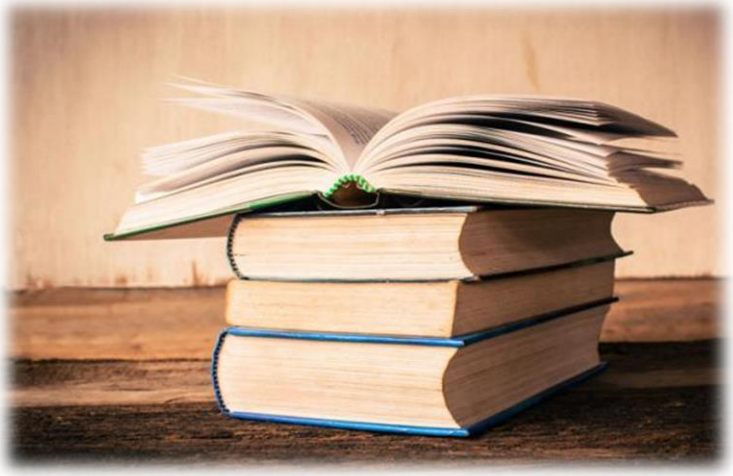
Chapter 2

REVIEW

Chromatographic methods for echinocandin antifungal drugs determination in bioanalysis



Based on

Beatriz Uribe, Oskar González, Boubakar B. Ba, Karen Gaudin, Rosa M. Alonso.

Chromatographic methods for echinocandin antifungal drugs determination in bioanalysis.

Bioanalysis, **2019**, 11 (12), 1215-26

Abstract

The increase of fungal resistance to drugs, such as azole family, gave rise to the development of new antifungals. In this context, echinocandins emerged as a promising alternative for antifungal therapies. Following the commercialization of caspofungin in 2001, echinocandins become the first-line therapy for invasive candidiasis in different patient population. The quantification of these drugs has gained importance since pharmacokinetic/pharmacodynamic and resistance studies are a paramount concern. This fact has led us to exhaustively examine the methodologies used for the analysis of echinocandins in biological fluids, which are mainly based on liquid chromatography coupled to different detection techniques. In this review, we summarize the analytical methods for the quantification of echinocandins focusing on sample treatment, chromatographic separation and detection methods.

1. Introduction

During the last decades, an increase of invasive infections has been noticed [1-3]. In this context, fungal infections have become a major cause of human disease, particularly in hospitalized and immunocompromised patients [4-6]. The increment of this kind of diseases and the high rate of mortality related to invasive fungal infections (often more than 50 %) together with the rising resistance of some species of *Candida* to antifungal drugs are a concern worldwide [1,7,8]. Therefore, the treatment against fungal infections is one of the biggest medical challenges nowadays.

For years, fungal infections have been mainly treated with azolic and polyene antifungal agents. The former target the inhibition of the synthesis of ergosterol and the latter cause the damage of the cytoplasmic membrane of fungi by binding to ergosterol [4,9,10]. Nevertheless, the appearance of acquired resistance to the widely used azoles has forced specialists to expand the targets of antifungals [3,4].

At the beginning of the XXI century, a new antifungal drug family emerged with the introduction of the first echinocandin (EC): caspofungin (CSF) [3,11-13]. Since then, ECs have become the first-line therapy for invasive candidiasis in different patient population [7,14]. The antifungal activity of this family of drugs is based on the inhibition of the β-(1,3)-D-glucan synthase, an enzyme responsible for fungal cell wall synthesis [3,9]. To date, three ECs have been approved (CSF, micafungin (MCF) and anidulafungin (ADF)) and the therapeutic effect of a fourth one (rezafungin (RZF)) is being investigated.

Due to the emerging development of antifungal resistance, the importance of ECs in the medical field is increasing. In this aspect, more efficient therapies need to be developed and resistance and pharmacokinetic/pharmacodynamics (PK/PD) studies should be performed. Therefore, analytical methods for the quantification of ECs are more and more necessary. Herein we present the first comprehensive review on the analytical methods developed for the quantification of ECs reported in literature. The analytical technique of choice for the determination of ECs is liquid chromatography coupled to different detectors. This review is especially focused on the separation conditions and the detection methods employed, as well as on the sample treatments carried out before the chromatographic analysis. Furthermore, a brief overview on the physicochemical properties of the molecules and their mechanism of action is offered, highlighting the resistance phenomenon observed in the last years.

2. Chemistry, antimicrobial activity and pharmacokinetics

2.1 Structure and chemical properties

ECs are semisynthetic lipopeptides with molecular weights between 1000 and 1300 g/mol (Table 1), derived from the fermentation broths of various fungi [3,12,15]. These molecules have a similar core hexapeptide structure composed by two

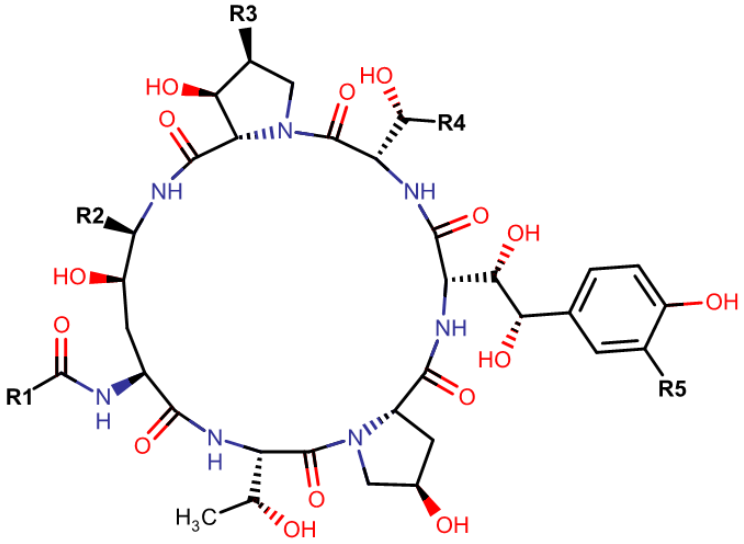
ornithines, two prolines and two threonines (Figure 1). The main structural difference among the different ECs is the N-linked acyl lipid side chain: a fatty acid chain in CSF, a 3,5 diphenyl-substituted isoxazole ring in MCF and an alkoxytriphenyl in ADF and RZF [7,12,16-19]. Indeed, RZF is an analogue of ADF but it has a choline group in the core structure which avoids the opening of the ring and increases the solubility and stability of the molecule in different matrices [7].

***Table 1**: Chemical properties of echinocandins*

Name	*M.W. (g/mol)*	*pKa* (most acidic)*	*pKa* (most basic)*	*Formula*
Caspofungin	1093.33	9.76	9.46	$C_{52}H_{88}N_{10}O_{15}$
Micafungin	1270.28	-2.25	0.28	$C_{56}H_{71}N_9O_{23}S$
Anidulafungin	1140.2	9.46	-1.20	$C_{58}H_{73}N_7O_{17}$
Rezafungin	1226.41	9.46	-1.20	$C_{63}H_{85}N_8O_{17}^+$

M.W.: Molecular weight ,*Values obtained from ChemAxon

CSF diacetate was the first EC approved by the Food and Drug Administration (FDA) in 2001. It is a semisynthetic derivative compound of pneumocandin B_0, a fermentation product isolated from the fungus *Glarea lozoyensis* [3,16]. In 2005 the FDA approved MCF monosodium salt. This pneumocandin A_0 is a semisynthetic lipopeptide synthesized from the chemical modification of a fermentation product of *Coleophoma empetri* [3,17]. The last EC approved by the FDA (2006) was ADF. This lipoprotein is derived from fermentation products of *Aspergillus nidulans* [3,18]. Finally, the investigational EC currently in development should be mentioned: RZF; also known as CD101 or Biafungin [2,7,20-22].



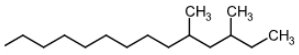
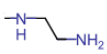
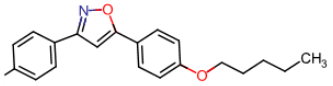
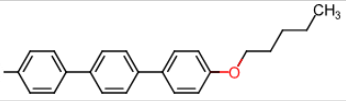
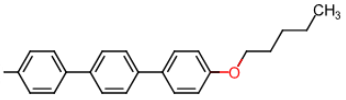
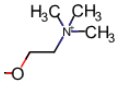
	R1	R2	R3	R4	R5
CSF			-H		-H
MCF		-OH	$-CH_3$		
ADF		-OH	$-CH_3$	$-CH_3$	-H
RZF			$-CH_3$	$-CH_3$	-H

Figure 1: *Chemical structure of CSF, MCF, ADF and RZF*

ADF: Anidulafungin; CSF: Caspofungin; MCF: Micafungin; RZF: Rezafungin

2.2 Mechanism of action and pharmacokinetics

Fungal cell-walls are rigid structures composed of large polysaccharides, α-glucans and glycoproteins [15]. The mechanism of action of ECs is based in noncompetitive inhibition of β-(1,3)-D-glucan synthase, an enzyme complex which synthetizes one of the large polysaccharides of the cell wall (β-(1,3)-D-glucan) [9,11,13]. This complex has two subunits: FKS1p and Rho1p. The former is responsible of cell wall remodeling and the latter of the regulation, driving or arresting the synthesis of (1→3)-β-D-glucan [12,23]. The inhibition of this enzyme results in fungicidal activity that affects in a different way to the fungal species depending on the glucan proportion they have in the cell wall [12,23]. EC antifungal potency is related to their spatial structure and lipophilicity. Even though all of them have a similar cyclic hexapeptide core structure, it has been observed that the differences in their side chains determine their inhibitory activity against different fungi [15,23].

ECs are administered by infusion and the standard pharmacological treatment is different for each drug and population [24-30]. For adults, CSF treatment starts with a 70 mg bolus followed by 50 mg/day doses. Under these conditions elimination half-life ($t_{1/2}$) of 10-14 h and a maximum plasmatic concentration (C_{max}) of 10-12.1 mg/L are obtained [24]. The drug has a 96 % protein binding and is metabolized by the liver with an area under the curve at 24 hours (AUC_{24h}) of 93.5-100.5 mg x h/L. MCF is also metabolized in the liver and its protein binding reaches 99.8 %. It is administered in 100 mg/day doses without bolus requirement. The C_{max} is 7.1-10 mg/L, the AUC_{24h} = 59.9-111.3 mg x h/L and the $t_{1/2}$ of 13-18 h. Finally, ADF is not eliminated by the liver but via biliary excretion after spontaneous degradation. Similarly to the other ECs, this molecule also has a high protein binding (>99 %). ADF treatment requires a higher dosification with a bolus of 200 mg in three hours followed by 100 mg/day doses. Under these conditions $t_{1/2}$ is 25.6 h and C_{max} of 3.44-7.5 mg/L and AUC_{24h} of 44.4-104.5 mg x h/L are obtained.

For using ECs in children population (2-17 years old), it has to be highlighted the difference in dosing between ECs [27]. MCF and ADF doses are associated to the weight of the child being 1-4 mg/Kg/day and 0.75 or 1.5 mg/Kg/day (with a loading dose of 1.5 or 3 mg/Kg), respectively. However, for CSF the dose remains as in adults.

2.3 Resistance

Antimicrobial Resistance is defined by the European Medicines Agency (EMA) as "When a microbe evolves to become more or fully resistant to antimicrobials which previously could treat it" [31]. This phenomenon was deeply studied for antifungal families in *Candida* species by Morace *et al*. [9]. As the authors explain, when exposing a microorganism to a drug, microorganisms compete each other to survive and they create new strategies to resist the action of these drugs. In this way, a mutation could occur and these microorganisms could become drug-resistant, being the main reason of the subsequent therapeutic failure. Several factors can influence the failure of antifungal treatments, among them the immunological status of the host, the drug dosage and the cellular organization of the fungus. Although the resistance of microorganisms to ECs is still infrequent, the incidence is alarmingly increasing since the first case for *Candida* was reported in 2005 (for some *Candida* species) [32,33]. Resistance mechanism is different depending on the antifungal mechanism of action. In literature, resistance to ECs is attributed to several possibilities: mutation in the FKS1 gene [3,13,34,35], presence of a drug efflux pump [3], overexpression of cell wall proteins [34] or intracellular buildup of long chain bases [34].

3. Analytical methodology

Considering that the main objectives of the determination of ECs are therapeutic drug monitoring and pharmacokinetic studies, most of the methods found in the literature for the determination of ECs are intended for matrices obtained from blood such as plasma or serum. Only one method for urine analysis of ECs (CSF) has

been reported probably due to the small amount of drug excreted in this biofluid (1.4 %) [36]. In the following sections the sample preparation, chromatographic analysis and detection methods used for the analysis of CSF, MCF and ADF are summarized (RZF is still in investigational drug and, so far, there is only one method described for its determination [37]). Additionally, in order to offer the information of interest for each EC individually, the parameters of the analytical methods reported for CSF, MCF and ADF have been gathered in **Table 2, Table 3** and **Table 4**, respectively.

3.1 Sample preparation

Sample treatment for plasma and serum samples is commonly a protein precipitation (PPT) procedure with acetonitrile (ACN) or methanol (MeOH) [38-48] using sample:solvent ratios that vary from 1:1 (v/v) to 1:8 (v/v). Besides plasma, Farowski *et al*. [42] also analyzed different types of blood cells (peripheral blood mononuclear cells, polymorphonuclear leukocytes and erythrocytes) using ACN)as extraction agent. The only analytical method for the determination of ECs in whole blood was developed by Cheng *et al*. [49] for the analysis of CSF using dried blood spot. This method includes the extraction of the analyte with a H_2O:MeOH (50:50, v/v) solution and a subsequent PPT with ACN.

Some authors have proposed the direct chromatographic analysis of ECs without any prior sample treatment. Uranishi *et al*. [50] developed a method for MCF analysis that only included a filtration step before direct injection of the plasma sample in the high performance liquid chromatography (HPLC) system. Also direct injection was applied by Egle *et al*. [51] for the determination of CSF in plasma, but including an online column switching procedure that allows matrix cleaning in the extraction column.

Table 2: *Chromatographic methods reported for caspofungin*

Matrix (amount)	*Sample treatment*	*Column*	*Mobile phases*	*Detection*	*LLOQ (mg/L)*	*Year and reference*
Plasma (100 µL)	PPT (CH_3CH_2OH)	Zorbax SB-C18	0.1% HCOOH (A) CH_3CN (B)	MS (ESI +) 1093.5>1033.0/286.7 547.4>538.2/136.9	0.2	2017 [38]
Plasma (50 µL)	PPT (CH_3CN)	BEH C18	0.1% HCOOH (A) CH_3CN (B)	MS (ESI+) 547.5>137.1/131.1	0.5	2017 [39]
Plasma (200 µL)	PPT (CH_3CN)	Hypersil GOLD C18	0.1% HCOOH (A) CH_3OH (B)	MS (ESI+) 547.5>137.3	0.5	2015 [40]
Plasma (100 µL)	PPT (CH_3CN)	Acquity UPLC C18	10 mM NH_4HCO_2 +0.1% HCOOH (A) CH_3CN +0.1% HCOOH (B)	MS (ESI+) 547.2>538.7/137.0/131.0/86.0	0.06	2010 [41]
Plasma (100 µL)	PPT(CH_3CN)	Symmetry Shield RP8	10mM $NH_4CH_3CO_2$ (1% CH_3COOH) (A) CH_3CN (1% CH_3COOH) (B)	MS (ESI+) 547.3>137.1	0.04	2007 [43]
Plasma (60 µL)	PPT (CH_3OH)	Kinetex F5	H_2O-IPA -HCOOH (90:10:0.1, v/v/v)- NH_4CH_3COO (2 mM) (A) MeOH-IPA-HCOOH (90:10:0.1, v/v/v)- NH_4CH_3COO (2 mM) (B)	MS (ESI+) 547.5>538.4/137.2/131.0	1.25	2018 [44]
Plasma (30 µL) ***Blood Cells (10^7 cells)***	Dilution in PBS PPT (CH_3CN) PPT (CH_3CN)	Beta Basic C4	0.1% HCOOH (A) CH_3CN (B)	MS (ESI+) 547.4>137.1	0.108 -	2010 [42]
Serum ***(5 µL injection V)***	Column switching	Vertex Eurospher 100 CN	0.1% HCOOH (A) CH_3CN (B)	MS (ESI+) 1093.9>547.7/365.4	0.2	2004 [51]
Blood (50 µL)	DBS extraction PPT (CH_3CN)	Hypersil GOLD aQ	0.1% HCOOH (A) CH_3OH (B)	MS (ESI+) 547.6>538.7	0.2	2018 [49]
Aqueous humor (30 µL)	Dilution with CH_3CN	Synergi Hydro-RP C18	0.1% HCOOH (A) 0.1% HCOOH in CH_3OH (B)	MS (ESI+) 547.5	0.01	2010 [57]
Plasma (100 µL)	SPE	Keystone Betasil C18	0.1% TFA (A) CH_3CN (B)	FLD λ_{ex} =220 nm λ_{em}=304 nm	0.125	2007 [55]

Table 2: *Chromatographic methods reported for caspofungin (continued)*

Matrix (amount)	*Sample treatment*	*Column*	*Mobile phases*	*Detection*	*LLOQ (mg/L)*	*Year and reference*
Plasma (100 µL)	SPE	Keystone Betasil C18	0.1% TFA (A) CH_3CN (B)	FLD λ_{ex} =220 nm λ_{em}=304 nm	0.125	2009 [53]
Plasma (100 µL)	SPE	Keystone Betasil C18	0.1% TFA (A) CH_3CN (B)	FLD λ_{ex} =220 nm λ_{em}=304 nm	0.125	2009 [54]
Serum (250 µL) ***Plasma (250 µL)***	SPE	Nucleosil 300 C8	0.1% TFA (A) CH_3CN (B)	FLD λ_{ex} =224 nm λ_{em}=300 nm	0.1 for serum and plasma	2007 [52]
Aqueous humor (100 µL) ***Vitreous humor (125 µL)*** ***Cornea (20-50 mg)***	Dilution with CH_3OH and CH_3COOH. Drying. Reconstitution					
Plasma ***Kidney***	-	Kinetex C18	0.1%TFA (A) CH_3CN (0.1%TFA) (B)	FLD λ_{ex} =224 nm λ_{em}=304 nm	0.125	2012 [69]
Plasma (1 mL) ***Urine (1 mL)***	SPE	Zorbax C8	0.1% TFA (A) CH_3CN (B)	UV/Vis FLD λ_{ex} =220 nm λ_{em}=304 nm	-	1997 [36]
Cancidas pharmaceutical product	Filtration	Zorbax Eclipse Plus C18	0.1% trimethylamine (A) CH_3CN (B)	UV/Vis λ=220 nm	-	2014 [62]
Vitreous humor (150 µL) ***Aqueous humor (150 µL)***	PPT (CH_3OH) Drying and reconstituted in 20% CH_3OH (0.1% TFA)	LiChrospher 100RP-18e	0.1% TFA (A) 0.1% TFA in CH_3OH (B)	UV/Vis λ=215 nm	-	2014 [56]
Bulk drug	Direct injection	PhenoSphere-NEXT C18	0.1% TFA (A) 0.1% TFA in CH_3OH (B)	UV/Vis λ=215 nm	-	2012 [61]
Human microdialysates (20 µL)	PPT (Ice-cold CH_3CN)	LiChroCART CN	0.05M Citric acid (pH= 6.3) (A) CH_3CN (B)	Amperometric detection	0.07	2006 [58]

Table 3: *Chromatographic methods reported for Micafungin*

Matrix (amount)	*Sample treatment*	*Column*	*Mobile phases*	*Detection*	*LLOQ (mg/L)*	*Year and reference*
Plasma (100 µL)	PPT (CH_3CH_2OH)	Zorbax SB-C18	0.1% HCOOH (A) CH_3CN (B)	MS (ESI +) 1270.2>1172.1	0.2	2017 [38]
Plasma (30 µL)	Dilution in PBS PPT (CH_3CN)	Beta Basic C4	0.1% HCOOH (A) CH_3CN (B)	MS (ESI+) 1270.9>1172.4	0.16	2010 [42]
Blood Cells (10^7 cells)	PPT (CH_3CN)				-	
Serum (100 µL)	PPT (CH_3CN)	Zorbax Eclipse XDB-C18	0.1% (w/w) CH_3COONH_4 (A) CH_3CN (B)	MS (ESI+) 1270.4>1173/1191	-	2011 [45]
Plasma (100 µL)				UV/Vis λ= 273 nm	-	
Plasma (200 µL)	PPT ($^{13}C_6$ MCF in CH_3CN: CH_3OH (83:17))	Waters Atlantis T3	0.02 M NH_4HCO_2 : CH_3OH (95:5, v/v) (A) CH_3CN (B)	MS (ESI -) SIM 1268.2	0.2	2018 [46]
Plasma (100 µL)	Direct injection	Cadenza HS-C18	0.05 M NH_4CH_3COO (A) CH_3CN (B)	FLD λ_{ex} =273 nm λ_{em}=464 nm	0.33	2011 [50]
Plasma	PPT (CH_3CN)	ODS-80TM	0.02 M KH_2PO_4 (A) CH_3CN (B)	FLD λ_{ex} =273 nm λ_{em}=464 nm	0.1	2001 [48]
Pentobarbital tissue ***Brain tissue*** ***Cerebrospinal fluid*** ***Choroid*** ***Vitreous humor*** ***Aqueous humor*** ***Lung*** ***Liver*** ***Spleen*** ***Kidney***	SPE		0.05 M NH_4CH_3COO (pH=4) (A) CH_3CN (B)	UV/Vis λ= 273 nm	0.05	

Table 3: *Chromatographic methods reported for Micafungin (continued)*

Matrix (amount)	*Sample treatment*	*Column*	*Mobile phases*	*Detection*	*LLOQ (mg/L)*	*Year and reference*
Plasma (50 µL)	PPT (CH_3CN)	ODS-80TS	0.02 M KH_2PO_4 (A) CH_3CN (B)	FLD λ_{ex} =273 nm λ_{em}=464 nm		2005 [64]
Plasma (100 µL) ***Liver*** ***Kidney*** ***Lung***	-	ODS-80TM	0.02 M KH_2PO_4 (A) CH_3CN (B)	FLD λ_{ex} =273 nm λ_{em}=464 nm	0.05 - - -	2004 [65]
Plasma ***Kidney***	-	Hypersil BDS C18	0.02 M KH_2PO_4 (A) CH_3CN (B)	FLD λ_{ex} =273 nm λ_{em}=464 nm	0.1	2012 [69]
Pharmaceutical drug product	Dilution with H_2O	C18 ODS Hypersil	NaH_2PO_4+$NaClO_4$ in H_2O (pH=9) (A) CH_3CN (B)	UV/Vis λ= 210 nm	-	2015 [63]
Bulk drug	Direct injection	Phenomenax Aeris peptide XB	0.01 M phosphate buffer (pH=2.9) (A) CH_3CN (B)	UV/Vis λ= 279 nm	0.05	2016 [66]

Table 4: *Chromatographic methods reported for Anidulafungin*

Matrix (amount)	*Sample treatment*	*Column*	*Mobile phases*	*Detection*	*LLOQ (mg /L)*	*Year and reference*
Plasma (100 µL)	PPT (CH_3CH_2OH)	Zorbax SB-C18	0.1% HCOOH (A) CH_3CN (B)	MS (ESI +) 1140.1>1122.1/388.1	0.2	2017 [38]
Serum (100 µL)	PPT (CH_3CN)	Zorbax Eclipse XDB-C18	0.1% (w/w) NH_4CH_3COO (A) CH_3CN (B)	MS (ESI+) 1141.5>1123	-	2011 [45]
Plasma (100 µL)				UV/Vis λ= 306 nm	-	
Plasma (200 µL)	Cell lissing (NaCl+ CH_3OH)	Xbridge C_{18}	1 M NH_4HCO_3:H_2O:CH_3OH:NH_4OH (1:90:10:0.6, v/v/v/v) (A) 1 M NH_4HCO_3:H_2O:CH_3CN (1:5:95, v/v/v) (B)	MS (ESI -) 1138.6>898.8	-	2009 [68]
Bronchoalveolar lavage (200 µL)						
Alveolar cells (200 µL)						
Plasma (100 µL)	PPT (CH_3OH)		1 M NH_4HCO_3:H_2O:CH_3OH:NH_4OH (1:90:10:0.6, v/v/v/v) (A) 1 M NH_4HCO_3:H_2O:CH_3CN (1:5:95, v/v/v) (B)	MS (ESI -) 1138.6>898.8	-	2011 [47]
			0.005 M $(NH_4)_3PO_4$ (A) CH_3OH (B)	UV/Vis λ= 310 nm	1	
Plasma	-	Hypersil BDS C18	0.02 M KH_2PO_4 (A) CH_3CN (B)	FLD λ_{ex} =273 nm λ_{em}=464 nm	0.05	2012 [69]
Kidney						
Plasma	-	Kinetex C18	0.1% TFA (A) CH_3CN (0.1% TFA)(B)	FLD λ_{ex} =273 nm λ_{em}=464 nm	-	2012 [70]
Aqueous humor						

More specific sample treatments such as solid phase extraction (SPE) have been proposed for CSF determination in serum, plasma and urine samples [36,52-55], using different phase sorbents (diol, reversed phase and mixed phase). For instance, Bi *et al*. [55] compared C8, diol and mixed phase (strong cation exchange/reverse phase) sorbents and they obtained the best recoveries using C8 sorbents. According to the literature, the solvent of choice for the elution of CSF in SPE is methanol in acidic conditions (using trifluoroacetic acid or acetic acid), probably because acidic pH values increase the polarity of the molecule [52,55]. For the elution from the diol or mixed phase sorbents also the use of ammonium hydroxide has been reported as necessary [36,55]. In the case of MCF, Groll *et al*. [48] developed a SPE sample treatment for the analysis of tissues and body fluids using a C8 sorbent and ACN-ammonium acetate mixtures.

Sample treatment procedures described for the analysis of ECs in vitreous and aqueous humor consist of a sample dilution either with MeOH (subsequently dried and reconstituted) [52,56], or ACN [57]. Similarly, Traunmüller *et al*. [58] proposed a human microdialysates sample deproteinization with ACN for determination of CSF followed by a drying step and reconstitution in 0.05M citric acid (pH=6.3) and ACN mixture (67:33, v/v). Determination of CSF in cornea tissue was achieved by treating cornea pieces with MeOH in acidic media (4% acetic acid) followed by evaporation of the supernatant and reconstitution in 0.1% trifluoroacetic acid (TFA) (pH=2):ACN (60:40, v/v) [52].

3.1.1 Analyte loss during sample treatment

Some authors have observed very poor recoveries for ECs that are probably related to the adsorption of ECs to different surfaces during the sample preparation. While CSF was still a drug in research phases, Schwartz *et al*. [36] observed adsorption to plastic surfaces during SPE procedure. In the same publication authors state that adsorption to glassware was already known. In order to improve the analysis, the effectiveness of bovine serum albumin (BSA) for avoiding CSF adsorption to plastic

was tested. An increment of recovery from 64% to 94% was observed when using a 0.25% of BSA. Traunmüller *et al*. [58] obtained low recoveries for CSF during the PPT step (less than 30%). In order to avoid the loss of CSF in protein pellets and polypropylene tubes, the effect of the pH and the addition of organic solvents were studied. The acidification of the sample (pH 4) before the deproteinization step increased the recovery until 70%, probably due to the fact that ionized CSF is better extracted from proteins. The addition of solvents such as 1-propranol to the sample extract improved the recovery until 81-89%, suggesting a detachment of CSF from the tube walls due to the higher affinity to the organic phase. Similar problems related to adsorption of drugs to different surfaces have been observed with MCF and ADF. For instance, Martens-Lobenhoffer *et al*. [45] obtained recoveries about 40% after the extraction procedure (SPE) of plasma samples. Besides, Sutherland *et al*. [47] observed that 50% of MeOH in the saline standard solutions and quality controls prevented the loss of the drugs.

3.2 Chromatographic Separation

ECs are relatively non-polar compounds and, therefore, all the chromatographic methods found in literature are based on reversed phase liquid chromatography. Nevertheless, it is important to highlight that analytical methods based on normal phase chromatography have been developed for pneumocandins, the precursors of ECs [59,60]. The most common stationary phases utilized for the analysis of ECs are non-polar stationary phases such as C18 or C8 either using classical bonded columns [36,38-41,45,52,55,56,61-63] or end-capped/embedded columns [43,48,49,57,64,65]. Among the few methods developed using other interaction mechanisms the one proposed by Uranishi *et al*. [50] for MCF determination using a hybrid column should be mentioned.

Chromatographic separation of ECs is mainly performed using gradient elution with aqueous phase and MeOH or ACN as organic modifiers, except in those cases when a single drug is analysed and isocratic elution is carried out [36,47,48,52,58,63-65].

Although run times are usually shorter than 15 minutes, they could reach 30 minutes when a better chromatographic separation is needed (direct injection analysis [50,61]) or a column switching procedure is carried out [51]. The preferred aqueous mobile phase for CSF analysis is acidified with formic acid or TFA [36,38-43,49,51,52,55-57,61,62] in order to improve peak shape. Acidic aqueous phases are also used for MCF analysis [38,63,66]. Nonetheless, Martens-Lobenhoffer *et al*. [45] studied the influence of the pH value of the mobile phase on the chromatographic analysis and they suggested a neutral pH value in order to get shorter retention times and more symmetric chromatographic peaks [46,50]. Finally, both acidic and neutral mobile phases have been employed for the analysis of ADF [38,41,45,47].

There are a few chromatographic separations that are based on interactions others than hydrophobic interactions and should also be mentioned. Interestingly, Hösl *et al*. [44] developed a method for CSF analysis with a pentafluorophenyl column using as mobile phases a mixture of water-isopropanol (IPA)-formic acid (90:20:0.1, v/v/v) and a mixture of MeOH -IPA-formic acid (90:20:0.1, v/v/v) both including 2 mM ammonium acetate. Both Traunmüller *et al*. [58] and Egle *et al*. [51] developed methods for the analysis of CSF using cyano based columns and ACN as organic modifier, but the former employed citric acid buffer at pH 6.3 and the latter 0.1% formic acid as aqueous phases.

3.3 Detection methods

Methods described for ECs determination are based on mass spectrometry (MS), fluorescence detection (FLD) or UV-Visible absorption spectroscopy (UV/Vis). The preferred choice is MSdetection because of its intrinsic selectivity and sensitivity. Most of the MS based methods described in the literature use triple quadrupole type instruments working in Multiple Reaction Monitoring (MRM) mode with electro spray ionization (ESI) source. A more simple method based on single quadrupole detection is described by Neoh *et al*. [57] using Single Ion Monitoring (SIM) mode. SIM mode was also used by Boonstra *et al*. [46] for MCF determination. As an

exception, the works of Kelly *et al*. [67] and Egle *et al*. [51] were performed with an ion trap mass analyzer. Positive mode ionization is used in all the cases for CSF analysis, but MCF and ANF determination has been performed also in negative mode [46,47,68]. In general, these methods use transitions of the single protonated $[M+H]^+$ or deprotonated molecular ions $[M-H]^-$. CSF is an exception where the double charged molecular ion $[M+2H]^{2+}$ is also used as a precursor. It is important to highlight the complexity of the isotope distribution pattern of ECs. Due to the high number of carbon atoms the intensity of $[M+1+H]^+$ ion is around 60% the intensity of the monoisotopic ion and there is also a significant signal for $[M+2+H]^+$ and $[M+3+H]^+$ ions. Due to this fact Martens-Lobenhoffer *et al*. [45] centered the quadrupole at $[M+1+H]^+$ m/z and set the quadrupole window at 2.5 amu.

Fluorescence detection is another usual choice for the quantification of ECs. CSF has an intense native fluorescence that according to Schwartz *et al*. [36] is due to the phenol group (Figure 1). At basic conditions, the fluorescence band is shifted and the intensity decays probably because of the ionization of this functional group. All the reported methods for the fluorimetric analysis of CSF are carried out using wavelengths around 220 nm for excitation and 304 nm for emission [36,52-55,69], even if CSF excitation spectrum shows another maximum of lower intensity around 275 nm [36]. Methods based on FLD have also been reported for MCF [48,50,64,65,69] and ADF [69,70] using in all the cases 273 nm and 464 nm wavelengths for excitation and emission, respectively. In this case, the native fluorescence of the molecules might be due to the aromaticity of the N-linked chain (R1: see Figure 1).

Besides MS and FLD methods, ECs have also been determined using UV/Vis, mainly by means of diode array instruments. This detection technique has been used for determination of EC in complex matrices such as plasma, serum or vitreous and aqueous humors after matrix cleaning steps as SPE or PPT with drying and reconstitution procedure [36,47,56]. Among ECs, CSF is the drug that has been more

widely analyzed employing this technique. Schwartz *et al*. [36] carried out a thorough study of the effect of the pH on the absorption spectra. Under acidic and neutral conditions, they observed an intense absorption peak around 200 nm with a shoulder at 225 nm and a less intense peak at 276 nm. As they reported, and similarly to fluorescence spectra, the absorption spectrum shows a significant change in basic conditions, with absorption maxima at 246 nm and 292 nm. This study explains why the UV/Vis methods for CSF are performed around 220 nm using acidic mobile phases [56,61,62]. Although the absorption spectra of MCF and ADF have not been so exhaustively studied, quantitative methods at longer absorption wavelengths have been reported: around 275 nm for MCF [45,66][66] and 308 nm for ADF [45,47].

As an exception to all the aforementioned spectrometric techniques, the amperometric determination of CSF in human microdialysates proposed by Traunmüller *et al*. [58] should be mentioned. They used an electrode of glassy carbon as working electrode and an Ag/AgCl as reference electrode. This determination was carried out checking as cell potential for oxidation a range from +650 mV to +1100 mV, choosing finally +950 mV due to its higher current intensity and no interference with the internal standard used.

3.3.1 Lower limit of quantification of the analytical methods

Regardless of the analytical methodology employed for the quantification of ECs, it has to be highlighted the wide variability in the lower limit of quantification (LLOQ) reported in literature. As expected, the lowest LLOQ for CSF in plasma was obtained using a MS detection. Neoh *et al*. [57] were able to quantify a concentration as low as 0.01 mg/L using a single quadrupole, although the reported value is calculated from the calibration curve built in a ACN:H_2O (50:50). Amperometric detection allowed to determine 0.07 mg/L concentration in human microdialysates samples [58]. Finally, with FLD detection 0.125 mg/L concentration was reached in plasma [52-54,69]. Surprisingly, for MCF the lowest LLOQ is not obtained by MS but by

fluorescence and UV/Vis detection. Indeed, Niwa *et al*. [65] reported a method for plasma analysis and Groll *et al*. [48] for biological fluids and tissues with a LLOQ of 0.05 mg/L, while the lowest reported value with MS detection for plasma is 0.16 mg/L. Similarly, the lowest LLOQ for ADF was obtained using a fluorescence detector. Arendrup *et al*. [69] reported a 0.05 mg/L LLOQ for plasma analysis, whilst Ventura *et al*. [38] obtained 0.2 mg/L by MS and Sutherland *et al*. [47] 1 mg/L by UV/Vis.

4. Conclusions

The introduction of new pharmaceuticals formulations of ECs requires the development of analytical methods that allow the quantitative determination of these drugs in biological fluids, especially in plasma. Therapeutic drug monitoring, stability of active components, pharmacokinetic studies and resistance mechanism studies are the main targets of these analytical methodologies. The analytical methods developed for the analysis of ECs are based on reversed phase liquid chromatography coupled to MS, FLD or, to a lower extent, UV/Vis detection. Although the reported LLOQ values cover a wide range (0.01-1.25 mg/L for CSF, 0.05-0.2 mg/L for CF and 0.05-1 mg/L for ADF), it is noticeable that a similar sensitivity can be obtained with MS and FLD. Furthermore, with some exceptions, sample treatment of choice is a simple PPT or sample dilution. Reliability of most of these methods is assured considering that they have been validated according to FDA or EMA guidelines. The review presented here can be extremely helpful for the members of scientific community who need to apply an analytical method to any study that requires the quantification of ECs.

5. Future Perspectives

Authors consider that liquid chromatographic methods will continue to be the technique of choice for ECs analysis, with an upward trend of ultra high performance liquid chromatography methods. It is likely that sample treatment procedures that require a lower amount of sample will be developed in order to deal with volumes even lower than 30 µL. Those sample treatments will probably be based on simple PPT procedures since the high selectivity offered by MS and FLD do not require an exhaustive elimination of endogenous compounds. In this sense, even if the latest methods published are based on MS, we consider that chromatographic methods using FLD will remain important taking into account that the sensitivity of the methods developed to date is comparable.

Due to the increasing invasive fungal infection cases and the acquired resistance of fungi, the development of new ECs is expected. Obviously, new analytical methods will be necessary for the quantification of those drugs. RZF is the first EC of the new generation with longer dosing times (daily doses for traditional ECs and weekly doses for RZF) and higher stability compared with its precursors. Furthermore, subcutaneous administration way is being explored in order to facilitate its treatment. Taking into account all these factors, it is likely that the analytical methods will need to adapt to a new scenario where a higher sensitivity is required and the analysis of different matrices is necessary.

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Chapter 3

Determination of antifungal caspofungin in RPMI-1640 cell culture medium by column-switching HPLC-FLD

Based on

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Determination of antifungal caspofungin in RPMI-1640 cell culture medium by column-switching HPLC-FLD.

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Abstract

The actual scenario in the fight against fungal infections, force researchers to carry through with resistance studies to improve the therapies. These studies, which are performed in cell culture media, need accurate and sensitive analytical methodologies. That is why, in this work, an analytical method for caspofungin (CSF) concentration determination in RPMI-1640 cell culture medium with on-line sample treatment was developed and validated. CSF concentration was determined by HPLC-FLD using a column-switching procedure. The chromatographic analysis was carried out in less than 10 min using a C8 column (4x4 mm, 5µm) as extraction stationary phase and a HSS T3 column (4.6 x 100 mm, 5µm) as the analytical column. The used mobile phases were mixtures of phase A: pH 2 (adjusted with TFA) aqueous phase and phase B: ACN. For the extraction, the composition was (95:5, A:B v/v) and for the analysis (60:40, A:B v/v), both done in isocratic elution mode. These chromatographic conditions allowed reaching a limit of quantification of 10 µg/L, using 100 µL of sample with an injected volume of 40 µL. The proposed method was successfully validated in terms of selectivity, carryover, linear concentration range, accuracy and precision according to the criteria established by the Food and Drug Administration. Stability of CSF in RPMI-1640 solution was found critical. CSF was stable up to 2 hours at room temperature. The developed method was applied for the direct analysis of CSF from *in vitro* experiment in presence of *C. glabrata* (CAGL18). The results highlight the decrease of cell proliferation associate caspofungin activity.

1. Introduction

Since the last decades, the incidence of fungal diseases has drastically increased. Severity of fungal diseases range from mild mucocutaneous infections to potentially life-threatening invasive infections. Serious invasive fungal diseases occur often as a consequence of underlying health problems such as AIDS, cancer, organ transplantation and corticosteroid therapies [1].

Candidiasis is one of the most frequent invasive fungal infection and an important cause of morbidity and mortality worldwide [2]. Among the 200 species described for *Candida* about 15 can cause human diseases and 5 of them are more frequent. In a retrospective study of candidemia at Nantes Hospital between 2004 and 2010, *C. albicans* was the predominant species (51.8%) followed by *C. parapsilosis* (14.5%), *C. glabrata* (9.8*%), C. tropicalis* (9.8%) and *C. krusei* (4.1%) [3]. Candidiasis are under growing interest due to the increase of incidence (particularly among immunocompromised patients), the emergence of new species (i.e. *Candida auris*) [4] and the rise of resistant and multi-resistant isolates to antifungal treatments [5].

The current armamentarium of antifungal drugs is limited to four families: azoles, polyenes, echinocandins and pyrimidines [6]. Over the years, the emergence of acquired resistance to azoles has forced the specialists to begin handling echinocandins as first-line therapy. According to Infectious Diseases Society of America (IDSA) [7,8], European congress of Clinical Microbiology & Infectious Diseases (ESCMID) [9] and European Conference on Infections in Leukaemia (ECIL) [10] guidelines, this therapeutic strategy was implemented for invasive and disseminated candidiasis and for candidemia in neutropenic and non-neutropenic patients.

The echinocandin class of antifungal drugs is composed by caspofungin (CSF), micafungin, anidulafungin [11,12] and rezafungin, a drug in phase 3 of clinical trial [13]. Echinocandins inhibit β-(1,3)-D-glucan synthesis by binding non-competitively to FKS p subunit of β-(1,3)-D-glucan synthase, a heteromeric glycosyltransferase enzyme complex present in the fungal cell membrane. Echinocandins are fungicidal against the majority of pathogenic *Candida* spp., even those resistant to azole compounds. Even though the actual difficulties in the treatment of fungal infections are partly due to the resistance phenomenon [14-16], resistance to echinocandin-class drugs remains low in most of *Candida* species except in *Candida glabrata* where an increase has been observed [17]. For example, at Duke Hospital between 2001

and 2010, the number of *Candida glabrata* isolates resistant to echinocandins increased from 4.9% to 12.3% and among that 14.1% were resistant to fluconazole [3,18]. In France, *in vitro* micafungin resistance among *C. glabrata* reached 3.9% [19]. Emergence of multidrug resistant (MDR) *C. glabrata* isolates is of concern with 36% of MDR *C. glabrata* isolates in the USA [15,20,21].

Due to the increasing medical importance of invasive candidiasis [2] and based on the reported therapeutic failures in particular with caspofungin [18], drug resistance studies are needed [3]. It is necessary to have adequate *in vitro* study approaches, which allow evaluating and comparing the antifungal efficacy of drugs. In laboratory of clinical microbiology, for example, echinocandin resistance is assessed by measuring MICs and comparing results to reference breakpoints or epidemiological cut-off value (ECOFF) established by European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical & Laboratory Standards Institute (CLSI). Unfortunately, because of significant interlaboratory variability in caspofungin MICs, EUCAST and CLSI do not propose interpretive criteria for this drug. Furthermore, echinocandin MICs have not been shown to correlate consistently with outcomes among patients with invasive candidiasis who are treated with these agents [22].

In vitro studies present important difficulties related to the need of appropriate analytical methodologies that allow the sensitive quantification of the drugs in culture media. Cell culture media are complex matrices composed by amino acids, salts and vitamins among others that usually require from a sample treatment before performing the analysis. Determination of CSF has been carried out in biofluids using different sample treatments such as solid phase extraction, protein precipitation and column-switching prior to liquid chromatography coupled to mass spectrometry (MS), photodiode-array (PDA) or fluorescence (FLD) detectors [23]. Nevertheless, to our knowledge, no method for the determination of CSF in cell culture media has been developed. In this sense, column-switching procedure can be useful for *in vitro* studies considering the high amounts of samples and the small volume that should

be handled. This kind of sample treatment has been already used for CSF determination in serum by Egle et al. [24], but the LLOQ obtained (200 µg/L) might not be low enough for its application to every *in vitro* studies [25-27].

Therefore, the aim of this work was the development and validation of an on-line sample treatment liquid chromatography method for the quantitative determination of caspofungin in RPMI-1640 medium. The column-switching-HPLC-FLD method proposed here requires only 100 µL of sample and was successfully applied to the quantification of CSF in samples obtained from *in vitro* studies of *C. glabrata* cell growth under treatment by CSF.

2. Materials and Methods

2.1 Reagents and solutions

Caspofungin acetate was purchased from Finetech (Wuhan, Hubei, China). Cell culture media used was RPMI 1640 with L-glutamine with MOPS buffer obtained from Capricorn Scientific (Ebsdorfergrund, Germany), Lonza (Aubergenville Cedex, France) and RPMI-1640 medium with L-glutamine and without sodium bicarbonate (Sigma, Saint Quentin Fallavier, France) with addition of 1% glucose and 0.0825 mol/L MOPS (Sigma, Saint Quentin Fallavier, France) with pH adjusted to 7.0.

[3-(2-Aminoethylamino)propyl] trimethoxysilane (AATMS) was bought from Sigma-Aldrich (St. Louis, USA). Sodium hydroxide (≥99%) was purchased from Merk (Darmstadt, Germany). Acetonitrile (LC-MS grade) and trifluoroacetic acid (for LC-MS) were supplied by VWR chemicals (Fontenay-sous-Bois cedex, France). Ultrapure analytical water was obtained from an ELGA PURELAB classic system from Veolia Water STI (Saucats, France).

2.2 Instruments and analytical conditions

Dionex Ultimate 3000 chromatograph System (Thermo Scientific, Waltham, Massachusetts, USA) coupled to a 2475 multi wavelength fluorescence detector

(Waters, Mildford, USA) was used for the analysis. Fluorescence detection was performed at 278 and 299 nm for excitation and emission wavelengths, respectively [23]. For the switching system, a Rheodyne 6 port valve (IDEX Health & Science, Erlangen, Germany) and a Spectra SYSTEM P1000 Isocratic (pump 1, **Figure 1**) were used (Thermo Scientific). System control, data collection and data processing were accomplished using Chromeleon software.

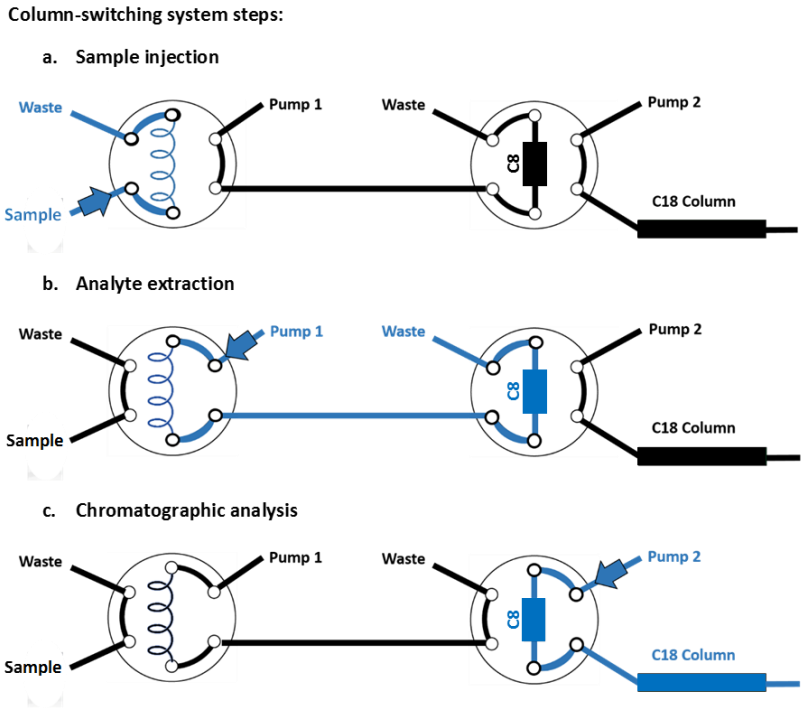

Figure 1: Column-switching system scheme with the different positions (a, b and c). In blue the connection where the analyte can be found in each moment

The column-switching for the on-line extraction was performed with a LiChroCART C8 column (4 x 4mm, 5 µm) (Merck, Darmstadt, Germany). The mobile phase consisted of an aqueous mobile phase of pH 2 adjusted with TFA (A) and acetonitrile as organic modifier (B) at 95:5 (A: B, v/v) proportion. A flow rate of 1 mL/min during

1.25 min was used in the extraction step. After, the analyte was eluted to the analytical column, HSS T3 (4.6 x 100 mm, 2.5 µm), from Waters, which was heated using a 100-040-220P column oven (Croco-cil, Cluzeau, France) to 40 °C. The mobile phase condition was at 60:40 (A:B, v/v) mixture at a flow rate of 0.8 mL/min. 40 µL of sample volume was injected.

2.3 Glassware treatment

Due to the sorption of caspofungin to glass surfaces [23, 28] all the glass material was treated with AATMS silanization agent following the procedure established in the work of Fukazawa *et al*. [29] for minimization of the peptide adsorption. In short, the material used was treated filling it with a solution of 1% AATMS in 1% of acetic acid. After few minutes, the liquid was removed and the material was dried at 50 °C for 4 hours.

2.4 Standard solution and spiked cell culture samples

Individual stock solutions were prepared each validation day by dissolving commercial CSF diacetate powder in Milli Q H_2O at a concentration of 800 mg/L of CSF. An intermediate solution of 50 mg/L CSF and the calibration standards, with concentrations from 10 to 1000 µg/L of CSF, were freshly prepared each day of analysis in RPMI-1640. The quality control (QC) samples were prepared by dilution to four levels of concentration: 750, 500, 30 and 10 (LLOQ) µg/L of CSF in RPMI-1640.

The different solutions in MOPS used were freshly prepared each experimentation day from the stock solution: 20 µg/L CSF for the switching optimization and QCs concentration for the recovery.

2.5 Column-switching optimization

Column-switching procedure is based on an on-line extraction of the sample (**Figure 1 b**) before eluting the analyte to the analytical column where the chromatographic separation is performed (**Figure 1 c**).

In preliminary trials, results showed a high variability in CSF chromatographic peak area when using a 20% of ACN in the mobile phase of the extraction step, probably due to an excessive elution strength of the mobile phase mixture. Based on this, the range studied in the optimization was limited from 5% until 15% ACN. The switching time (from 0.5 min to 2 min) was also optimized. A 20 µg/L CSF diacetate solution in RPMI-1640 was used for this study. Chromatographic peak areas and their variation (RSD %) were taken into account to decide the optimum conditions.

To test the loading capacity of the extraction column, two columns with different dimensions were studied: 4 x 4 mm and 4.6 x 25 mm (Lichrospher, Interchim, Montluçon, France). A 750 µg/L CSF in RPMI-1640 sample was analysed and five replicates were injected in each extraction column. Chromatographic peak areas were compared to study if there were significant differences. Additionally, the recovery of the on-line sample treatment was calculated at the different QC levels by analysing samples in MOPS buffer at pH 7 with and without column switching. The recovery was calculated by the ratio of the chromatographic peak areas obtained in the different conditions.

2.6 Validation of the column-switching HPLC-FLD method

The method was validated in terms of selectivity, carryover, linear concentration range, stability, accuracy and precision following the criteria listed in the last guideline on bioanalytical method validation of the Food and Drug Administration (FDA) [30].

The RPMI-1640 cell culture medium is a matrix prepared in laboratory, not a biological matrix as blood or urine, with a small variability and scarce sources. In consequence, authors decided that three sources were enough to ensure the reliability of the selectivity of this methodology. Selectivity of the method was assessed comparing different RPMI-1640 cell culture media in order to evaluate if

there were interferences. A selective method should not have interference of more than 20% the response of the lower limit of quantification (LLOQ).

The absence of carryover was checked injecting RPMI-1640 blank samples after the injection of a high concentration sample (750 µg/L CSF). The response in the blank was compared with the response in the LLOQ. For acceptance, the signal obtained in blank samples should be lower than 20% the response of CSF at the LLOQ.

The selected range for the calibration was from 0.01 mg/L to 1 mg/L in order to be able to use this method in different contexts. This range was based on CSF pharmacokinetic data [25-27]. In order to study the effect of the matrix, calibration curves in MOPS buffer and in RPMI were built and compared. For validation, the calibration curve was built in RPMI using 6 calibration levels (10, 50, 90, 300, 600 and 1000 µg/L CSF) repeated at three different days. The curve was adjusted using a regression weighted by a factor of $1/x^2$. As established in the guidelines, non-zero calibrators should be ± 15% of the nominal concentration except at LLOQ where the calibrator should be ± 20% of the nominal concentrations in each validation run. LLOQ was defined as the lowest nonzero standard on the calibration curve. The sensitivity of the method was evaluated regarding the intended use and ensuring the reliable quantification in terms of accuracy and precision.

Accuracy and precision were evaluated at LLOQ (10 µg/L) and three QCs at 30 (LQC), 500 (MQC) and 750 (HQC) µg/L CSF. Each validation day, five replicates of LLOQ and QCs were injected. Acceptance of accuracy and precision was expressed in terms of relative error (RE) of the nominal concentration and relative standard deviation (RSD), respectively. The acceptance criterion for accuracy was that the mean value should be within 15% of the nominal value (20% for the LLOQ) and precision was that % RSD should be ± 15% (20% for LLOQ).

The long-term stability of the analyte was studied in the stock solution (800 mg/L) in H_2O. LQC and HQC samples' (in RPMI) stability was checked at room temperature

(autosampler conditions). Concentrations obtained in the different conditions were compared with the nominal concentrations of the fresh ones. The mean concentration in each case should be ± 15% of the nominal concentration to be considered acceptable.

2.7 Culture assay and analysis of CSF in cell culture medium

One clinical isolate of *C. glabrata* (CAGL18) was selected into IICiMed collection to perform the study of cell growth under caspofungin treatment. CAGL18 is a susceptible to caspofungin with a MIC of 0.5-1 µg/mL, determined by the broth microdilution method with RPMI 1640 medium according to the procedures of the Clinical and Laboratory Standard Institute described in document M27-A2 [31]. The isolate was stored at -80 ^{o}C for long-term storage and grown on Sabouraud dextrose agar at 37 ^{o}C for 24h prior to experiments.

10 mL of yeast suspension containing 1 Mac Farland were prepared in RPMI medium (with L-glutamine and without sodium bicarbonate, 1% glucose, 0.0825 mol/L MOPS and with pH = 7.0) from 24 h cultures on Sabouraud dextrose agar at 37 ^{o}C. 2 mL of yeast suspension were inoculated into 18 mL of RPMI medium in a 50 mL erlenmeyer pre-treated with AATMS. When necessary 50 µL of fresh caspofungin stock solution (50000 µg/L in DMSO) were added to a final concentration of 125 µg/L. Erlenmeyers were incubated at 35 ^{o}C under agitation (110-150 rpm) in a non-CO_2 incubator. Samples were collected from the erlenmeyers from 0 to 4 hours each hour and after at 6, 8 and 24 h. 50 µL were counted on Malassez cells. Growth curves were obtained and displayed as log (number cells) = f (time of culture). In each sampling time (0, 1, 2, 3, 4, 6, 8, and 24 h), 100 µL of sample were transferred to vials for the direct injection of 40 µL in the chromatographic system.

3. Results and discussion

3.1 Column-switching optimization

When optimizing the column-switching procedure, the organic modifier percentage and the switching time were studied simultaneously. The results shown (**Figure 2**) that a higher CSF peak area was obtained when using 5 % of ACN. This proportion was fixed as the optimum for the analysis. Regarding the switching time, no significant difference was seen in the chromatographic peak area when increasing it. However, a minimum of 1 min was required to remove a compound eluting just after the injection peak. Taking this into account, a switching time of 1.25 min (the equivalent to 20 times the volume of the extraction column) was chosen for the routine analysis.

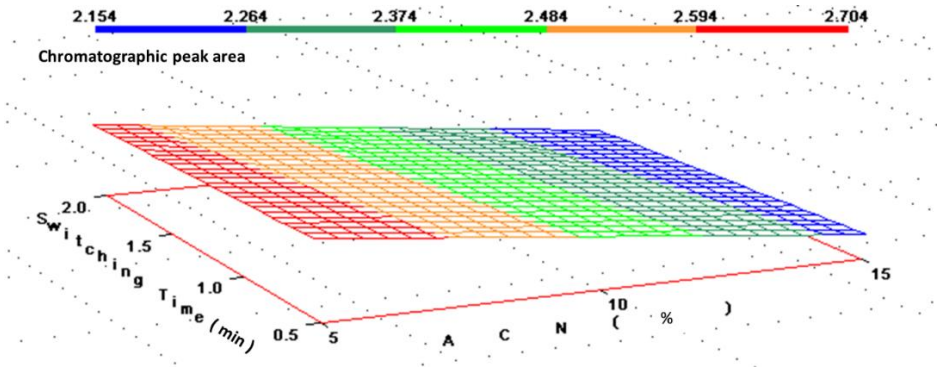

Figure 2: Response surface of CSF chromatographic peak area. Switching time range from 0.5 to 2 min and ACN % range from 5% to 15%

Due to the previously mentioned matrix complexity of cell culture, two different extraction column dimensions were checked for the on-line analyte extraction with the aim of studying a potential saturation of the column, which may lead to a loss of CSF. No significant differences were observed ($p>0.05$) when comparing the responses of different extraction columns. Consequently, the loading capacity of the smaller column (4 x 4 mm) was found sufficient for the retention of caspofungin at high concentration

The recovery of CSF using the sample treatment by column switching was calculated (**Table 1**). These results demonstrate that this on-line approach allows a sample treatment with a high recovery for CSF.

Table 1: Recovery results (± standard deviation) of the on-line sample treatment (4 x4 mm extraction column, n=5) for different QC samples and LLOQ.

Sample	HQC	MQC	LQC	LLOQ
Recovery (%) ± s	109 ± 3	108 ± 4	107 ± 5	108 ± 13

3.2 Validation of the column switching HPLC-FLD method

3.2.1 Selectivity

Different RPMI-1640 cell culture media solutions were compared. Thanks to the on-line sample preparation, no interference from the different constituents of the RPMI was observed at CSF retention time with the different cell culture solutions analysed. **Figure 3** shows the RPMI analysis in this method.

3.2.2 Carryover

No chromatographic peak at the CSF retention was observed in the blank analysed after injecting the HQC sample.

3.2.3 Lower limit of quantification and linear calibration range

Calibration curves performed in both MOPS and RPMI-1640 matrices at a pH value of 7, were compared. The linear regressions were $y = 0.0277 x - 0.2068$ ($R^2 = 0.9986$) and $y = 0.0241 x – 0.3460$ ($R^2 = 0.9992$) for MOPS and RPMI, respectively. A significant difference in the slope value was found proving that there is an effect of the matrix. For this reason, the validation was totally performed with CSF samples in RPMI-1640 matrix.

Data obtained from the chromatographic peak area of CSF were treated using a weighted linear regression ($1/x^2$). In **Table 2**the linear regression equations of the different calibration curves built are given (Response = slope · concentration + intercept).

Table 2: Weighted ($1/x^2$) calibration curves and the coefficient of determination (r^2) obtained in the different validation days

Day	Curve equation	r^2
1	$y = 0.0217x - 1.1225 \cdot 10^{-5}$	0.9973
2	$y = 0.0232x - 6.6396 \cdot 10^{-5}$	0.9998
3	$y = 0.0241x - 5.4115 \cdot 10^{-5}$	0.9992

The lowest nonzero calibrator was established as the LLOQ with less than 20% of variability (8.1%) in the nominal value. **Figure 3** shows chromatograms obtained with the injection of the RPMI medium and the injection of this concentration level of CSF prepared in RPMI. At the retention time of CSF, no signal was observed for the blank analysis.

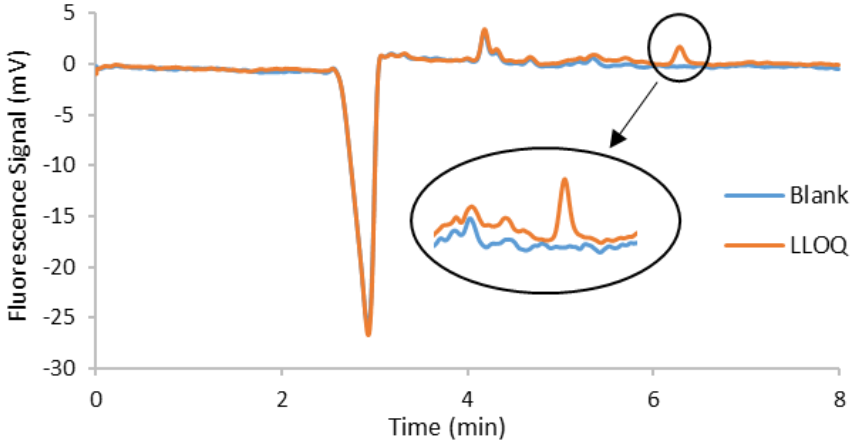

Figure 3: Chromatograms of RPMI-1940 blank and CSF at LLOQ concentration (10 µg/L CSF)

3.2.4 Accuracy and precision

The results obtained for accuracy and precision are shown in **Table 3**. The mean value of the % RE of the nominal concentration was lower than 15 % at all concentration levels in both assays (intra-day and inter-day). The high variability in the accuracy within days has to be highlighted. In **Table** , apart from the mean value of the three days, the minimum (min) and maximum (max) values obtained are shown.

Table 3: Intra- and inter-day accuracy and precision results (n= 3 days x 5 replicates) in terms of % RE and % RSD, respectively, at four concentration levels (10, 30, 500 and 750 µg/L CSF)

Concentration level (µg/L)	**Intra-day**		**Inter-day**	
	Accuracy (% RE) Mean (min-max)	**Precision** (% RSD) Mean (min-max)	**Accuracy** (% RE)	**Precision** (% RSD)
10 (LLOQ)	13.6 (12.5-15.3)	8.5 (7.5-10.4)	13.6	8.1
30 (LQC)	10.8 (2.2-16.4)	5.6 (4.5-6.1)	10.7	8.8
500 (MQC)	9.6 (0.35-16.1)	7.8 (7.5-8.4)	9.4	9.8
750 (HQC)	7.6 (1.0-12.7)	6.7 (2.6-8.7)	7.6	8.4

3.2.5 Stability

The stock solution of 800 mg/L CSF in H_2O was stable at -80 ^oC for at least 1 month. LQC and HQC samples were stable for 2 hours at room temperature in RPMI. From that time on, a significant loss of CSF was observed. Based on these results, samples must be analysed in a short period of time after sampling and stability is considered a critical parameter for CSF determination in cell culture media.

The results obtained for selectivity, carryover, linear concentration range, accuracy and precision met the acceptance criteria of the guideline followed, hence the method was successfully validated.

3.3 Analysis of CSF in cell culture medium

Yeast cells were cultured with or without caspofungin (125 µg/L) for 24h at 35°C under agitation. Cell counting was performed at different times (see sampling times in section 2.7) to obtain growth curves. In addition, free caspofungin contained in the cell culture medium was quantified. As observed in **Figure 4**, cell proliferation was reduced in the presence of caspofungin. The partial and transitory blocking of proliferation is coherent with the choice of a subinhibitory concentration of caspofungin (MIC/4). In parallel, a decrease of the concentration of free caspofungin was shown during the experiment. In the control or in the presence of cells, the concentration reduction was similar. This phenomenon could be due to the poor stability of caspofungin or to the adsorption phenomenon described before [23]. However, maybe due to the use of a column switching, we can eliminate some degradation products.

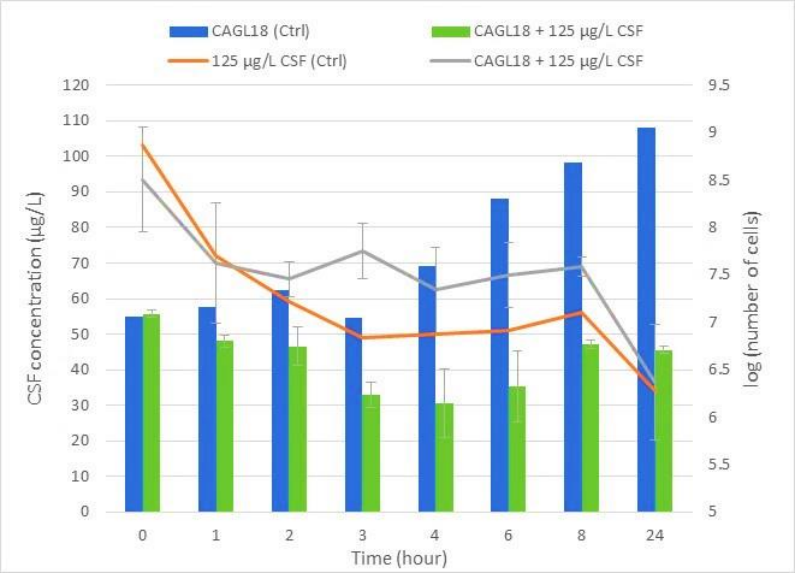

Figure 4: Variation of CSF concentration in absence of fungal cells (orange curve) and in the presence of fungal cells (grey curve, CSF concentration mean of two experiments with error bars representing min and max values), and cell proliferation without CSF (blue histogram) and with a dose of 125 µg/L of CSF (green histogram, log number of cells mean valueof two experiments with with error bars representing min and max values)

4. Conclusions

The column-switching HPLC-FLD method developed allows the simple and rapid quantification of caspofungin antifungal drug in RPMI-1640 cell culture media. The effect of the matrix in the chromatographic response obliges to use RMPI to build the calibration curve. Nevertheless, no significant differences were observed among the calibration curves built on different days nor among the different RPMI matrices employed. The method showed a high recovery and was successfully validated according to the criteria established in the guideline on bioanalytical method validation of the FDA [30] in terms of selectivity, carryover, linear concentration range, accuracy and precision. The loss of CSF signal over time in the medium was found as a critical variable that could have a negative impact in the accuracy of the method. Due to this reason, the analysis should be performed immediately after sampling. The observed effect of the matrix obliges to use the same medium for calibration, due to the difference in chromatographic peak area.

Comparing the method developed with the work of Egle *et al.* [24] for the determination of CSF in serum using a column-switching system, this work presents a faster analysis with a total run of 8 min instead of 30 min. Furthermore, the extraction stationary phase is C8 instead of diol. Above all, the LLOQ decrease has to be mentioned, from 200 µg/L to 10 µg/L.

Results from the *in vitro* experiment confirmed that the method we described in this study allows to follow free CSF concentration in RPMI-1640 in classical conditions of cell culture without any prior sample treatment. This method will be useful to study CSF in different *in vitro* approaches in order to be able to study *C. glabrata* strains and emergence of multidrug resistance in clinical isolates, which led to more and more therapeutic failures.

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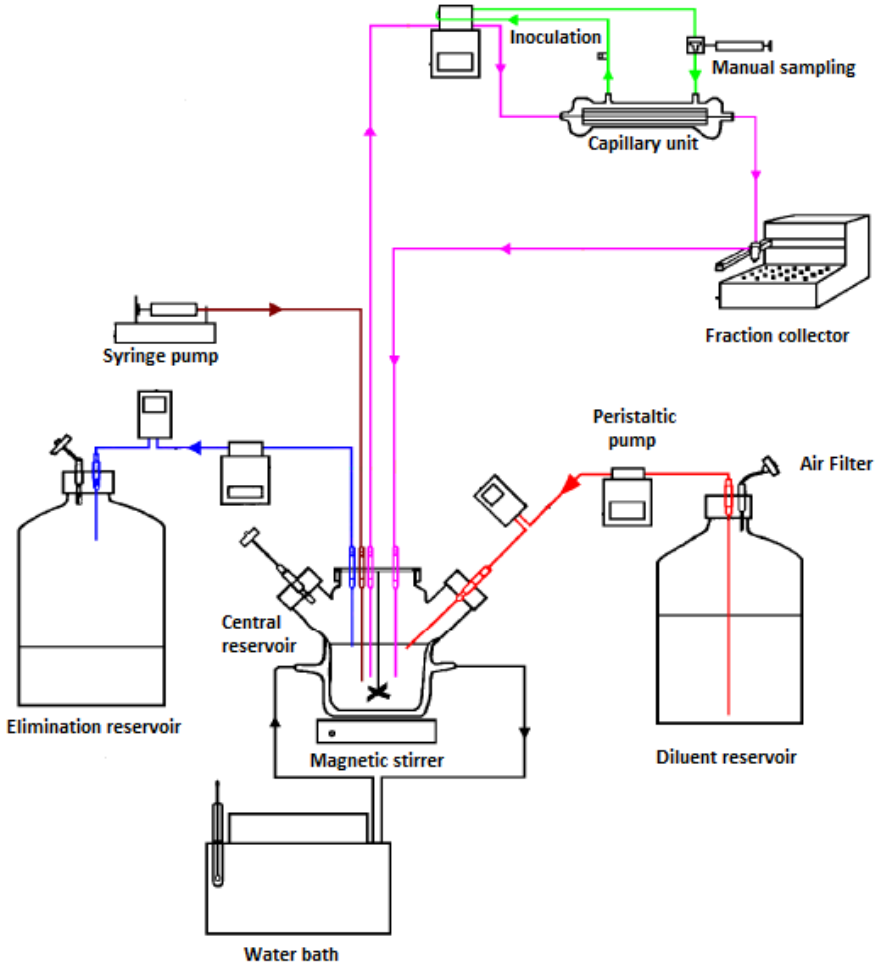
4. Kapitulua

Kandidaren kaspofunginarekiko erresistentziaren in vitro ikerketa SIMULI-PHARM 1 eredua erabiliz



1. Sarrera

Azken urteotan, gorakada nabarmendu da infekzio fungiko inbaditzaileen (IFIen) kasuetan, batez ere, ospitalizaturik dauden eta immunitate arazoak dituzten pertsonen artean [1-3]. Kandidiasia IFIrik ohikoena izan ez ezik, giza erikortasunaren eta hilkortasunaren arrazoi garrantzitsu bat ere bada [4]. Egun, infekzio fungikoei aurre egiteko lau farmako-familia erabili ohi dira: azolak, polienoak, ekinokandinak eta pirimidinak [5]. Dena den, azken urteetan onddo askok azolekiko garatutako erresistentziaren ondorioz, ekinokandinak kandidiasiaren tratamendurako lehen aukera bihurtu dira [6-9].

Ekinokandinek infekzio fungikoei aurre egiteko aukera berri bat eman zuten ezagutzera XX. Mendearen amaieran. Droga familia horrek akzio mekanismo berri bat jarri zuen agerian; β-(1,3)-D-glukan sintasaren inhibizioa [10,11], hain zuzen ere. Ekinokandinen familian garatutako lehen farmakoa kaspofungina (CSF) izan zen, 2001. urtean [12-14]. Ekinokandinak fungizidak dira kandida espezieen gehiengoarekiko, baita azolekiko erresistentzia erakusten duten zepekiko ere.

Urteak pasa ahala ekinokandinekiko onddoen erresistentzia baxu jarraitu arren, kandidaren lehen kasua antzeman zenetik, 2005. urtean, erresistentzia kasuen kopurua nabarmen handitu da [15]. Kandidiasi inbaditzaileak medikuntza arloan hartzen ari duen garrantzia, gertatzen ari diren akats terapeutikoekin batera (batez ere CSFrekin), farmakoen erresistentzia ikerketen premia jarri dute agerian [4,16,17]. *In vitro* erresistentzia-ikerketa egokiak behar dira antifungikoen eraginkortasuna ebaluatu eta konparatu ahal izateko.

Antifungikoek droga baten aurrean duten jarduera aztertzeko, hainbat *in vitro* metodo garatu dira urteetan zehar. Metodo horietan, ikertu beharreko onddoa, legamia edo lizuna farmakoarekin jartzen da kontaktuan. Ondoren, farmako horrek denboran zehar mikroorganismoan duen eragina aztertzen da, hala nola, zelulak hiltzen diren edo zelulen hazkundea oztopatzen den. Garatutako lehenengo

metodoetan droga kontzentrazio finkoa erabiltzen zen (sarrera orokorrean aipaturiko “sistema estatikoak”). Horien artean, denbora-heriotza ikerketak, disko sentikortasun testak eta kontzentrazio inhibitzaile eta bakterizida minimoen determinazioak aurkitzen dira [18].

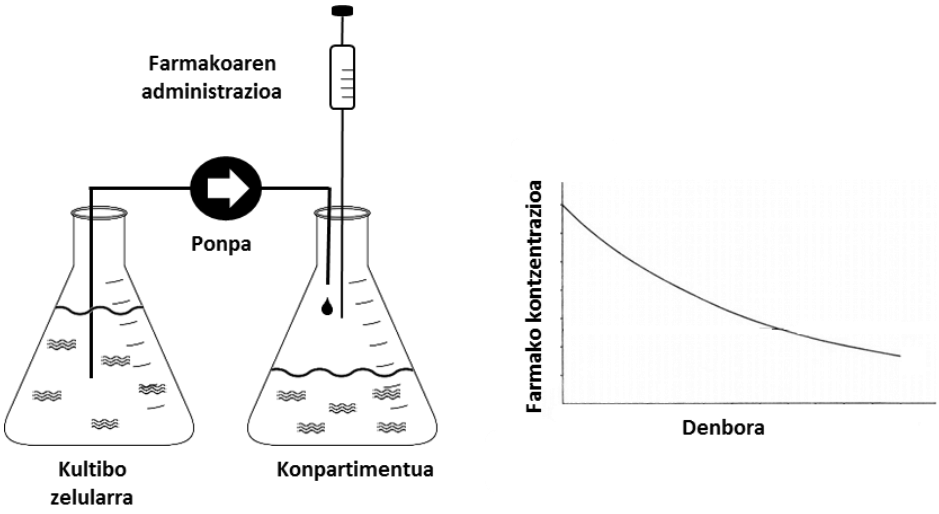
Dena den, antimikrobiano bat hartzean, gorputzeko farmakoaren kontzentrazioa aldatzen doa denboran zehar, gertatzen diren drogaren absortzio, distribuzio eta eliminazio prozesuak direla eta. Horregatik, azken hamarkadetan, bakterioak farmakoen kontzentrazio aldakorretara aurrez aurre jartzen dituzten *in vitro* metodoak garatu dira. Metodo farmakozinetiko/farmakodinamiko (PK/PD) dinamikoak deritze horiei (sarrera orokorrean azaldutako kontzeptua). Gehiengoak, aho, zain barneko edo muskulu barneko administrazioaren ondoren, serum, ehun edo gernuan aurki daitezkeen farmako kontzentrazioak simulatzen dituzte [18].

Metodo horiek baliabide erabilgarriak dira antibiotikoen eraginkortasuna aztertzeko, parametro bakoitza bereizita kontrolatu daitekeelako. Parametro horien artean dosia, parametro farmakozinetikoak eta kultibo zelularraren baldintzak aurkitzen dira. Antibiotikoen kontzentrazioa eta bakterioen populazioa denboran zehar ebaluatzeko erabiltzen dira eredu dinamikoak. *In vitro* eredu PK/PD dinamikoak konpartimentu bakarreko (diluzio metodoak) edo bi konpartimentuko ereduetan (difusio metodoak) banatu ohi dira [18-21]. Horien arteko ezberdintasun nagusia da, konpartimentu bakarrekoan farmakoaren adizioa eta mikroorganismoekiko interakzioa konpartimentu berdinean ematen dela. Bestean, bi konpartimentu ezberdinetan gertatzen da hori.

1.1 Konpartimentu bakarreko ereduak

San Filippok eta Morvillok [22] plasman aurkitzen diren antibiotikoen kontzentrazioak erreproduzitzen zituen lehen eredua garatu zuten 1968. Urtean (**1.Irudia**). Sistema horretan, bi Erlenmeyer erabiltzen dira, tutu baten bidez elkarri konektatuak. Batean, konpartimentu deritzona, bakterioak kultibo zelularraren

baitan daude eta antibiotikoa gehitzen zaio kultiboari esperimentuaren hasieran. Bestean, ordea, kultibo zelular garbia dago eta, ponpa peristaltiko bat baliatuta, beste Erlenmeyerrera gehitzen zaio likido hori. Horrela, konpartimentuan dagoen antibiotikoaren kontzentrazioa diluitzen da, gorputzean gertatzen den farmakozinetika simulatzeko asmoarekin. Esperimentuan zehar tenperatura kontrolpean mantentzen da, gorputz tenperaturan hain zuzen ere (37 ^{o}C).



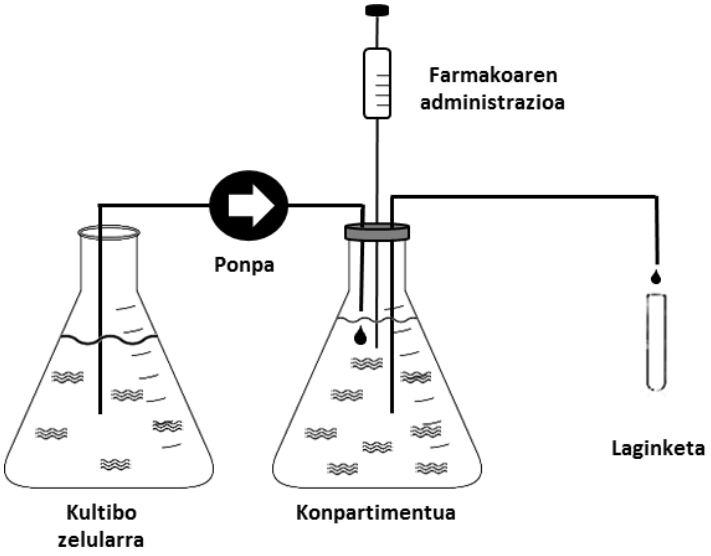
1. Irudia: San Filippo eta Morvilloren konpartimentu bateko ereduaren eskema eta bertan lortutako farmako kontzentrazioen aldaketaren profila

Antimikrobiano gehienen kasuan, serumak giza gorputzean duen zinetika egoki simulatu daiteke lehen ordenako ekuazio zinetikoari jarraituz [20], farmakoaren jaitsiera esponentziala gertatzen da denboran zehar (**2. Irudia**). Eredu horretan, kultibo zelular garbia modu linealean gehitzen denez, ezinezkoa da drogaren profil zinetiko errealaren simulazioa egitea. Hau, ordenagailu batez kontrolatutako ponpa erabiliz konpondu daiteke, bertan behar izaneko fluxu aldaketak programatu ahal direlako..

2. Irudia: Odolean ematen den farmakoen kontzentrazio aldaketa denborarekiko

Horretaz gain, eredu horrek beste arazo bat aurkezten zuen; likidoa gehitu ahala konpartimentuan dagoen bolumena handitzen doa. Simulatu beharreko kontzentrazio minimoak hasierakoak baino 10 edo 100 aldiz txikiagoak izan ahal direnez, esperimentuaren amaieran eduki ahal den bolumena oso handia izan daiteke. Batzuetan, ikuspuntu praktiko batetik maneiatzeko zaila izan daitekeena: konpartimentuko ontzia oso handia izan behar da, kultibo zelular asko behar da… [18].

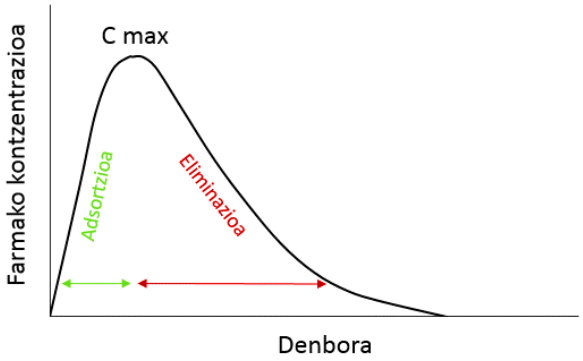
Azken arazo horri konponbidea emateko, Otaya *et al.* [23] eta Grasso *et al.-ek* [24], farmakoaren kanporatzea simulatzeko sistema gehitzen zuten ereduak garatu zituzten. Horrela izanik, Grasso *et al.*-ek, aurreko ereduaren eskemari, beste tutu bat gehitu zioten eliminazioa edota laginketa aurrera eramateko (**3. Irudia**). Ereduan, likidoaren zirkulazioa ziurtatzeko, konpartimentua itxita dago eta ia likidoz betea. Modu horretan, kultibo zelular garbia konpartimentura gehitzen den ahala, presioa dela eta, likidoa ere konpartimentutik eliminatzen da. Esan bezala, *in vitro* esperimentuak tenperatura kontrolatuan egin behar direnez, sistema (**3. Irudia**) 37 ^{o}C ziurtatzen dituen esparru edo kutxa batean jartzen da.



3. Irudia: Grasso *et al*.- ren [24] konpartimentu bateko eredua

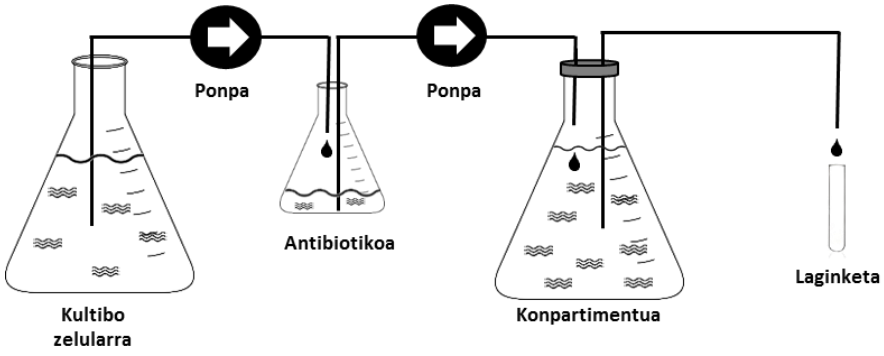
Esperimentua hasten den momentuan hazkunde fasean dagoen bakterio suspentsioari antibiotikoa gehitzen zaio. Kultibo zelular berria gehitzen den heinean, nahastea kanporatzeko prozesua ere hasiko da. Gehitzen den kultibo zelular garbiaren fluxuak eta drogaren eliminazio fluxuak berdinak izanez, farmakoaren erdibizitza simulatzea lortu zuten.

Grasso *et al*.-en lan horretan [24], bigarren eredu bat proposatu zuten. Sistema horretan, hirugarren Erlenmeyer bat gehituz, antibiotikoen lehen ordenako bi prozesu jarraian lortzen dira, bat farmakoaren adsortzioa prozesuari dagokiona, eta bestea, eliminazio prozesuari (**4. Irudia**). Hori antibiotikoak muskulu-barnean edo ahoz administratzen direnean gertatzen da.



4. Irudia: Aho edo muskulu barneko administrazioaren ondoren odolean ematen den farmakoaren kontzentrazio aldaketa denborarekiko, adsortzio (orlegiz) eta eliminazio (gorriz) prozesuekiko zatiak ezberdinduz

Hiru Erlenmeyerrak eta laginketa sistema seriean daude konektatuta zenbait tuturen bitartez **5. Irudian** ikus daitekeen moduan. Aurreko ereduarekin konparatuz, antibiotikoa konpartimentura gehitu beharrean Erlenmeyer batean jartzen da kultibo zelular garbian disolbatuta. Ponpa peristaltiko baten bidez, kultibo zelular garbia gehitzen zaio esperimentua hasten den momentutik hasita. Denbora pasa ahala antibiotikoaren diluzio esponentziala gertatzen doa konpartimentura heldu baino lehen. Ponpa peristaltiko baten bidez antibiotikoa gehitzen da bakterioak dauden Erlenmeyerrera



5. Irudia: Grasso *et al*.- ren [24] 2. eredua, ahoko eta muskulo barneko administrazioak simulatzeko

Hala ere, eredu horietan eragozpen handi batekin egin zuten topo *in vitro* esperimentuak era egokian aurrera eramateko: farmakoaren diluzioa egitean, bakterioen kontzentrazioa ere diluitzen zen. Egile batzuek zuzenketa matematiko bat proposatu zuten diluzio efektu honen eragina ekiditeko [25,26].

Bakterio kontzentrazioaren diluzio efektua esperimentalki minimizatzeko asmoarekin, Lowdin *et al.*–ek [27] mintz erdiiragazkor bat gehitu zioten haien ereduari. Mintz horrek kultibo zelularra iragazten uzten zuen eta, ondorioz, bakterioak Erlenmeyerrean isolatzen ziren. Era berean, Schneider *et al.*-ek [28] iragazkiaz gain, fotometro bat ere gehitu zioten [29,30]. Konpartimentuaren alde batetik bestera transmititzen zen argia neurtzen zuten, uherdura kalkulatuz. Uhertasunaren aldaketa bakterioen hazkundea aztertzeko erabili daiteke, zelulen zenbaketarekin batera. Pareko eredu baten automatizazioa garatu zuten Ledergerber *et al.*-ek [29], gentamizinaren dosi anitzeko 72h-ko erregimenen simulazioa egin ahal izateko.

Nahiz eta mintz erdiiragazkorrak bakterioak Erlenmeyerrean isolatu, denbora pasa ahala mintzak asetzen doaz. Batez ere, bakterio kontzentrazio altuak behar dituzten esperimentuetan [18], mintza asetzean sistemaren presioa igotzen da eta zaila izaten da, ondorioz, fluxua konstante mantentzea. Hori horrela izanik, konpartimentuko bolumena aldatzen da eta esperimentuaren emaitzak kolokan jartzen dira.

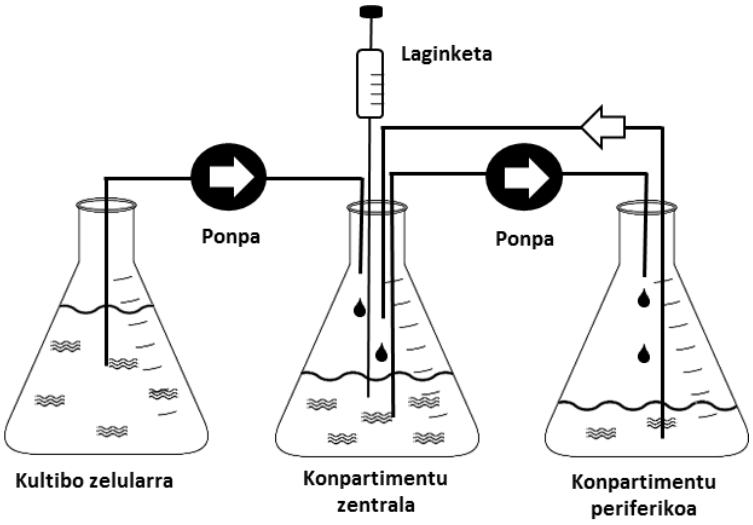
1.2 Bi konpartimentuko ereduak

Farmakoen profil farmakozinetikoak lehen ordenako zinetika jarraitzen duela esan dugu orain arte lan honetan zehar. Hala ere, egia esan, antibiotikoa administratu ondoren gorputzean zehar distribuzio prozesu labur bat gertatzen dela aurkitu zen, eta denbora tarte horretan gorputzean gertatzen den profil zinetikoa bigarren ordenako zinetikak hobeto azaltzen zuela ikusi zen [18].

Hori kontuan hartuta eta aurretiaz garatutako ereduen arazo ezberdinei aurre egiteko, bi konpartimentuko ereduak garatu ziren. Konpartimentu horiei, zentrala (KZa) eta periferikoa deritze (KPa) [18,20,21]. Konpartimentu periferikoaren gehipenak distribuzio prozesua simulatzea ahalbidetzen du..

1.2.1 Murakawa-ren eredua

1980. urtean Murakawa *et al.*-ek [31] bi konpartimentuko eredu bat garatu zuten droga zain bidez administratu ondoren odolean lortzen ziren antibiotiko kontzentrazio aldaketak simulatzeko helburuarekin. Hiru Erlenmeyer erabiliz (**6. Irudia**) hasierako distribuzio prozesuaren ondorioz gertatzen den bigarren ordenako zinetika simulatu zuten. KZa bi tuturen bidez dago konektatuta KPra nahastearen joan-etorria bermatzeko. Esperimentuan zehar konpartimentu zentralera kultibo zelular garbia gaineratzen zaio ponpa peristaltiko baten bitartez.



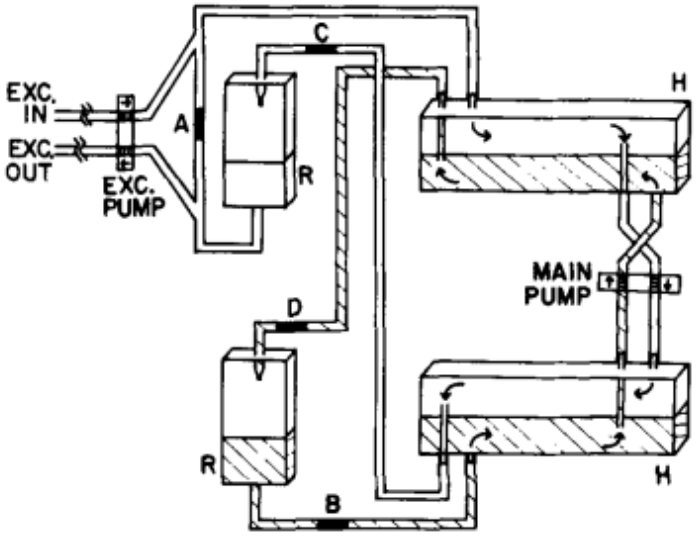
6. Irudia: Murakawa *et al*.-ren [31] eredu bi konpartimentalaren eskema

Eredu horretan, bigarren ordenako zinetika lortzen da bi konpartimentuen arteko nahasketa eta kultibo zelular garbia gaineratuz. Farmakoaren profil egokia lortu arren, bertan ez dago bakterioak konpartimentu zentralean isolatzeko sistemarik (ez

zioten mintz erdiiragazkorrik gehitu) eta bakterio kontzentrazioa diluitzen doa denbora pasa ahala. Ez hori bakarrik, eredu horretan odolean gertatzen den farmako kontzentrazioaren aldaketa simulatzeko diseinatuta dago, eta infekzio askotarako egokiagoa da ehunetan edo likido interstizialean ematen diren kontzentrazio profilak simulatzea.

1.2.2 Toothaker-en eredua

Ehunetan gertatzen den zinetika simulatzeko gai zen bi konpartimentuko eredu berri baten emaitzak aurkeztu zituzten Toothaker *et al*.-ek [32] 1982. urtean. Sistema horretan, konpartimentuak, mintz erdiiragazkor batez konektatutako hemodialisiko bi unitate dira (**7. Irudia**). Konpartimentu batean bakterioak jartzen dira eta farmako disoluzioa bestean. Droga mintzaren alde batetik bestera mugitzen da, gorputzeko ehunetako profil zinetikoa simulatzen.

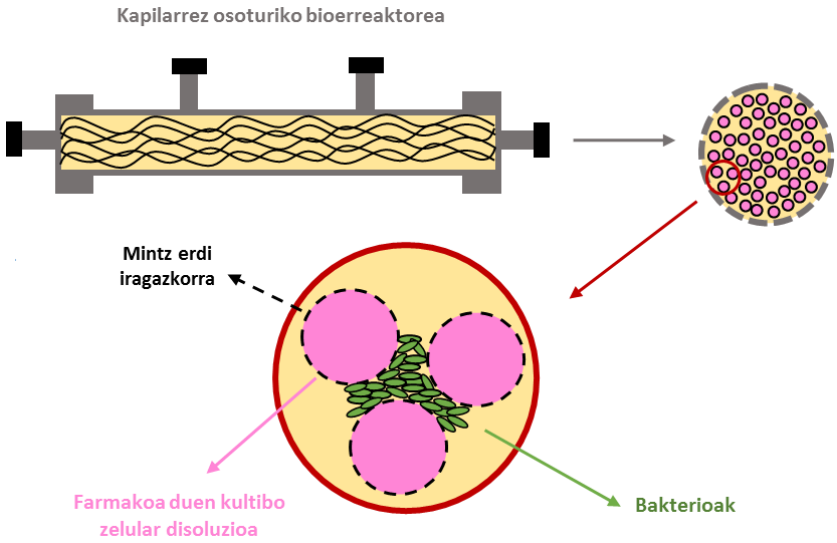


***7.* Irudia:** Toothaker et al.-en ereduaren eskema [32]. H letraz identifikatutako ontziak hemodialisiko unitateak dira

Blaser *et al*.-en [18] ustez, eredu horrek erabiltzen zituen bolumenak direla eta, muntaia handiegia behar zen eta, ondorioz, sistema txikitzea gomendatzen zuten [18,33]. Haien ustez, txikiagoak ziren orokorrean simulatu beharreko infekzioetan infektatutako bolumenak eta muntaia txikituz, ere, haren maneiua erraz zezakeen.

1.2.3 Kapilar hutseko eredu bikonpartimentalak

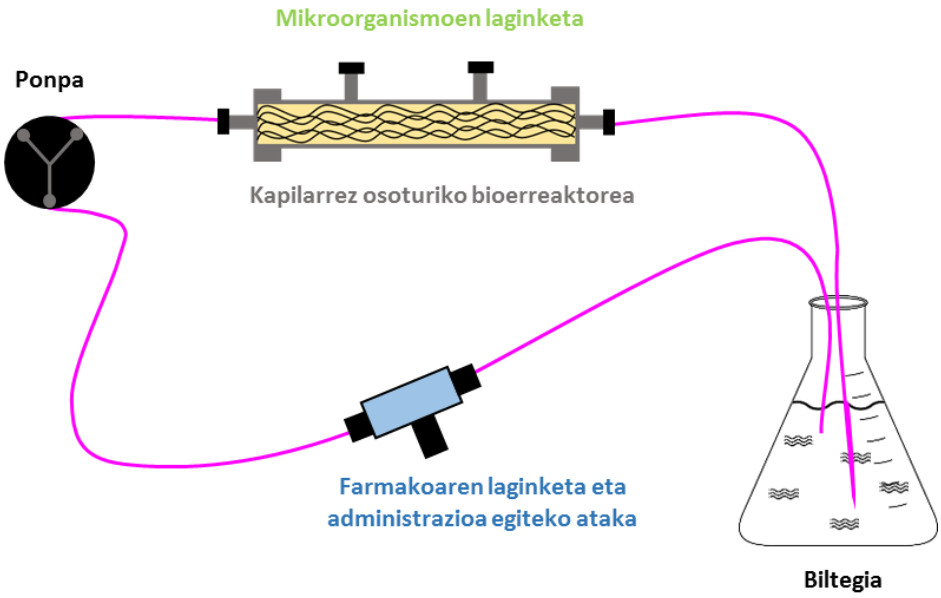
In vitro ereduen simulazioa hobeagotzeko ideiari jarraituz, Zinnerrek *et al*.-ek [34] kapilarrez osaturiko bioerreaktore bat gehitzea proposatu zuten antibiotikoen PK/PD ikerketa aurrera eramateko [20,35]. Hots, biologikoki aktiboa den ingurune bat mantentzeko gai den ontzi edo sistema. Eredu hori, antibiotikoak administratzeko bidearen ondorioz (zainetik, ahoz edo muskulu barnean) likido interstizialean gertatzen den profil zinetikoa simulatzeko gai da. Eredu horietan, KZa bioerreaktoreko mintzek, biltegia eta hainbat tutuek osotutako zirkuitua dira (**8. Irudia**). KPa, ordea, mintz horien ateko tartea osatzen dute.



8. Irudia: Bioerreaktore kapilarraren eskema

Biltegitik ateratzen den kultibo zelular garbiaren fluxuan, administrazio atakaren bitartez farmakoa gehitzen da, eta nahastea bioerreaktoretik pasarazten da. Behin farmakoa bioerreaktorera heltzen denean, mintz erdiiragazkorrez egindako kapilarretatik pasaraztean, farmakoa KPera igarotzen da eta bakterioekin kontaktuan jartzen da (**9. Irudia**). Mintzen arteko tartean bakterioen eta farmakoaren arteko interakzioa gertatzen da. Esperimentuan zehar laginketa KZean egiten da farmakoaren kontzentrazioa determinatzeko eta KPan mikroorganismoen hazkundea ikertzeko.

Mintzaren poroen tamainari esker, bakterioak ezin dira kapilarren barnera pasatu eta bioerreaktore horietan isolatuta gelditzen dira. Horrela, bakterioen kontzentrazioa ez da diluitzen eta bakterioek sistema kutsatzea ekiditen da.



9. Irudia: Zinner *et al*.-ren [34] bi konpartimentuko ereduaren eskema

Lortutako emaitzen egokitasuna zela eta, garrantzia hartu zuten kapilar hutsetan oinarritzen ziren bi konpartimentuko *in vitro* ereduek [36-39]. Egindako ikerketen artean, administrazio denbora luzea behar duten antibiotikoak (ordu bat), bolo bidez edo ahoz ematen direnak simulatu dira [37]. Aipatu beharra dago, droga administratzeko modu ezberdinak egiteko, ordenagailuz kontrolatutako xiringak ere erabili direla nahi izandako ordutegiari jarraitu ahal izateko [39,40].

1.2.3.1 Ba et al. –en kapilar hutsezko in vitro PK/PD bi konpartimentuko eredua

SIMULI-PHARM 1 deritzon sistema Bordeleko unibertsitateko Farmazia Fakultatean garatu zuten Ba *et al*.-ek [41]. Sistema honetan, KZa beirazko erreaktore termostatizatu batek, bioerreaktoreko mintzek eta hauek konektatzeko erabilitako zenbait tutuk osatzen dute (**10. Irudia**). KPa, bioerreaktoreko mintzen arteko espazioa eta bertara konektatutako tutu batzuk osatzen dute. Eredua Blaser *et al.*-en [36] kapilare hutsezko bi konpartimentuko sisteman oinarrituta dago, baina zenbait ataletan hobekuntzak egin zaizkio:

- Administrazioa:

Blaser *et al*.-ren [37] ereduan aho administrazioa simulatzeko absortzio konpartimentu bat erabiltzen da. Aho administrazioa egin beharreko kasuetan, farmakoa erlenmeyer batean gehitzen da non kultibo zelularrarekin nahastatzen den KZera pasa baino lehen. Horrela, lehen ordenako adsortzio zinetika simulatzea lortzen da, **5. Irudiko** ereduan bezala. Ba *et al*.-ren ereduan, ordea, xiringaren maneiua ordenagailuarekin kontrolatu daiteke. Horrela, sistema berberarekin droga administratzeko hainbat modu simulatu daitezke, hala nola, ahoz, zainez, zain barneko bolo bidez edo muskulu barnean txertatuz.

- Diluzio- eta kanporatze-sistemak:

In vitro eredu PK/PD gehienek bi ponpa ezberdin erabiltzen dituzte: bata, kultibo zelular garbia gehitzeko, eta bestea, nahastea kanporatzeko. Nahitaezkoa da konpartimentuetan bolumena konstantea izatea. Horretarako, gehitzeko eta kanporatzeko sistemek fluxu berdina izan behar dute. Luzaro irauten duten esperimentuetan (48 h baino gehiago) zaila izaten da baldintza hori betetzea, ponpa peristaltikoetan plastikozko tutuek arrabolekin izaten duten marruskadura dela eta, tutuen diametroa apurka-apurka aldatzen joaten delako. Ondorioz, fluxua aldatzen doa. Beraz, ikerlariek fluxu neurgailu bat jarri zuten ponpa bakoitzaren ondoren. Neurgailu horiek benetako fluxua neurtu eta informazioa ordenagailura pasatzen dute, ondoren, ordenagailuak ponpa peristaltikoaren abiadura haren arabera aldatzen doa.

Bibliografian azaldutako ereduetan, antibiotikoaren profil plasmatikoa simulatzen da KZean eta KPan eduki beharreko kontzentrazioak lortzen direla suposatzen da. Eredu hau diseinatuta dago profil zinetikoa bai KZean, non farmakoa gehitzen den, bai KPan egoki simulatzeko. Software-ak ahalbidetzen du parametroen konfigurazioa dela eta, farmakoaren adsortzio, distribuzio eta eliminazio prozesuak simulatzea diluzio eta kanporatze sistemen laguntzaz.

- Konpartimentuen arteko kultibo zelularraren fluxua:

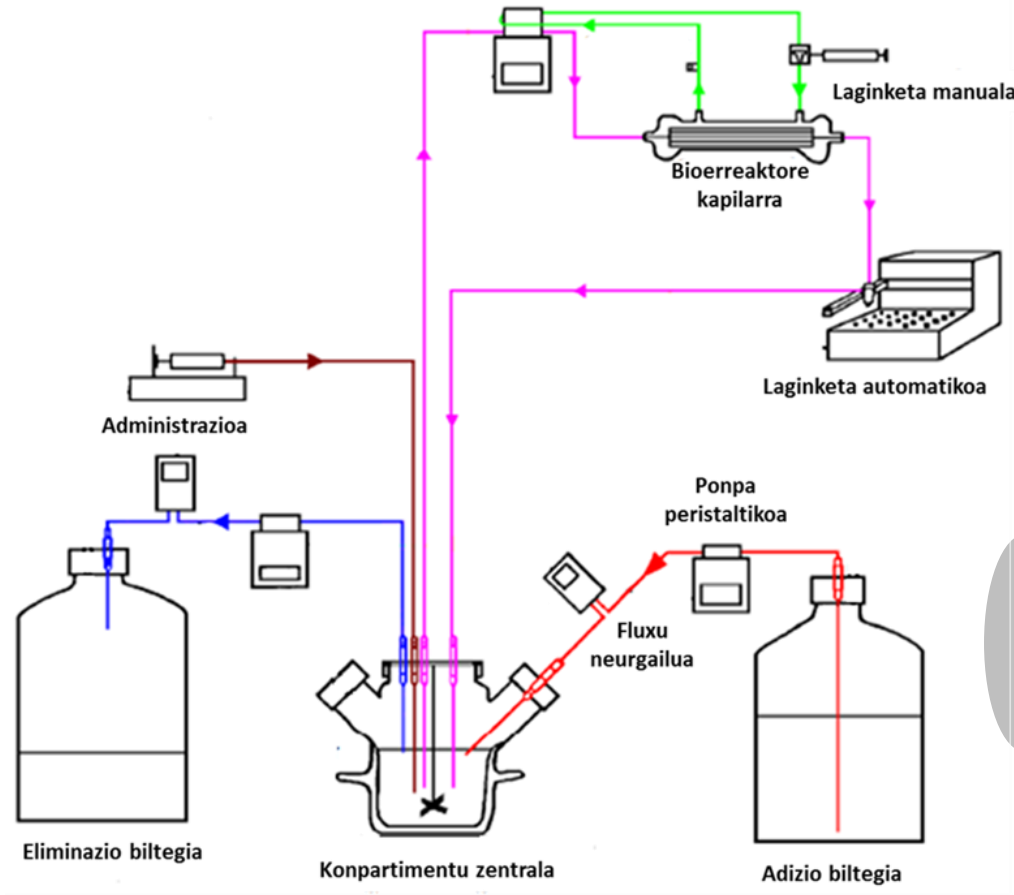
Bi konpartimentuen arteko fluxua ziurtatzeko bi kanaleko ponpa peristaltiko bat erabiltzen du SIMULI-PHARM 1 ereduak. KZeko tutuak diametro handiago bat edukiz, presioa dela eta, drogaren garraioa KZetik KPra azkar ematen da orekara helduz, 5 minutu baino gutxiagotan (laginketa tarteak baino txikiago). Gorputzean ematen den farmakoaren oreka oso azkarra denez, garrantzitsua da ereduetan horrela ere izatea simulazioa fidagarria izan dadin.

- Laginketa:

Bi konpartimentuen arteko profil zinetikoak egiaztatzeko eta sistemaren funtzionamendu egokia ziurtatzeko, automatikoki egin daiteke laginketa eredu honetan. Sistema guztia maneiatzen duen software-an laginketa denborak konfiguratu daitezke eta momentu horietan bi konpartimentuetan kokaturiko hiru portuko balbulen (bat konpartimentu bakoitzeko) posizioa aldatzen sisteman dagoen likido kantitate txiki bat (300 µL) bi saiodietara botaz. Horrela, bi konpartimentuen laginketa momentu berdinean ematen dela ziurtatu daiteke. Lagin hauek ez direnez esterilak izango, mikroorganismoekin lan egiterako orduan eskuzko laginketa egingo da (odol laginketarako erabiltzen diren saiodiak erabiliz). Lagin horiek bai farmakoaren kontzentrazioa zehazteko bai mikroorganismoen zelulen zenbaketa egiteko erabili daitezke.

Hobekuntza horiek direla eta, SIMULI-PHARM 1 eredua besteekin konparatuz maneiu errazagoa eta fidagarriagoa du. Halaber, administrazio sisteman egindako aldaketei esker hainbat terapia simulatzeko gai den sistema da.

Sistemako tresna guztien maneiua I.D.I.I.S enpresak (Toulouse) sortutako software bat baliatuz egiten da.



10. Irudia: SIMULI-PHARM 1 *in vitro* ereduaren eskematik, [41] eraldatua

2. Helburua

Kaspofungina, kandidiasi inbasiboaren tratamendurako gomendatutako farmakoa da. Hala ere, azken urteetan *C. glabrata* tratatzean huts egiten duten kasuen kopurura igo da. Hori dela eta, beharrezkoa da *in vitro* erresistentzia ikerketak egitea tratamendu eraginkorragoak bermatu ahal izateko.

Hori da, hain zuzen, lan honen helburua:

“Kaspofunginarekiko erresistentzia azaltzen duten kandida glabrata zenbait zepen *in vitro* ikerketa egitea, SIMULI-PHARM 1 eredua erabiliz, dosi erregimen berriak garatu ahal izateko “

Horretarako, esperimentuan zehar zelulen zenbaketa egin ez ezik, aurretiaz garatutako eta balidatutako column-switching-HPLC-FLD metodo kromatografikoa erabiliko da kaspofungina farmakoaren kontzentrazioaren monitorizazioa egiteko.

3. Atal esperimentala

3.1 Erreaktiboak eta materialak

Kaspofungina azetatoa Finetech Industry Limited-etik (Wuhan, Hubei) eskuratu zen. Azido 3-(N-Morpholino)propanosulfonikoa (MOPS) eta [3-(2-Aminoetilamino) propil] trimetoxisilanoa (AATMS) Sigma-Aldrich (Darmstadt, Alemania) marka komertzialari erosi zitzaien. Dialox (erreaktibo bakterizida) Bioxal SA (Chalon-sur-Saône, Frantzia) marka komertzialari erosi zitzaion.

Azetonitriloa (LC-MS grade) eta azido trifluoroazetikoa (% 99, for synthesis) VWR chemicals-ek hornitu zuen. Ur ultrapurua Veolia Water STI-k fabrikatutako (Saucats, Frantzia) ELGA PURELAB sistema klasiko batetik eskuratu zen.

3.2 Kaspofungina kontzentrazioa zehazteko instrumentazioa eta baldintza analitikoak

Dionex Ultimate 3000 (Thermo Scientific, Waltham, Massachusetts, USA) sistema kromatografikoa erabili da kaspofunginaren analisia egiteko, uhin luzera anitzeko fluoreszentzia detektagailu (2475 modeloa, Waters, Mildford, USA) bati akoplatuta. Analitoaren detekzioa egiteko 278 eta 299 nm kitzikapen eta igorpen uhin luzerak erabili ziren, hurrenez hurren. Online SPE erauzketa egiteko Rheodyne (IDEX Health & Science, Erlangen, Germany) markako sei atakako switching balbula erabili zen, Spectra SYSTEM P1000 ponpa batekin konektatuta (Thermo Scientific). Chromeleon software-a erabili zen sistema kontrolatzeko eta datuak prozesatzeko (Thermo Scientific). pH-a neurtzeko, Crison pH Meter GLP 22 markako pH-metroa erabili zen.

Analisi kromatografikoan erabilitako fase mugikorrak; TFAz finkatutako pH 2 ko fase urtsua (A) eta azetonitriloa (B) izan ziren. Laginaren garbiketa egiteko, online SPE erauzketaren bidez, LiChroCART C8 (4 x 4mm, 5 µm) (Merck, Darmstadt, Alemania) zutabea erabili zen, fase mugikorren 95:5 (A:B, v/v) proportzioa erabiliz. Erauzketa 1,25 minutuz egin zen 1 mL/min-ko fluxua erabiliz. Ondoren, banaketa

kromatografikoa Waters-eko HSS T3 (4.6 x 100 mm, 2.5 µm) zutabe analitikoan egin zen. Zutabea 100-040-220P berogailu batez (Croco-cil, Cluzeau, Frantzia) 40 °C-tan mantendu zen. Determinazio analitikoa burutzeko fase mugikorra 60:40 (A:B, v/v) nahastea izan zen, 0,8 mL/min-ko fluxuarekin. Injekzio bolumena 40 µL-koa izan zen.

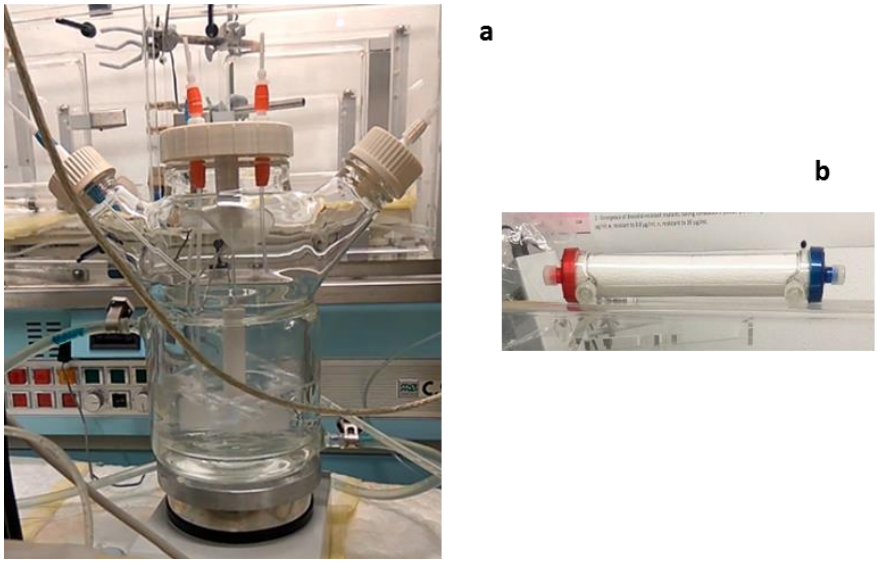
3.3 Disoluzio estandarrak

Erabilitako 800 mg/L CSF stock disoluzioa prestatzeko, CSF kantitatea ur ultrapururan disolbatu zen. Lanerako, 10 mg/L eta 20 mg/L-ko CSF disoluzioak ur ultrapuruan prestatu ziren esperimentazioko egun bakoitzean.

3.4 Kandida glabrataren erresistentziaren *in vitro* ikerketa

3.4.1 Ereduaren deskribapena

KZ, irabiagailu magnetiko baten gainean zegoen 1 L-ko erreaktore termostatiko bat (**11.Irudia, a**) (Wheaton, Polylabo, Strasburg, Frantzia) eta bioerreaktoreko kapilar hutsek (**11. Irudia, b**) (F40S, Fresenius, Sèvres, Frantzia) osatzen zuten, hainbat tuturen bitartez konektatuta zeudenak [41].



11. Irudia: SIMULI-PHARM 1 ereduko atal nagusiak: a)konpartimentu zentraleko erreaktore termostatikoa eta b)Kapilar hutsezko bioerreaktorea

Bioerreaktorearen kapilarren arteko tarteak eratzen zuen KP. Ponpa peristaltiko batek lagunduta (Minipuls 3M/HF, Gilson MEdical Electronics, Villiers le Bel, Frantzia), farmakoaren kontzentrazioaren oreka azkarra lortzen zen bi konpartimentuen artean. Kultibo zelularraren gehitze- eta kanporatze-fluxuak kontrolpean zeuden ordenagailura konektatutako RS 232 interfase baten bitartez (Phase separations France, Saint Quentin en Yvelines, Frantzia). Bioerreaktoreko tenperatura finko mantendu zen 37 °C-tan, esperimentu osoan zehar [41].

3.4.2 CSFren profil zinetikoaren kalkulua

SIMULI-PHARM 1 eredua zelulekin erabili baino lehen, CSFren profil zinetikoa ordenagailuan programatu eta bi konpartimentuetan berdina zela frogatu behar izan zen esperimentalki.

CSFren giza kontzentrazioak [42] eta proteinei lotzeko duen gaitasuna (% 96) [43] jakinik, plasman aske aurkitzen diren kontzentrazioak kalkulatu ziren. Kalkulu horiek Stone *et al*.-en [42] lanean aurkitutako datuen arabera egin ziren. Kontzentrazioak grafikoki zehaztu ziren 70 mg-ko dosiarentzako, ohiko karga dosia baita giza terapian. Ondoren, eta plasman aurki daitezkeen profil zinetikoek erlazio lineala jarraitzen dutenez [44], gainerako dosien kontzentrazio askeak kalkulatu ziren proportzionaltasunean oinarrituta (**1.Taula**)

1. Taula: Stone et al-en [45] lanean oinarrituta, kalkulatutako kaspofungina kontzentrazio totalak eta askeak 30, 50, 70, 90 eta 100 mg-tako erregimen terapeutikoentzat

	Dosia (mg)									
	30		50		70		90		100	
	Kaspofungina kontzentrazioa (µg/mL)									
Denbora (h)	Totala	Askea	Totala	Askea	Totala	Askea	Totala	Askea	Totala	Askea
0	0	0	0	0	0	0	0	0	0	0
0,50	0,04	0,12	5,07	0,20	7,10	0,28	9,13	0,36	10,14	0,40
0,75	4,28	0,17	7,14	0,28	10,00	0,40	12,86	0,51	14,28	0,57
1,00	5,57	0,22	9,28	0,37	13,00	0,52	16,71	0,67	18,57	0,74
1,25	5,14	0,20	8,57	0,34	12,00	0,48	15,43	0,62	17,14	0,68
1,50	4,71	0,18	7,85	0,31	11,00	0,44	14,14	0,56	15,71	0,63
2,00	4,24	0,17	7,07	0,28	9,90	0,39	12,73	0,51	14,14	0,56
3,00	3,43	0,13	5,71	0,23	8,00	0,32	10,28	0,41	11,43	0,46
4,00	3,04	0,12	5,07	0,20	7,10	0,28	9,13	0,36	10,14	0,42
6,00	2,48	0,10	4,14	0,16	5,80	0,23	7,46	0,30	9,28	0,37
9,00	1,93	0,08	3,21	0,13	4,50	0,18	5,78	0,23	6,43	0,26
12,00	1,63	0,06	2,71	0,11	3,80	0,15	4,88	0,19	5,43	0,22
24,00	0,64	0,02	1,07	0,04	1,50	0,06	1,93	0,08	2,14	0,08

3.4.3 Sistemaren prestaketa

Mikroorganismo eta kultibo zelularrekin lan egiteko ingurune eta material esterilak erabili behar dira. Horregatik, *in vitro* eredua erabili baino lehen, atal edo osagai guztien esterilizazioa bermatu behar da, garbiketa bat eginez. Horretarako, sistema guztian zehar Dialox:H_2O 60:40 (v/v) nahaste bat igaroarazi zen 3 orduz. Ondoren, sistema guztia baldintza esteriletan zegoela, Dialox erreaktiboaren aztarnak kentzeko, ur esterila sistematik igaroarazi zen pH neutroa lortu arte.

3.4.4 Profil zinetikoaren simulazioaren ebaluaketa

70 mg-ko dosia simulatzea erabaki zen, espezialistek gomendatzen duten karga dosia delako. Simulazioan bi gauza lortu behar dira: batetik, konpartimentuetan dagoen farmako kontzentrazioa berdina izatea eta, bestetik, bi horiek teorikoarekin konparagarriak izatea. Horrela bada, profilaren simulazioa egokitzat hartzen da eta ondoren, onddoekin egin beharreko esperimentua burutzen da.

Laginketa automatikoaz baliatuz, denboran zehar analitoaren kontzentrazioa determinatu zen profil zinetikoa egokia zela egiaztatzeko. Laginketa KZean eta KPan egin zen.

3.4.4.1 Baldintza normaletan egindako esperimentua

Lehenengo eta behin, sistema piztu eta berotzen jarri zen. Sistema 37 °C-tan zegoela, konpartimentu zentraleko Erlenmeyer termostatizatuan zelulen kultiboaren (RPMI 1640) 500 mL jarri ziren. 10 mg/L-ko CSF disoluziotik, Erlenmeyerrera 27 mL gehitu ziren, fluxu konstantean, ordu batean zehar ordenagailuz kontrolatutako xiringa baten laguntzaz. KZ eta KPetan laginketa egin zen 1. Taulan adierazitako momentuetan. Laginak column-switching-HPLC-FLD metodo analitikoarekin analizatu ziren CSFren kontzentrazioa kuantifikatzeko.

3.4.4.2 Asetasun baldintzetan egindako esperimentua

Sisteman zehar CSFren adsortzioa ekiditeko, esperimentua egin baino lehen, sistema CSFz asetzea erabaki zen. Sistemaren asetzea, Ba *et al*. -en [45] ikerkuntza taldeak gatifloxacin farmakoaren determinazioaren lanean aurrera eramandako prozeduran oinarrituz egin zen. Laburbilduz: sistema baldintza esteriletan egonik, 20 mg/L-ko disoluzioaren 50 mL gehitu ziren KZan. Sistematik disoluzioa 4 orduz igaroarazi zen (zirkuitu itxian). Laginketa 15 minuturo egin zen, asetze puntua zehazteko.

Esperimentua aurrera eramateko, sistema asetu egin zen 10 mg/L-ko CSF disoluzioko 17 mL konpartimentu zentralean jarriz eta 45 minutuz sisteman zehar igaroz, zirkuitua itxita zegoelarik (kanporatzeko biderik gabe). Denbora tarte hori pasa ondoren, zirkuitua zabaldu eta disoluzioa sistematik atera egin zen. Behin disoluzio guztia kanpoan, 4.4.1 atalean azaldutako prozedura aurrera eraman zen.

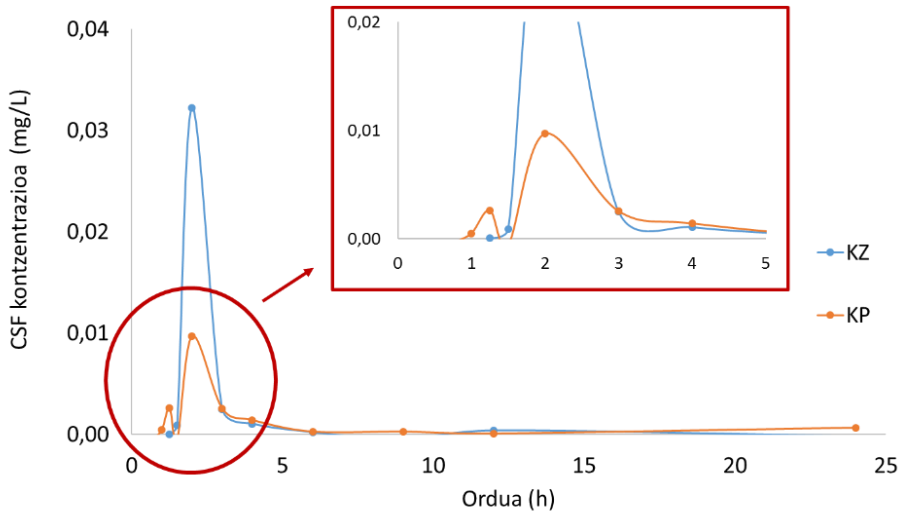
4. Emaitzak eta eztabaida

4.1 Profil zinetikoaren simulazioaren ebaluaketa

70 mg-ko dosia simulatzea erabaki zen, espezialistek gomentdatzen duten karga dosia delako. Simulazioan bi gauza lortu behar dira: batetik, konpartimentuetan dagoen farmako kontzentrazioa berdina izatea eta, bestetik, bi horiek teorikoarekin konparagarriak izatea. Horrela bada, profilaren simulazioa egokitzat hartzen da eta ondoren, onddoekin egin beharreko esperimentua burutzen da.

4.1.1 Baldintza normaletan egindako esperimentua

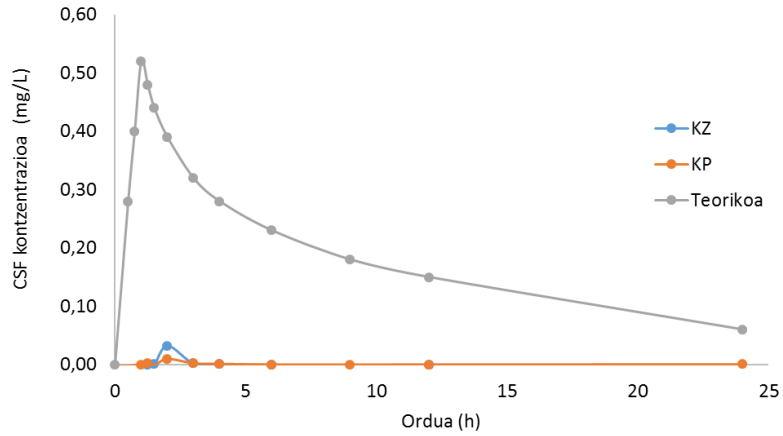
CSFren determinazio kuantitatiboaren emaitzetan (**12. Irudia**) ikus daiteke lehenengo laginketa momentuetan bi konpartimentuetako kontzentrazioa ez zela berdina. Ezberdintasun hori nabarmena zen 2. orduan egindako laginketan, non, KZan 0,032 mg/L-ko kontzentrazioa kuantifikatu zen, eta KPan, aldiz, 0,01 mg/L-koa. Erabilitako ereduan, konpartimentuen arteko kontzentrazio oreka minututako tartean lortzen da normalean.



12. Irudia: Baldintza normaletan simulatutako 70 mg-ko erregimenerako profil zinetikoaren emaitzak (24 h)

Lortutako emaitzak profil zinetiko teorikoarekin konparatu ziren (**13. Irudia**). Ezinezkoa izan zen profil zinetikoaren simulazio egokia lortzea, emaitzetan argi ikus daitekeen bezala, esperimentu bidez lortutako kontzentrazioak teorikoak baino bost aldiz txikiagoak izan baitziren.

CSFk plastikozko eta beirazko materialetara adsorbatzeko joera duela aurkeztu dute ikertzaile batzuk bibliografian zehar, non, lagin tratamenduetan analito galera esanguratsua ikusi den [43]. Hori jakinik eta kontuan edukita eredu osoan zehar erabilitako tutuak plastikozkoak direla (PVC) CSF sisteman zehar adsorbatu izan zitekeela ondorioztatu zen.



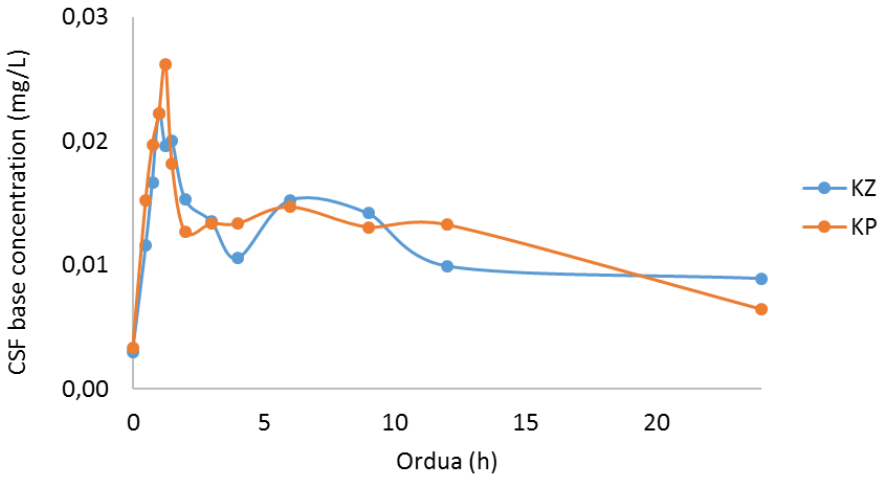
13. Irudia: Baldintza normaletan simulatutako 70mg-ko erregimenerako profil zinetikoaren emaitz esperimentalak teorikoarekin batera errepresentatuz

4.1.2 Asetasun baldintzetan egindako esperimentazioa

Sistematik CSF disoluzioa igaroarazten hasten denean analitoa sistemako material ezberdinetan adsorbatzen hasten dela suposatzen da. Horrela izanik, asetze puntua bi konpartimentuetan adsorbatu ahal den analito guztia adsorbatzen denean lortzen da. Esperimentalki, analitoa bi konpartimentuetako lagin batean agertzen den momentuan sistema asetuta dagoela esan daiteke.

SIMULI-PHARM 1 ereduan CSFren asetze puntua 0,5 h-an determinatu egin zen. Erabilitako fluxua eta CSF disoluzioaren kontzentrazioaren laguntzaz sistema asetzeko CSF kantitatea kalkulatu zen, 0,27 mg CSF. Hori dela eta, profil zinetikoaren simulazioa egin aurretik, 10 mg/L-ko disoluzioko 27 mL jarri ziren sisteman zirkulatzen.

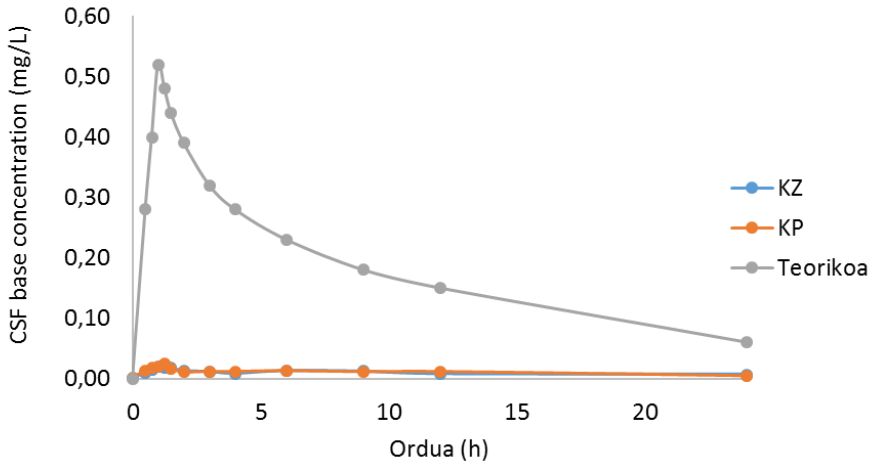
Baldintza horietan egindako simulazioan lortutako emaitzak ikusita (**14. Irudia**), eta aurrekoekin konparatuz, konpartimentuen arteko kontzentrazio ezberdintasuna txikiagotu zela ikus daiteke.



14. Irudia: Asetasun baldintzetan simulatutako 70mg-ko erregimenerako profil zinetikoaren emaitzak (24 h). KZ eta KPko CSF kontzentrazioak b) KZ eta KPko CSF kontzentrazioak teorikoarekin konparatuz

Dena den, bai prozedura normala jarraituz, bai sistema asetze baldintzetan erabiltzean, lortzen diren KZ eta KPko kontzentrazioak antzerakoak direla nabarmendu daiteke (**12. Irudia** eta **14. Irudia**).

Hala ere, lortutako CSF kontzentrazioak neurri berean mantendu dira eta ez dira teorikoarekiko konparagarriak (**15. Irudia**). Asetze prozedurak emaitza esperimentalak profil teorikoarekin konparagarriak izatea zuen helburua. Lortutako emaitzei erreparatuz, badirudi asetze prozedura ez dela eraginkorra izan. Baliteke, CSFren adsortzio prozesua beste farmako batzuena baino motelagoa izatea eta erabilitako asetze puntuaren determinazioa desegokia izatea. Era berean, posible litzateke, analito galerak (nabarmendutako seinale jaitsiera) adsortzioarekin erlaziorik ez edukitzea eta beste prozesu baten ondorio izatea, degradazioa adibidez.



15. Irudia: Baldintza normaletan simulatutako 70mg-ko erregimenerako profil zinetikoaren emaitza esperimentalak teorikoarekin batera errepresentatuz

5. Ondorioak eta etorkizuneko ikuspuntuak

Lan honetan, kapilar hutsezko PK/PD bi konpartimentuko *in vitro* ereduaren erabilera proposatzen zen *C. glabrata*-ren zepa ezberdinen CSFrekiko erresistentzia ikertzeko, ondoren dosi erregimen berriak proposatzeko helburuarekin. Burutako esperimentazioan zehar, analito galera garrantzitsu bat ikusi zen. Fenomeno hori aurretiaz literaturan deskribatutako CSF galerarekin [43] erlazionatuta egon zitekeela pentsatu zen. Adsortzio arazo bat zelakoan, sistema asetzearen saiakera egin zen, aurretiaz gatifloxacin konposatuarekin erabilitako prozedura berberari jarraituz [45]. Emaitzetatik ondoriozta daiteke ez zela analito galera ekiditea lortu. Lortutako emaitzak direla eta, SIMULI-PHARM 1 ereduan CSFrekin lan egiteko erabilitako baldintzak egokiak ez zirela ondorioztatu da. Ikusitako analito galeraren zergatian sakonago ikertzea proposatzen da.

Adsortzio arazo bat izatekotan, sisteman zehar dauden plastikozko tutuen eta kapilar hutsezko bioerreaktorearen arteko elkarreragina ikertu beharra dago. 6. kapituluan aurrera eramandako ikerketaren emaitzek argi uzten dute adsortzio fenomenoa asko aldatzen dela plastiko mota batetik bestera. SIMULI PHARM 1 ereduko tutuak PVCzkoak direla jakinik, merkatuan eskura daitezkeen plastikozko tutu ezberdinen adsortzioak ikertzea proposatzen da. Izan ere, esperimentuak egiteko egokiak diren materialezko tutuak erabiltzea gomendatzen dute ikerlariek.

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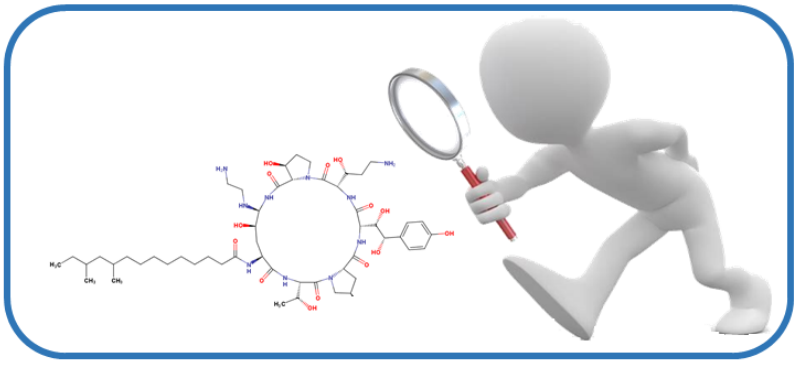
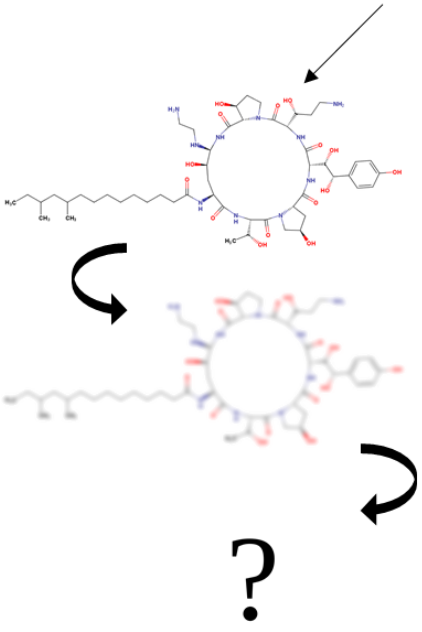
Chapter 5

Degradation study of caspofungin antifungal drug





1. Introduction

The increasing importance of echinocandins (EC) is well established in the scientific community for the treatment of invasive fungal infections as invasive candidiasis. However, ECs have raised some questions among specialists. As it has been said before in this work, some researchers found a loss of analyte while doing its analytical determination [1] and, in addition, the results obtained in the previous chapter had shown the loss of caspofungin (CSF) when using it for *in vitro* resistance studies.

It is known that CSF is adsorbed in different laboratory materials, and until now, its concentration decrease seen in analysis has been related to this phenomenon [1]. Nevertheless, the change in CSF amount could be a result of a degradation process and at this moment, there is no degradation study of CSF available in literature. Kartsonis *et al*. [2] reported the peptide hydrolysis, N-acetylation and an spontaneous degradation due to an opening of the CSF core ring. This statement was based on the previous work of Balani *et al*. [3] in 2000, where authors explained the metabolic pathway of MK-0991 (CSF) and the metabolites found in plasma and urine samples. In this work, four metabolites were described (**Figure 1**) using radio labelled CSF and HPLC-MS analysis.

Compounds described in **Figure 1** were taken into account in the degradation study carried out in this chapter. The stability of the molecule is a key point in the assessment of a pharmaceutical active ingredient. In particular, in our study, the degradation of CSF can be an explanation of loss of CSF quantity during *in vitro* applications.

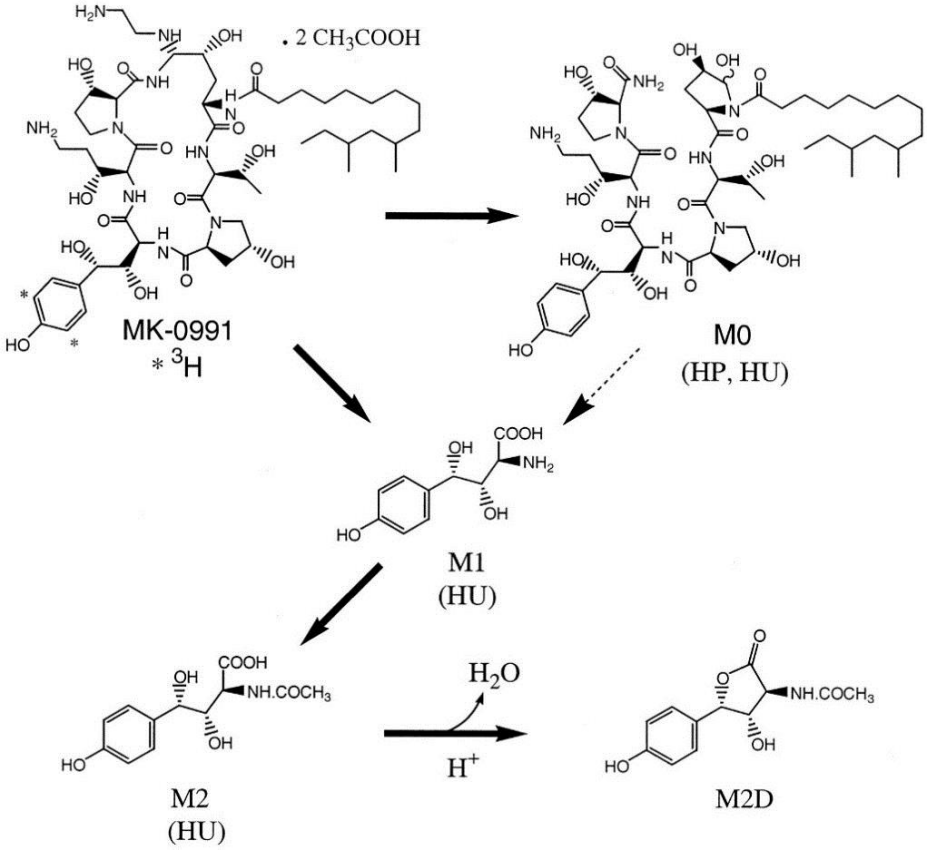


Figure 1: Metabolic pathways of MK-0991 in men (Human plasma (HP), Human urine (HU)). The asterisks denote the positions of the ^{3}H-label in [^{3}H]MK-0991" (Balani *et al*. [3])

The International Council on Harmonisation (ICH) Q1A (R2) is a guidance for stability testing in drug substances and products. The aim of stability testing is to evaluate how the quality of a drug varies with time under different factors. A forced degradation process contributes to the identification of the possible impurities and thus drug stability evaluation. Document supported by the European Medicines Agency (EMA) [4] describes forced degradation approach. A stress testing should include the study of the effect of different conditions as temperature or oxidation among others. This approach requires an analytical technique able to determine the chemical structure of the degradation products.

The increase of sensitivity and resolution achieved has turned nuclear magnetic resonance (NMR) spectroscopy into one of the most important instrumental measuring techniques in chemistry [5]. It is one of the most powerful technique for characterization and identification studies. It has to be mentioned, that most of the new NMR techniques were designed initially for protein and peptide analysis [6]. This fact transforms NMR in a useful tool for the stability study of the lipopeptide CSF with time.

In this work, a degradation study of CSF is presented by means of HPLC-FLD and NMR techniques. In addition, a complementary analytical approach, liquid chromatography coupled to mass spectrometry (MS) detector, will be used with the aim of corroborating the findings of the previous techniques.

2. Materials and Methods

2.1 Reagents

CSF acetate was purchased from Finetech (Wuhan, Hubei). Sodium hydroxide (NaOH) EMSURE (Millipore's premium grade) was bought from Merck (Darmstadt, Germany).

Acetonitrile (ACN) (HPLC LC-MS grade), hydrochloric acid (HCl) (37 %), hydrogen peroxide (3 %), dimethyl sulfoxide (DMSO) (ultra-pure grade) and trifluoroacetic acid (TFA) (for LC-MS) were supplied by VWR chemicals (Linars del Vallés, Spain). Formic acid (F.A) and acetic acid (A.A) both of LC-MS grade were purchased from Fluka (Buchs, Switzerland). Ultrapure analytical water (H_2O) was obtained from a Milli-Q Element A10 system (Millipore, Milford, USA).

2.2 Stock solutions

CSF stock solution was prepared at 1600 mg/L in DMSO. It was aliquoted and kept at -20 ^{o}C until working solutions preparation. 40 mg/L working solution was used for HPLC-(Q)-TOF-MS and 50 mg/L working solution for HPLC-FLD. Both were freshly prepared each day of analysis in H_2O.

2.3 Instrumentation and analytical conditions

2.3.1 HPLC-FLD analysis

Dionex Ultimate 3000 chromatograph System (Thermo Scientific, Waltham, Massachusetts, USA) coupled to a 2475 multi wavelength fluorescence detector (Waters, Mildford, USA) was used for the analysis. Fluorescence detection was performed at 278 and 299 nm for excitation and emission wavelengths, respectively [7]. For the switching system, a Rheodyne 6 port valve (IDEX Health & Science, Erlangen, Germany) and a Spectra SYSTEM P1000 Isocratic were used (Thermo Scientific). System control, data collection and data processing were accomplished using Chromeleon software.

The column-switching for the on-line extraction was performed with a LiChroCART C8 column (4 x 4 mm, 5 µm) (Merck, Darmstadt, Germany). The mobile phase consisted of an aqueous mobile phase of pH 2 adjusted with TFA (A) and acetonitrile as organic modifier (B) at 95:5 (A: B, v/v) proportion. A flow rate of 1 mL/min during 1.25 min was used in the extraction step. After, the analyte was eluted to the analytical column, HSS T3 (4.6 x 100 mm, 2.5 µm), from Waters, which was heated using a 100-040-220P column oven (Croco-cil, Cluzeau, France) to 40 °C. The mobile phase condition was at 60:40 (A:B, v/v) mixture at a flow rate of 0.8 mL/min. 40 µL of sample volume was injected.

2.3.2 NMR analysis

All 1D and 2D NMR experiments were performed on a Bruker Avance 600 MHz NMR spectrometer (Bruker, Wissembourg, France) operating at 600.3 MHz and equipped with a 5 mm TXI probe. Data were processed using TOPSPIN software version 3.2 (Bruker Biospin, Germany). All NMR spectra were acquired in $H_2O/D_2O/C_2D_4O_2$ (88/10/2) (v/v). NMR experiments were recorded at 293 K and 313 K. 60 µL of a reference solution containing CaF (calcium formate) as quantification standard, TMSP-*d4* (trimethylsilylpropanoic acid sodium salt) as chemical shift reference have been added.

The proton acquisition parameters were set up as follows: the Free Induction Decays were collected into a time domain of 64 K data points, with a spectral width of 16 ppm, an acquisition time of 3.40 s, and relaxation delay of 5 s per scan.

Partial molecule assignments were achieved by two-dimensional ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{1}H ROESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC experiments. All 2D-experiments were carried out with 2048 data points x 400 increments and a spectral width of 8417 Hz and 33209 Hz in proton and carbon dimension, respectively and 1.5 s for relaxation delay. Mixing time was 300 ms and the spinlock time was 100 ms for ROESY and TOCSY experiments, respectively.

2.3.3 HPLC-(Q)-TOF-MS analysis

HPLC system coupled to a 6530 series hybrid quadrupole time-of-flight mass spectrometer (Q-TOF-MS) from Agilent Technologies (Santa Clara, CA, USA) equipped with an Agilent Jet Stream electrospray (ESI) source was used. Chromatographic separation was carried out injecting 5 µL of sample on a HSS T3 (4.6 × 100 mm, 2.5 µm) reverse column from Waters at 40 °C and a flow rate of 0.5 mL/min was used during analysis. A binary solvent system consisting of 0.1 % F.A in H_2O (phase A) and 0.1 % F.A in ACN (phase B) was used for the elution. After 1 min of 10 % B, a gradient elution from 10 % B to 90 % B was performed in 9 min. It was

kept at 90 % B for 2 minutes and returned to starting conditions in 1 min. Finally, the column was re-equilibrated for 1 min.

The mass spectra data were acquired in positive ionization mode with a capillary voltage of +4000 V. The other ESI source parameters were set as follows: dry gas (nitrogen) temperature, 325 °C; dry gas flow, 10 L/min; nebulizer gas (nitrogen) pressure, 40 psig; sheath gas temperature, 350 °C; sheath gas flow, 10 L/min; fragmentor, 150 V.

The MS detector operated in low mass range (50 - 1700 *m/z*) and 2 GHz extended dynamic range, and centroid acquisition mode was used for data collection and storage. A reference solution was directly infused into the source to ensure mass accuracy, reproducibility and continuous internal calibration during the analysis. Two reference masses at *m/z* 121.0509 (purine, $[C_5H_4N_4+H]^+$) and *m/z* 922.0098 (HP-921, $[C_{18}H_{18}O_6N_3P_3F_{24}+H]^+$) were used. Pseudo MS/MS analysis were performed adding a cell collision energy of 20 V.

The data was acquired using the Agilent MassHunter Workstation version B.05.01 and the raw data was processed with the MassHunter Qualitative version B.07.00, both from Agilent Technologies.

2.4 Degradation study

2.4.1 HPLC-FLD analysis

CSF solutions at pH and oxidative stress conditions were tested by column-switching-HPLC-FLD. Stress solutions used were: 1 M NaOH for alkaline condition, 0.1 M HCl for acidic condition and 3 % of H_2O_2 for the oxidative.

25 mg/L CSF solutions were prepared in stress conditions adding 200 µL of 50 mg/L CSF working solution and 200 µL of stressing agent in each case. For injection, a final concentration of 2.5 mg/L was achieved by 1:10 dilution. Samples were analysed

freshly prepared, after 6 h in the bench at room temperature and after 6 h in the oven at 50 ^{o}C.

2.4.2 NMR analysis

CSF powder was weighed and dissolved in H_2O to a 1000 mg/L CSF concentration. It was freshly analysed. NMR analysis were done in acidic conditions (2 % A.A).

2.4.3 HPLC-(Q)-TOF-MS analysis

CSF solutions at pH and oxidative stress conditions were tested by HPLC-(Q)-TOF-MS. Stress solutions used were: 1 M NaOH for alkaline condition, 1 M HCl for acidic condition and 3 % of H_2O_2 for the oxidative.

Working solutions of 40 mg/L CSF were freshly prepared in chromatographic vials, by dilution of 10 µL of stock solution in 190 µL of H_2O and adding after 200 µL stress solution NaOH, HCl or H_2O_2 in each corresponding case. Vials were heated at 50 ^{o}C in the oven. For analysis, working solutions were removed from the oven and left 5 min in the bench before sampling. 10 µL of the working solution was taken and diluted with H_2O to 4 mg/L final concentration (90 µL of H_2O were added).

Samples were analysed freshly prepared (non-heated) and after 3 h and 5 h in the oven. One replicate of each condition was analysed in each sampling time.

3. Results and discussion

3.1 HPLC-FLD analysis

CSF compound was forced to degradation in acidic, alkaline and oxidative conditions and results were carefully studied. All the stress conditions shown a decrease in CSF signal obtained when comparing fresh samples and the heated ones (6 h at 50 ^{o}C), more than 50% of CSF. However, for alkaline and oxidative conditions no degradation product was observed in the analysis performed (no new chromatographic peak was seen in the chromatogram).

Acidic stress conditions resulted in a 58 % decrease of the CSF chromatographic peak area (t_R= 6.268 min) and the appearance of a new chromatographic peak (t_R= 6.682 min) representing a 28 % of the CSF peak area obtained when analysing the freshly prepared CSF solution.

3.2 NMR analysis

For the structure elucidation of the new compound seen in acidic stress conditions NMR analysis were carried out at room temperature.

Firstly, CSF spectra was obtained from freshly prepared sample and 2D NMR analysis were performed to carry out the partial assignment of the different protons of the molecule. ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC results were used. As can be seen in **Figure 2** aliphatic protons, aromatic protons and NH protons were distinguished in the ^{1}HNMR spectra corresponding to the CSF structure.

Degradation process of CSF was monitored during 6 days at room temperature. A decrease in proton resonances was observed during time, as expected. The difference in area of the aromatic protons at 6.90 and 7.16 ppm is shown in **Figure 3**. The results obtained from the aromatic proton area, shown an area decrease of 20 % within the first day. Furthermore, ^{1}H NMR signals of a new compound appeared. However, the signal obtained for this new compound was not high enough for any interpretation and integration (peak was not so intense and too wide).

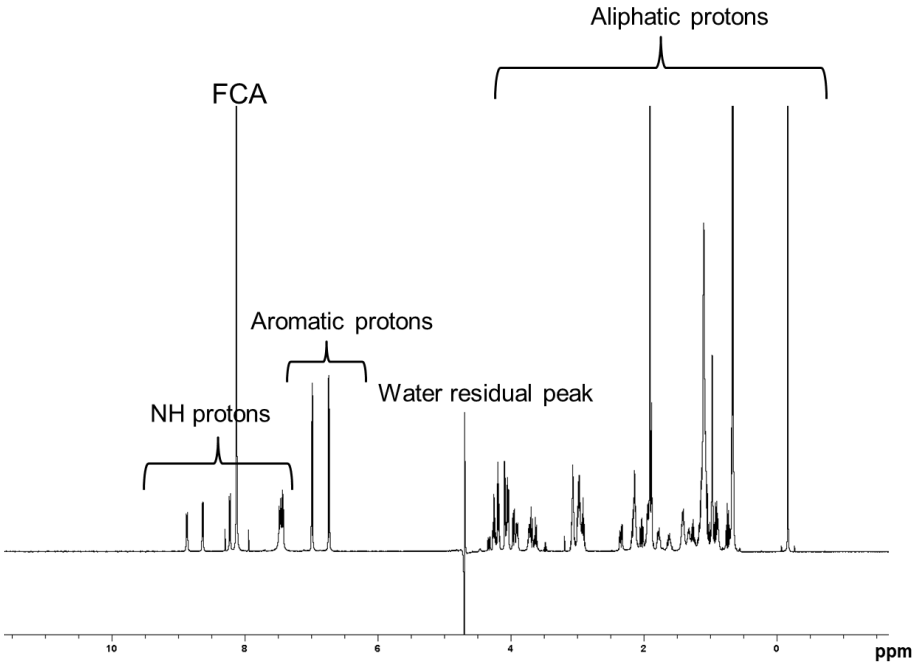


Figure 2: ^{1}H NMR spectra of caspofungin with assignment of molecule protons

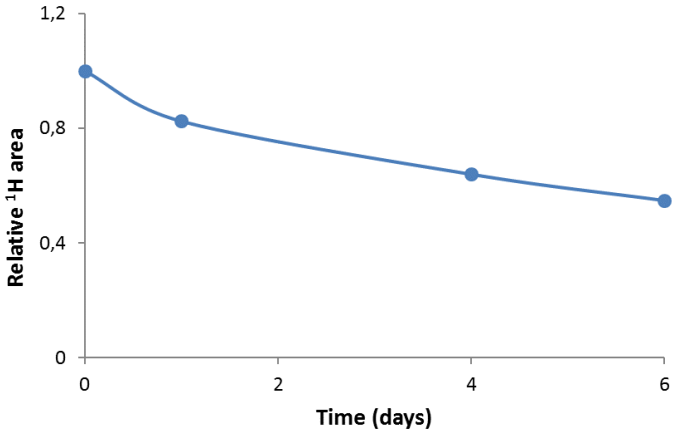


Figure 3: Change of relative area of the CSF aromatic protons (obtained in ^{1}H NMR) with time

In order to identify the degradation product, a strongest stress was performed to obtain higher amount of degradation product. A sample was heated at 60 ^{o}C for two hours and compared with a fresh one. Results shown a decrease in CSF protons and new protons' signals appearing in the heated sample (**Figure 4**, new protons indicated with a star in **Figure 4 B**).

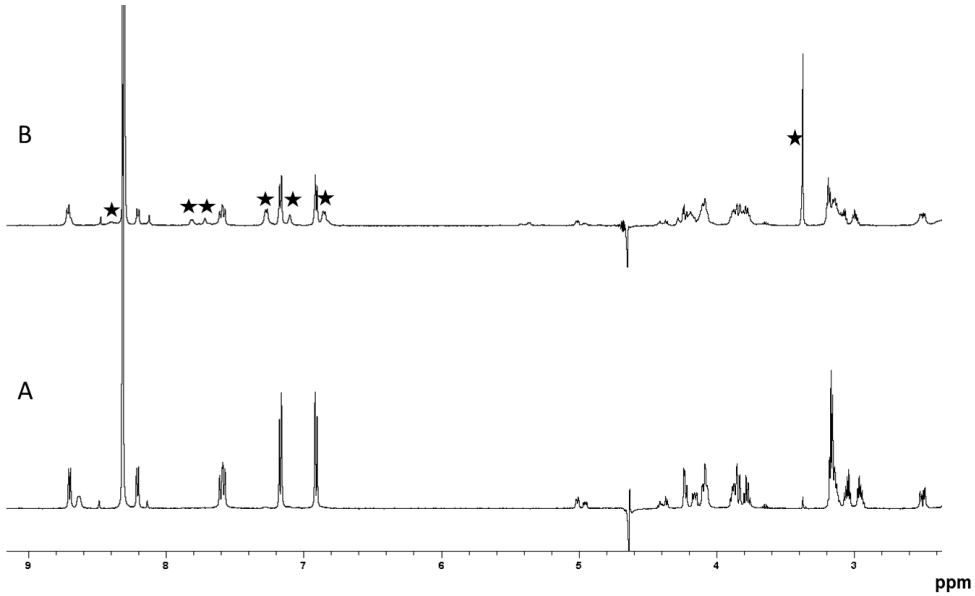


Figure *4*: ^{1}H NMR spectra of Caspofungin freshly prepared (20 °C) (A) and after 2 hours at 60 °C temperature (B). Proton NMR signals of degradation compound are indicated by a star

For the elucidation of the structure of the degradation compound (**Figure 4**), 2D NMR techniques were carried out. Unfortunately, the structural complexity of the molecule and the scarce amount of degradation compound obtained hampered its complete structural elucidation. However, the presence of signals probably attributable to amide protons (5.0 – 9.0 ppm) and aromatic protons (6.0 – 8.7 ppm) seem to indicate a cyclic structure close to that of caspofungin. In addition, there appears a signal probably due to an O-CH_3 at 3.37 ppm which is consistent with the classical chemical shift and the number of 3 protons obtained by signal integration.

Results obtained, the O-CH_3 group appearance, do not match with any CSF metabolite proposed by Balani *et al*. [3]. To complete the identification of this compound, additional chemical analyses will be necessary.

3.3 HPLC-(Q)-TOF-MS analysis

MS analysis hyphenated with HPLC was used as a complementary technique for trying to elucidate the possible degradation products. First of all, analysis of freshly prepared 4 mg/L CSF in H_2O was done. CSF peak was identified (t_R= 9.129 min) confirming it with the MS spectra obtained (m/z for $[M+2H^+]^{2+}$ (547.3288), $[M+H^+]^+$ (1093.6503) and $[M+Na^+]^+$ (1115.6333) were identified). MS/MS spectra of the CSF peak was carried out (**Figure 5**) to know the possible CSF molecule fragments that could be used for degradation compound identification. The most intense m/z ions found were the adduct with sodium (1115.6333) and the m/z ion of the molecule obtained after the loss of an ethylenediamine group (1033.5823). Some m/z ions obtained (highlighted in **Figure 5**) were linked with the possible molecules derived from CSF (**Figure 6**).

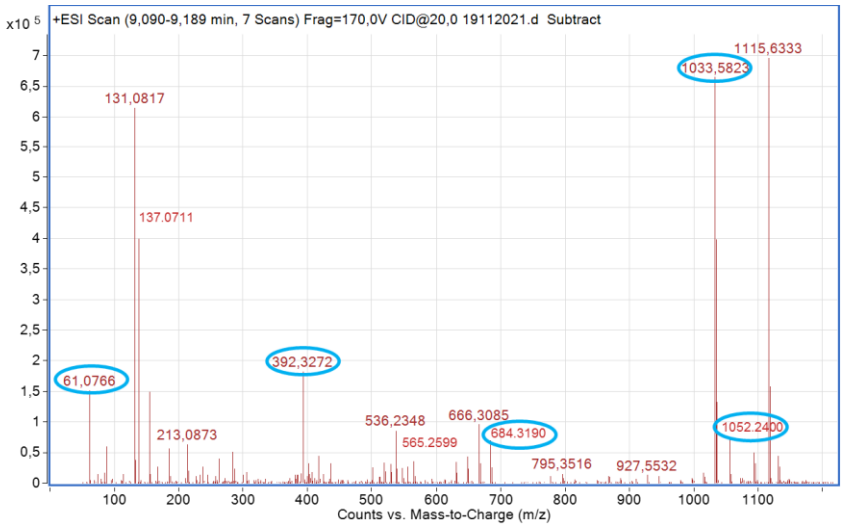


Figure 5: MS/MS spectra of CSF obtained in ESI+ ionization mode (blank spectra substracted). 4 mg/L CSF in H_2O sample. Possible molecular structures of the m/z ions highlighted have been proposed in the next figure

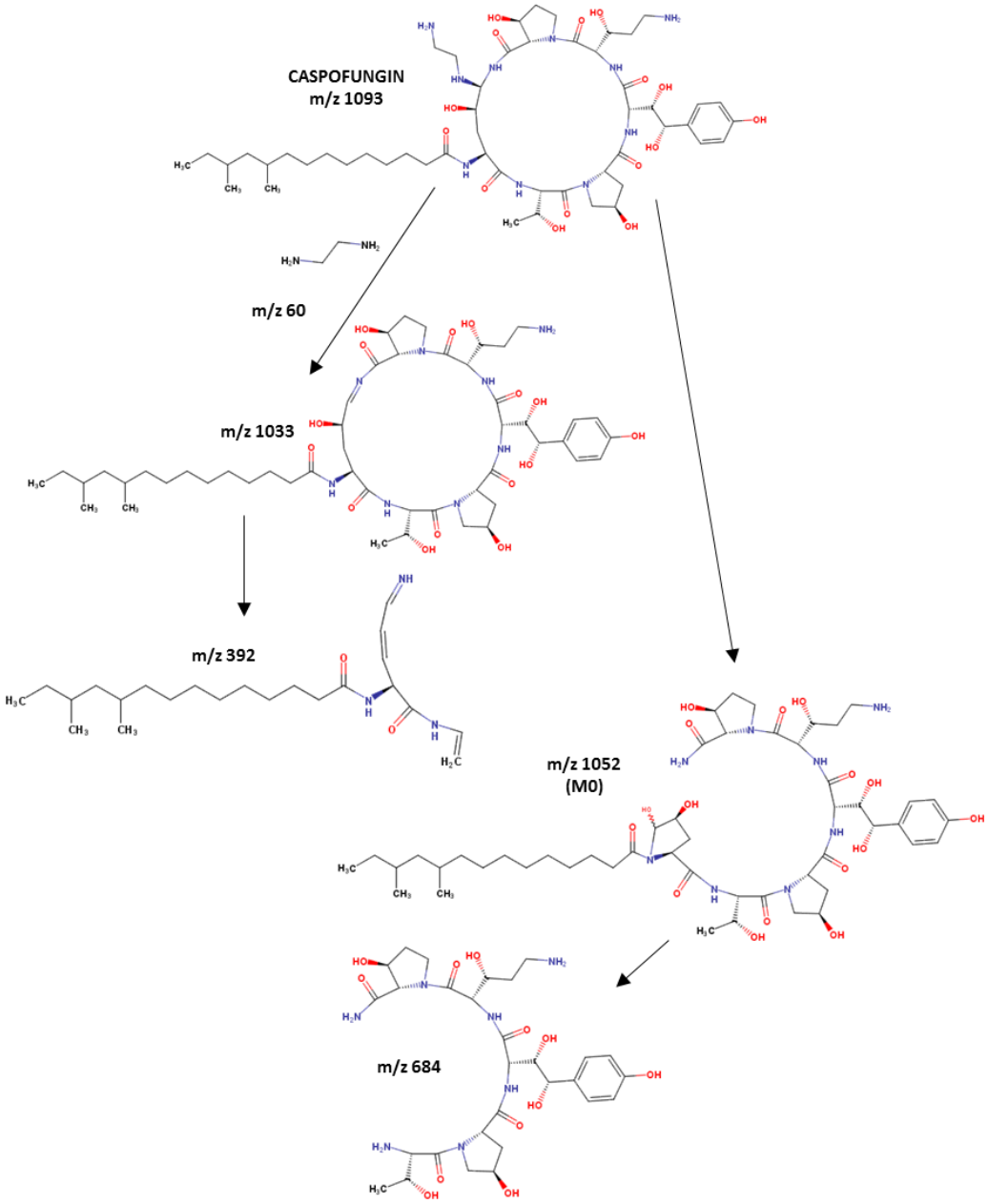


Figure 6: Fragmentation pattern of CSF molecule proposed for the m/z ions highlighted in Figure 5

Taking this information into account possible degradation products were checked in degraded samples. With this aim, freshly prepared and stressed samples extracted ion chromatograms (EICs) were compared. The m/z corresponding to $[M+2H^+]^{2+}$ ion (547.3200) of CSF was compared among the analysis of the fresh samples, the heated ones during 3 h and the heated for 5 h (50 ^{o}C) (**Figure 7**). Results shown an intense decrease of the signal obtained in the EIC for this CSF m/z ion with time in alkaline conditions (**Figure 7 b**).

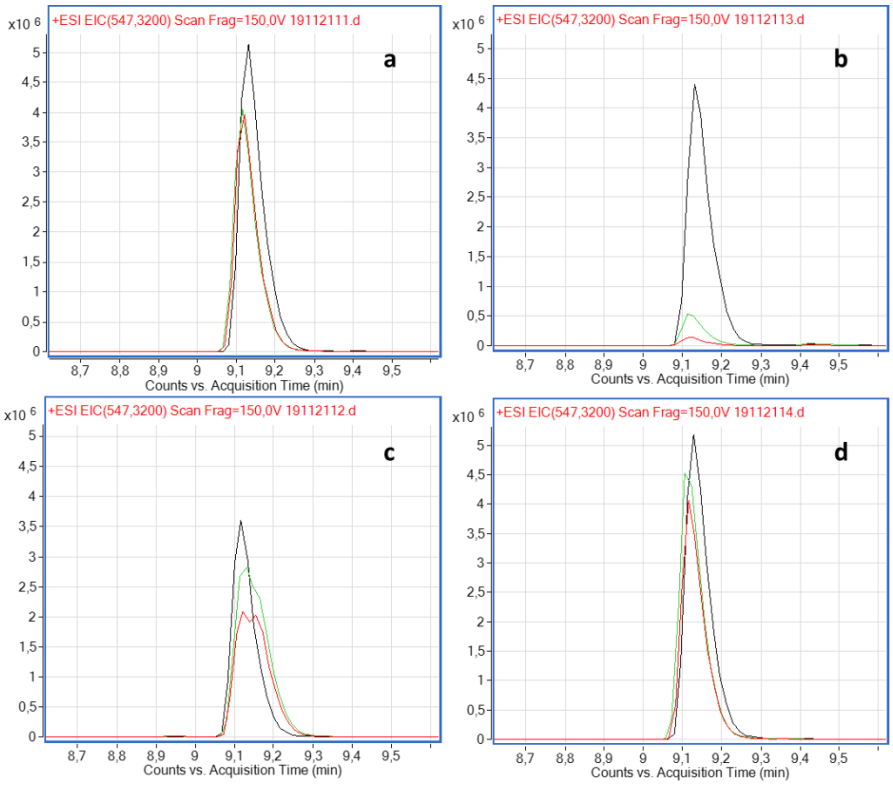


Figure 7: 547.3200 m/z ion's EIC for 4 mg/L CSF samples in H_2O condition (a), alkaline conditions (b), acidic conditions (c) and oxidative conditions (d). Comparison of freshly prepared samples (Black) and heated ones during 3 (Green) and 5 h (Red)

Interestingly, the CSF peak intensity obtained is different, having the most intense peaks for H_2O and H_2O_2 condition samples and being the less intense, the peak

observed in the acidic conditions sample. Balani *et al*. [3] reported the rapid and spontaneous ring-opening process of the peptidic core of CSF with the loss of an ethylenediamine. To confirm the possibility of the existence of this M0 molecule (**Figure 1**), m/z 1052.2400 ion was extracted and compared for different condition fresh samples. In the EIC obtained (**Figure 8**) the presence of this m/z ion could be seen at two different retention times 1.302 and 10.456 min. Paying attention at 1.302 min, huge differences in peak intensities could be clearly observed. Most intense peak was obtained for acidic stress condition sample, which could explain the low intensity obtained in the molecular ion checked in **Figure 7**. Probably, acidic conditions favoured the hydrolysis responsible of the M0 molecule creation.

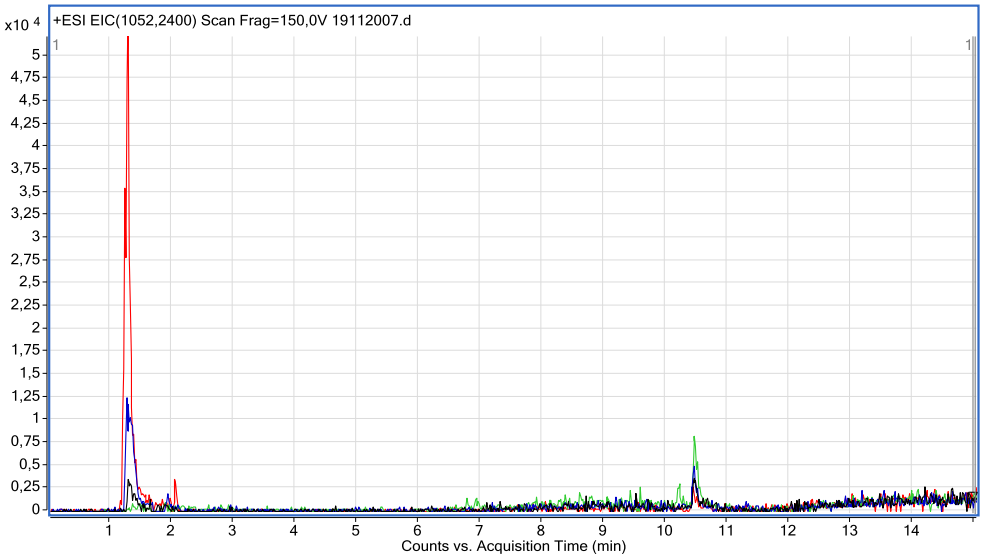


Figure 8: EICs of m/z 1052.2400 ion from the 4 mg/L CSF freshly prepared samples. H_2O condition (black), alkaline conditions (green), acidic conditions (red) and oxidative conditions (blue)

To look for degradation products appearing with time in the stressed conditions, the following m/z ions were checked among chromatograms obtained: 61.0766, 131.0817, 137.0700 and 392.3272. To consider a new chromatographic peak as a degradation product it was expected to see an increase of its intensity together with the decrease of CSF peak (t_R= 9.129 min) intensity.

In alkaline conditions, peaks obtained for the different ions studied were the same in fresh sample and the one heated during 5 h, and no increase during time was observed even though the CSF peak was nearly disappeared in the degraded one.

In acidic conditions, when comparing EIC obtained for 61.0766 m/z in the different samples (fresh, 3 and 5 h at 50 ^{o}C), a peak increasing during time was observed at 1.64 min. Different m/z ions obtained when extracting the MS spectra of this peak did not have, apparently, any structural correlation with CSF molecule.

In oxidative conditions, no peak degradation product has been seen.

4. Conclusions and future perspectives

Different techniques were used for the study of CSF degradation. In column-switching-HPLC-FLD analysis a possible degradation product was observed in acidic conditions. Due to the limitation of the method (column-switching cleaning and isocratic elution) and the detector used, probably, degradation products of oxidative and alkaline conditions were not seen in the analysis performed. Additionally, in NMR analysis proton signals of a new compound were seen. However, the scarce quantity of degradation product obtained in NMR hampered the identification or structural elucidation of the compound. With HPLC-Q-TOF-MS a lot of information was found. Firstly, with this technique it was observed that alkaline conditions pushed the disappearance of CSF chromatographic peak. Secondly, a degradation product of CSF (M0) was successfully identified in acidic conditions that was probably the responsible of the decrease in CSF molecular ion intensity. Oxidative conditions studied were not found powerful to degrade CSF molecule.

CSF has not been seen as unstable as thought before. The stress conditions used in this chapter were extreme and the molecule did not suffer huge degradation, except in alkaline conditions. Based on the results, and knowing the conditions used in the *in vitro* PK/PD studies where so much softer, degradation has not appeared to be the responsible of the analyte loss observed.

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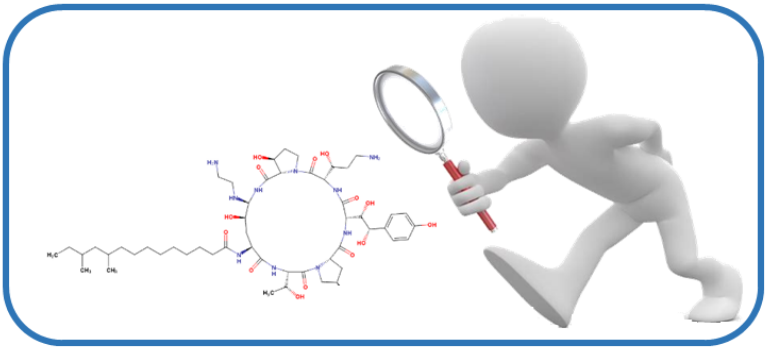
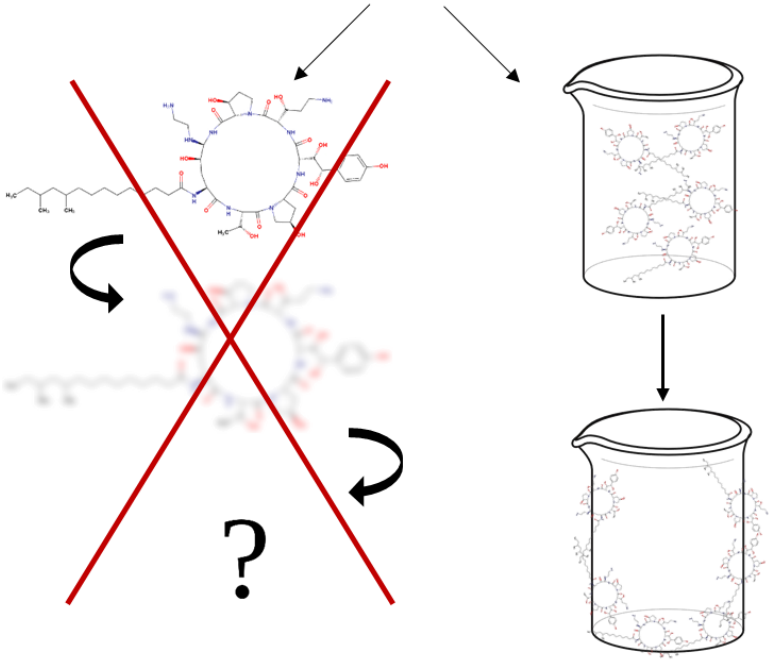
Chapter 6

Study of antifungal agent caspofungin adsorption to laboratory materials





Abstract

The increasing importance of improving therapies for invasive fungal infections, puts pressure on the comprehension of the therapeutic failures with the first-line therapy antifungal agents, caspofungin (CSF). In literature, some authors described a loss of this drug during experiments due to an adsorption on different material surfaces. However, no study focused on this phenomenon, has been published.

In this work, a systematic study of the impact of different parameters on CSF adsorption is presented. The effect of sample container, aqueous solution pH and organic solvent proportion were studied. In addition, the possibility of using a coating agent to minimize the adsorption to HPLC glass vials and 96-well plates was essayed and evaluated.

Results obtained shown the importance of taking care of the material used when CSF handling. The use of acid pH aqueous solution or the addition of acetonitrile and methanol proportions (50% and 70%, respectively) were found relevant to avoid adsorption. The treatment of HPLC glass vials and 96-well plates with N-(2-aminoethyl)-3-aminopropyltrimethoxysilane was observed to reduce the adsorption. The huge adsorption observed in this work in plastic materials, questions the results obtained before in different assays. Therefore, essay for minimum inhibitory concentration (MIC) determination was tested.

1. Introduction

Invasive fungal infections have risen as one of the most concerning human diseases, especially in hospitalized and immunocompromised population [1-5]. In this context, echinocandins (ECs) have become the first-line therapy for invasive candidiasis in different patient groups [6,7].

Kartsonis *et al.* [8] reported inconsistency among calculated minimum inhibitory concentration (MIC) values and the clinical or microbiological results obtained for patients with invasive candidiasis treated with the EC caspofungin (CSF). MIC reference values are established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical & Laboratory Standards Institute (CLSI). Nevertheless, the EUCAST does not provide any reference value for caspofungin MIC calculation for *Candida spp.* The lack of established MIC values is attached to an enormous interlaboratory variability in experimental results obtained. Furthermore, due to the emerging resistance to CSF of different *Candida spp.*, especially *Candida glabrata*, resistance studies are required for improving antifungal therapies [9,10].

One of the reasons of this lack of coherent microbiological test results could be associated to the EC loss during sample handling [8,11-13]. Already in the early beginning of ECs development, Schwartz *et al.* [14] highlighted that adsorption of CSF to glassware was apparently known. They also mentioned the adsorption of CSF to plastic materials during the solid phase extraction procedure of urine samples. Similarly, Traunmüller *et al.* [15] observed a CSF low recovery when protein precipitation sample treatment was applied. They shown that the addition of organic solvents or the change of pH of the solution, improves the results obtained.

CSF is a synthetically modified molecule derived from the fermentation of the fungi *Glarea lozoyensis* [16] (**Figure**). The core is a peptidic ring composed by two ornithines, two prolines and two threonines. The amphipathic nature of the core could be responsible of the adsorption phenomenon, as it is described for peptides and proteins in literature [17,18]. Furthermore, attached to the peptidic core a N-linked fatty acid chain gives to the molecule a hydrophobic part which could contribute to the interactions, and increase the adsorption to a wide range of materials. In addition, that decreases its solubility in aqueous media.

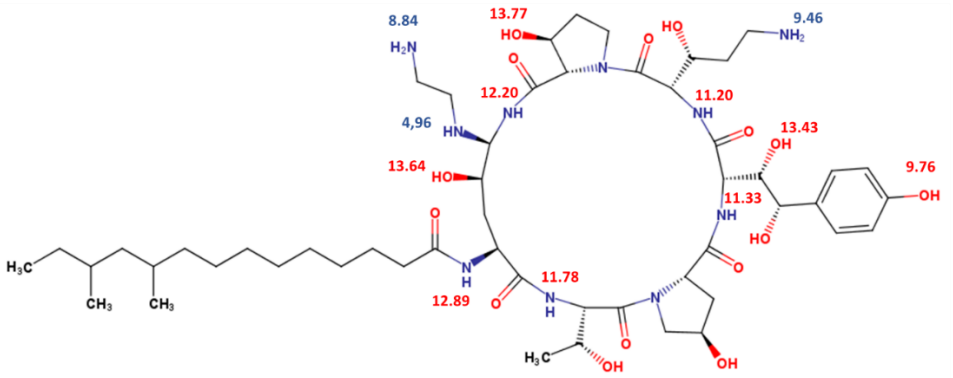


Figure 1: Chemical structure of caspofungin with pKa values (from 0 to 14 range) in red acidic ones and in blue alkaline ones. Atoms' colors follow CPK color code. Source: MarvinSketch v17.22

Non-specific adsorption (NSA) of peptides is well known in the scientific community [17-29], but due to the high variability in composition no general behavior has been identified yet [27,30]. The importance of the plastic (polypropylene, polyester, polyethylene, …) or glassware material used when peptide manipulation has been deeply studied [17,23,24,30] in literature. Additionally, different approaches for the minimization of non-specific adsorption have been proposed by researchers [19,20,31]. Among these strategies, the modification of the surface using coating agents or proteins can be found [19,31-34]. Although this strategy seemed to work, Suelter *et al*.[31] demonstrated the low effectiveness of modifying the surface compared with the change on the nature of the solvent used. In this way, some authors studied the effect of the addition of organic solvent or the acidification of the diluent [20,22,30]. Furthermore, surfactants and bovine serum albumin addition have been also studied for peptide adsorption minimization [20,35].

The increasing importance of CSF in the therapy against fungal infections obliges scientists to strongly study its adsorption phenomenon due to the possible impact on MIC determination, on the reliability of analytical methods performed for resistance studies, or on the efficiency of the antifungal treatment itself. Therefore,

the aim of this paper is the study of the different parameters affecting the adsorption of CSF to demonstrate this phenomenon. For that, variables as the sample container material or solvent nature (pH value and organic solvent proportion) were studied. In the same way, and focused on assays where sample manipulation is not possible, as *in vitro* assays, a way to prevent or minimize this adsorption was evaluated. A treatment for glass and plastic materials was investigated using different coating agents.

2. Materials and methods

2.1 Reagents

CSF acetate was purchased from Finetech (Wuhan, Hubei). Cell culture media used was RPMI 1640 with L-glutamine and MOPS obtained from Capricorn Scientific (Ebsdorfergrund Germany). 3-(N-morpholino)propanesulfonic acid (MOPS) and ammonium formate (for HPLC) were provided by Fluka (Burch, Switzerland). Sodium hydrogen carbonate (pro analisi) and potassium carbonate (GR) were supplied by Merck (Darmstad, Germany). The (N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AATMS), vinyltrimethoxysilane (VTMS) and (3-glycidyloxypropyl)trimethoxysilane (GPTMS) coating agents were purchased from Sigma-Aldrich (St. Louis, USA). Acetonitrile (ACN) (HPLC LC-MS grade), methanol (MeOH) (for HPLC-Isocratic grade), hydrochloric acid (37%), dimethyl sulfoxide (DMSO) and trifluoroacetic acid (for LC-MS) were obtained from VWR chemicals (Linars del Vallés, Spain). Acetic acid (LC-MS grade) was purchased from Fluka (Burch, Switzerland). Ultrapure analytical water was obtained from a Milli-Q Element A10 system (Millipore, Milford, USA).

2.2 Standard and working solutions

CSF stock solution was prepared at 1600 mg/L weighing CSF powder using an analytical balance Sartorius CP224 and dissolving it in DMSO. It was aliquoted and kept at -20 ^{o}C until working solutions preparation.

All the studies were performed at 0.25 mg/L CSF final concentration. The 0.5 mg/L CSF working solution, except in the experiment with MeOH where it was 0.83 mg/L, was prepared in MOPS buffer at pH 7. After 30 min, samples were prepared directly in vials by the transfer or dilution of the working solution.

2.3 Materials

Snap top normal glass vials (borosilicate clear glass type 1) and the corresponding plugs (with septum PTFE/silicone precut) were purchased from Scharlau (Barcelona, Spain). Commercially silanized glass vials (VWRI548-1366) were obtained from VWR (Fontenay-sous-Bois, France). Polypropylene vials (250 µL) and polyethylene terephthalate blood tubes (Vacutainer, label Z, 7 mL, 13x100mm) were supplied by Thermo Scientific (Waltham, Massachusetts, USA) and BD (Eysins, Switzerland), respectively. Eppendorf polypropylene 1.5 mL tubes were obtained from VWR. Universal pipette tips of 2-200 µL (Cat. No. 612-5755) and 100-1000 µL (Cat. No. 612-5756) of low-density polyethylene were bought from VWR chemicals. Nunclon delta surface 96-well plates (polystyrene) were purchased from Thermo Scientific.

2.4 Material treatment solutions

Plastic and glass materials were treated with AATMS, VTMS and GPTMS coating agents, following the procedure described by Fukazawa *et al*. [19]. In short: Using a 1% (v/v) acetic acid solution as solvent for preparing a 1% (v/v) coating agent solution. Coating solution was stirred for 1 hour. Afterwards, the material was filled with this solution. Five minutes after, the solution was removed and the material was left during 6 hours at 50^{o}C for drying.

2.5 Chromatographic equipment and conditions

Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, USA) coupled to a Fluorescence detector (FLD) was used. The chromatographic column used was an HSS T3 (50x2.1mm, 1.8µm) from Waters. System control, data collection and data processing were accomplished using Empower 2 software.

Chromatographic conditions were adapted from a previous work [36] and consisted of an aqueous mobile phase of pH 2 (pH Meter GLP 22, Crison, L'Hospitalet de Llobregat, Spain) adjusted with TFA (A) and acetonitrile as organic modifier (B) at a flow rate of 0.5 mL/min. The elution gradient was from 20 to 100% of B in 6 min and after a re-equilibration step of 2 min. 5 µL of sample were injected. During the chromatographic analysis, the column was thermostated at 35°C and samples were kept at 22°C in the autosampler. The excitation/emission wavelengths of FLD detector were 229/300 nm. Five replicates corresponding to five different vials were analyzed in each series.

In preliminary trials, adsorption process was observed and differences in the signal obtained for the same solution inter-days were noticed. To avoid any misinterpretation of the results due to these differences, chromatographic peak areas obtained of each day were normalized to the maximum of each experiment.

2.6. Non-specific adsorption in different laboratory materials

The solution of 0.25 mg/L CSF in MOPS buffer (10 mM, pH 7) was prepared in a volumetric flask and transferred to the different containers. Five vials of each one were analyzed: glass vials, polypropylene vials, blood tubes (polyethylene terephthalate) and Eppendorf tubes (polypropylene).

2.7 NSA in glass chromatographic vials

2.7.1 Effect of coating agent treatment

Normal vials, commercially silanized vials, vials coated with AATMS, vials coated with GPTMS and vials coated with VTMS, were compared using the same CSF solution (0.25 mg/L CSF in MOPS buffer at pH 7). Injections were performed at time 0 and after 24 h. Five different vials were filled for each condition which constituted five replicates analyzed randomly.

2.7.2 Kinetic study of the NSA

0.25 mg/L CSF in MOPS solution was placed either in a normal glass vial or coated with AATMS. Vials were left in the autosampler and injected several times during 12 h. During the first hour they were injected each 10 min, after, from 1 h to 3 h each 30 min and from 3 h to 6 h each hour. Finally, a 12 h sample was analyzed.

2.7.3 Effect of organic solvent proportion

CSF working solutions (0.5 mg/L CSF and 0.83 mg/L CSF) were prepared and left in the volumetric flask for 30 min. Samples were prepared in vials as described in Table 1 and Table 2 for ACN and MeOH studies, respectively. The preparation targeted a final concentration at 0.25 mg/L CSF whatever the dilution solvent used. First the solvent, after the MOPS and finally the CSF volume were added.

Table 1: CSF: MOPS: ACN volumes (v/v/v) for the study of ACN influence on adsorption phenomenon

ACN	0 %	10 %	20 %	30 %	40 %	50 %
0.50 mg/L CSF in MOPS (µL)	500	500	500	500	500	500
MOPS (µL)	500	400	300	200	100	-
ACN (µL)	-	100	200	300	400	500

Table 2: CSF: MOPS: MeOH volumes (v/v/v) for the study of MeOH influence on adsorption phenomenon

MeOH	0 %	10 %	20 %	30 %	40 %	50 %	60 %	70 %
0.83 mg/L CSF in MOPS (µL)	300	300	300	300	300	300	300	300
MOPS (µL)	700	600	500	400	300	200	100	-
MeOH (µL)	-	100	200	300	400	500	600	700

Samples in vials treated with AATMS and non-treated ones were analyzed and and results compared. Five vials of each one were injected randomly at 0 and 24 h.

2.7.4 Effect of pH

A working solution of 0.50 mg/L in MOPS (10 mM) was freshly prepared. A volume of 0.5 mL CSF solution was added to each vial already filled with the consequent 0.5 mL of buffer solution. pH 3 (formic acid/formate), pH 7 (MOPS) and pH 11 (carbonate/bicarbonate) buffers were prepared at 10 mM. Five vials of each one were injected randomly.

2.8 Adsorption effect in microbiology 96-well plates and application to EUCAST protocol

Samples were prepared following the broth dilution procedure established by the EUCAST [37] and inoculated with *Candida albicans* reference strain 54550. In this technique, a two-fold serial dilution of CSF is made in the liquid medium from one well plate to the next one. Starting at 8 mg/L CSF in RPMI cell culture medium at pH 7 buffered by MOPS, a range from 4 mg/L to 0.015 mg/L was analyzed. Three replicates were injected of each condition (treated and non-treated well plates with AATMS deactivated agent).

3. Results and discussion

The chromatographic method used, was adapted from one previously published [36]. The column change was removed to avoid the elimination of potential CSF degradation products and the elution was swapped to an elution gradient to be sure of their elution.

3.1 NSA in different laboratory materials

For analytical or microbiology laboratories, a lot of different materials are commonly used, consisted of glassware, plastic such as vials, pipette tips, Pasteur pipettes, blood tubes, Eppendorf tubes, … For that reason, firstly the study of the adsorption phenomenon of CSF was carried out with different types of materials.

The effect of adsorption when transferring the CSF solution with a glass pipette (Pasteur) or plastic pipette tips was not seen significant in the preliminary experiments and this variable was not taken into account for the rest of the experiments.

A clear effect of the sample container was observed (**Figure 2**), which is a main issue when using CSF solutions. Polypropylene HPLC vials result to be a non-feasible container for the studied analyte due to the low signal obtained. The effect of using blood tubes (polyethylene terephthalate) or Eppendorf tubes (polypropylene) could be also observed.

Based on the results obtained, the impact of using glass or plastic material must be considered when working with CSF solutions. Interestingly, a difference among the plastic types has been seen. In pipette tips (low-density polyethylene) the effect was negligible, whereas in HPLC vials (polypropylene), Eppendorf tubes (polypropylene) and blood tubes (polyethylene terephthalate) adsorption was clearly observed. So, even if we can conclude that "plastic" materials induce greater CSF adsorption compared to glassware, it is not enough to consider the type of plastic like polypropylene for example. This designation only takes into account the pattern used in the polymerization process. But a polymer is a complex structure: degree of polymerization, polymorphism, additives, … The huge difference between the two kinds of polypropylene (HPLC vials and Eppendorf tubes) has to be highlighted. Differences in physical properties can be easily observed when using them: while polypropylene HPLC vials were soft and malleable, Eppendorf tubes were so much rigid. The opacity is also a feature between these containers, Eppendorf tubes were transparent whereas the HPLC vials were totally opaque. Probably the crystal structure of them is not the same and this had a meaningful impact in CSF adsorption.

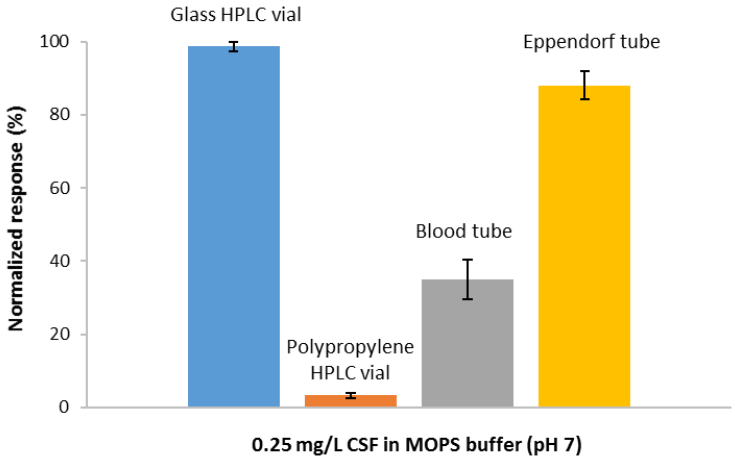


Figure 2: Normalized response for CSF solution (0.25 mg/L in MOPS) in different sample containers with their standard deviation (n=5)

3.2 NSA in glass chromatographic vials

3.2.1 Effect of coating agent treatment

Different coating agents were tested with the aim of minimizing the adsorption of CSF in material. HPLC glass vials were treated with different coating agents (AATMS, VTMS and GPTMS) and compared with non-treated ones. Commercially silanized glass vials were also assayed in this study. No statistically significant difference ($p>0.05$, 95% confidence level) was observed between the different conditions using freshly prepared samples due to the high variability of the results observed within each series (**Figure 3**). This variability could be related to a fast adsorption kinetics which can be observed due to the time difference between the analysis of the first and the last replicates.

After 24h, even if the signal decreased significantly in all cases, AATMS treatment samples shown the less adsorption compared with the other conditions. However, in general, glassware coating is not a solution to prevent adsorption of CSF.

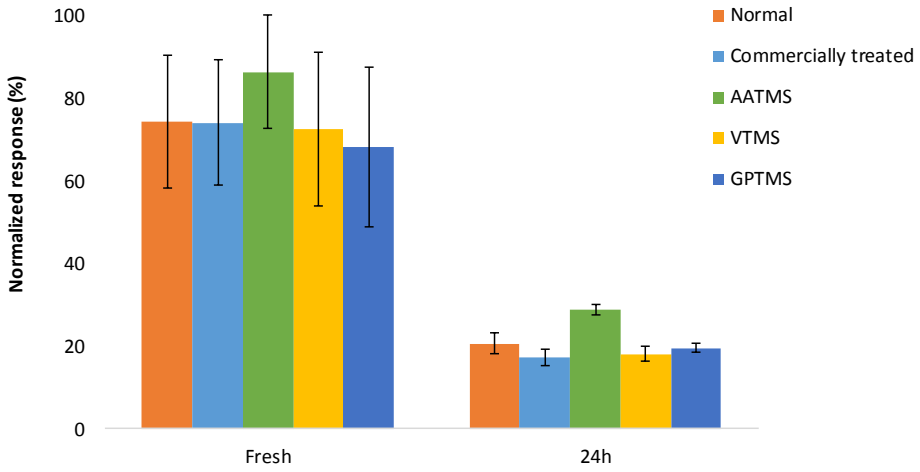


Figure 3: Effect of different vial treatment on CSF response using freshly prepared samples and 24h after preparation (n=5)

The decrease of CSF response at 24 h could also be attributed to other reasons such as analyte degradation. In order to study this possibility, acetonitrile was added to the samples after 48 h to a final concentration of 30%. Samples were analyzed again and the signal obtained was more than the double of the fresh one. This recovery, shown that ACN reversed the process of adsorption.

3.2.2 Kinetic study of the NSA

In order to study the evolution of CSF concentration with time, the kinetic profile of the adsorption phenomenon was investigated. Kinetic profile of CSF 0.25 mg/L in MOPS solution in normal and treated vial (with AATMS) is shown in **Figure 4**. Results demonstrated the change in the kinetic profile when treating the glass vial with the coating agent. The adsorption in the normal vial is much more drastic in the second hour compared with the treated one, CSF concentration decreases until 51%. Meanwhile, in the treated one, the normalized chromatographic response of CSF was maintained within 90% of the initial concentration for at least 3h. This variation confirmed the hypothesis made to explain the reason of the high standard deviation

seen in the previous experiment (Section 3.2), due to the length of the chromatographic sequence (around 3 hours between first and last replicate of each series). In this way, if the different conditions are studied by random injections during the chromatographic sequence, around 3 hours can be used for injection of the five replicates of each series, between first and last replicate.

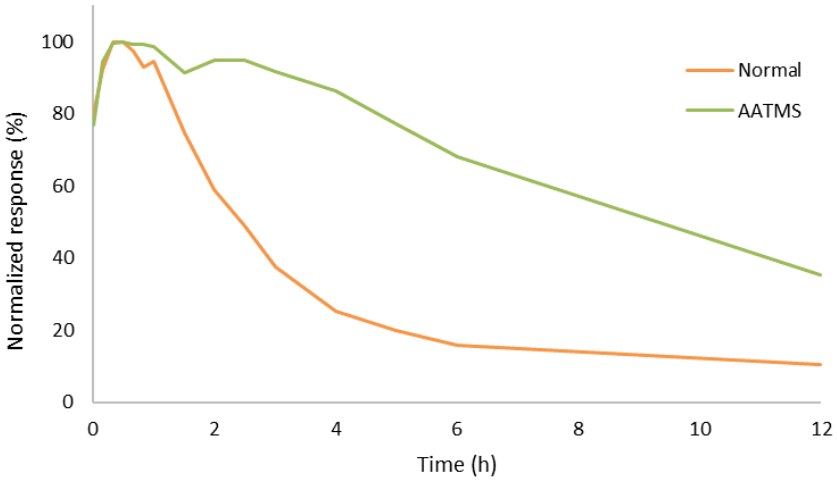


Figure 4: Kinetic profile of CSF adsorption in normal and treated with AATMS glass vials. 0.25 mg/L CSF in MOPS at pH 7

In the adsorption kinetic profile obtained for the normal vial, a plateau was reached after 6 hours remaining only a 10% of the initial concentration. However, in the vial treated with AATMS, at 12 h the remaining concentration of CSF was 35%, and adsorption phenomenon was still occurring slowly. This result supports and explained the previous work published by this group [36], where a decrease in CSF concentration was seen in real samples analysis. In literature, this plateau has been also described for proteins. In protein adsorption studies has been reported that a plateau is reached at some point of response-time variation, in some cases faster (60 min) and in others slower (24 h) [30]. Thus, glassware coating treatment has always been applied with AATMS for the preparation of CSF sample, as this slows down the kinetic of adsorption.

3.2.3 Effect of organic solvent proportion

The presence of organic solvent in CSF solution may prevent interaction with the material of the container and can avoid adsorption phenomenon. Methanol and acetonitrile were tested. Results obtained (**Figure 5**) shown the addition of organic solvent as a valuable way to minimize or avoid CSF adsorption to glass vials. It can be observed, that as soon as 10% of MeOH or ACN is added, a significant increase of the signal is measured, except for MeOH in normal vials where obtained response kept comparable.

The use of 30% of ACN apparently avoids the adsorption in freshly prepared samples, but it is not enough to suppress the adsorption kinetic during 24 h (**Figure 5 a**). In this way, 40% of ACN is required to ensure no effect of adsorption in 0.25 mg/L CSF samples. Comparing treated and non-treated vials (exception 20% ACN), no statistically significant difference has been observed ($p>0.05$, 95% confidence level).

MeOH was proved to be less effective to avoid adsorption, in this way, a wider range was used for the study of the MeOH effect. As seen in **Figure 5 b**, the percentage of MeOH required to avoid analyte adsorption is higher than the percentage of ACN. At least 60% of MeOH is needed to avoid the adsorption of CSF in freshly prepared samples and to ensure a reliable quantification during 24 h in treated vials. For vials non treated with AATMS, a 70% of MeOH was found as needed minimum proportion to ensure comparable results in freshly and after 24 h analyzed samples ($p>0.05$, 95% confidence level). In addition, it has to be mentioned the difference appreciated between treated and normal vials in the analysis of fresh samples with MeOH. With this organic modifier, the treatment of vials increases its effectiveness obtaining higher chromatographic signal.

Results obtained in this section, are supported by some studies found in literature where they proved the effectiveness of organic solvent addition to increase the solubility of peptides in solution and, by this way, to minimize adsorption [20,24,38].

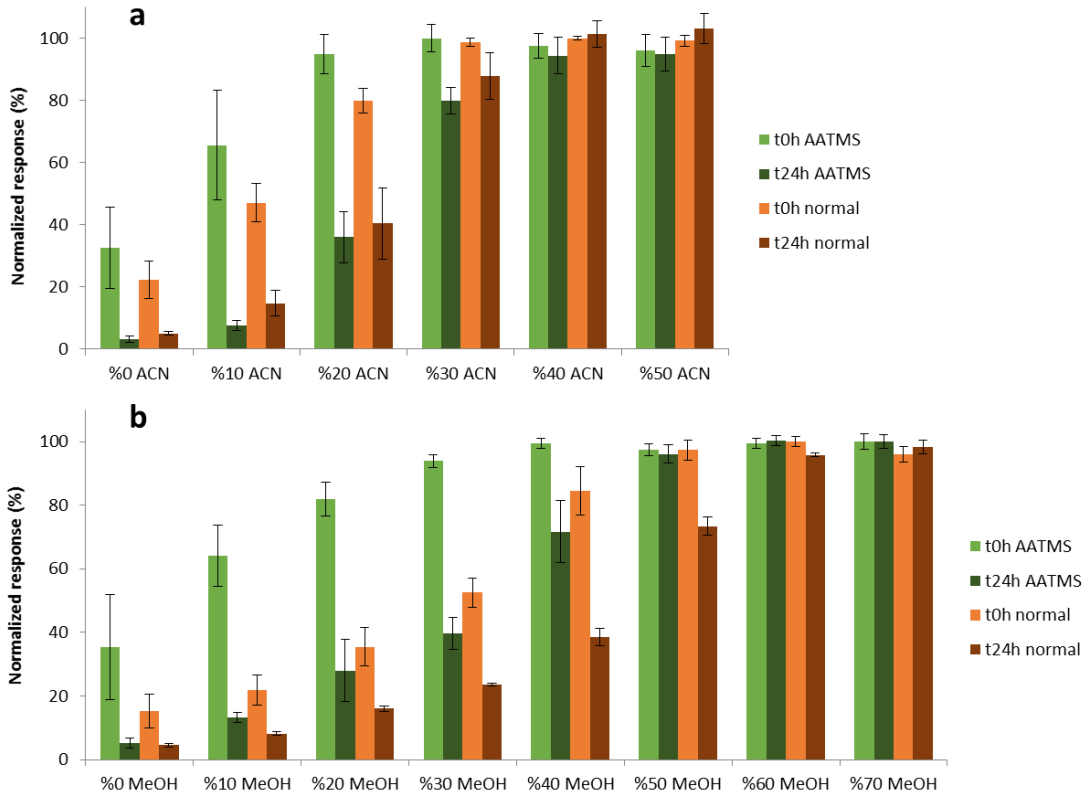


Figure 5: Effect of ACN (a) and MeOH (b) on the response of CSF MOPS solution (pH 7). Treated with AATMS and normal vials, 0.25 mg/L CSF final concentration (normalized response, n=5)

3.2.4 Effect of pH

The effect of pH on the adsorption process was studied. pH values of 4, 7 and 10 were selected in order to cover a wide range of pH where CSF is in different ionization states (**Figure 1**).

In **Figure** , a huge difference in normalized chromatographic response was observed depending on the pH value assayed. Results demonstrated a significant signal decrease at pH > 4 condition. In addition, at pH 4 no time dependence has been seen between freshly injected solution at 0 h and 24 h.

At pH 7 and pH 10, a decrease in signal was seen comparing with acid conditions. In addition, whereas at pH 4 the signal remained stable during 24h whatever the vials. The adsorption rate between vials treated with AATMS and normal ones, were not comparable at pH > 4 and with not treated vial, the decrease is more important and faster as previously observed. While at pH 7 in fresh samples, AATMS treatment minimizes the adsorption compared with the normal ones. Alkaline conditions seemed to be the worst.

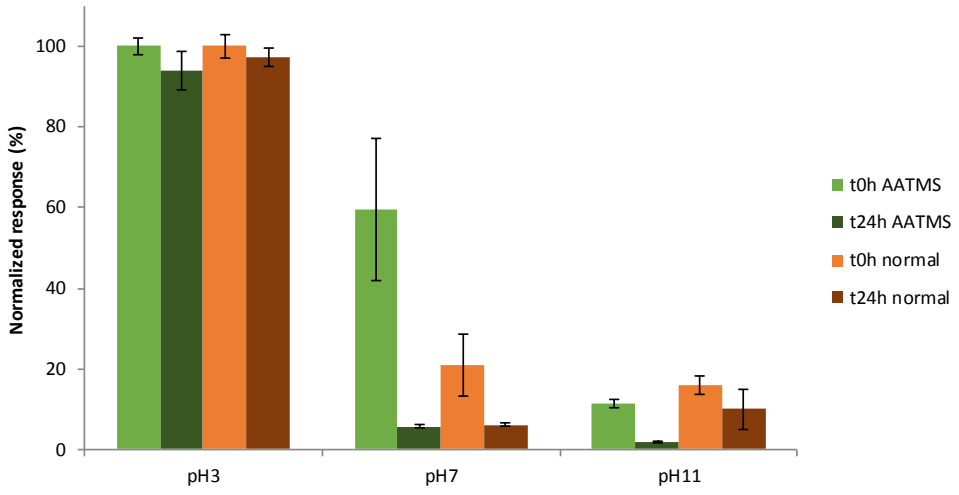


Figure 6: Normalized response for CSF analysis at different buffered solutions and H_2O, and with or without vials treated with AATMS. CSF concentration: 0.25 mg/L (n=5)

For a better comprehension of the results, it must be taken into account the different protonation states of the molecules involved in the adsorption process: the analyte, the silica network in glass vial surface and the AATMS in treated glass vial.

Based on the predicted pKa values (**Figure 1)**, CSF molecule's major species would be positively charged (+3) at pH 4, positively charged (+2) at pH 7 and negatively charged (-2) at pH 10. The silica network in glass vials is neutral except in alkaline conditions where it is deprotonated and charged negatively (-1) [38]. For vials treated with AATMS, first, the treatment done to the material has to be understood. In brief, in the silanization process the methoxy groups of AATMS (**Figure 7**) are hydrolyzed, condensed and finally bonded to the substrate (borosilicate glass vial surface) [32-34]. In this way, the interaction with the analyte happens with the end of the molecule of the coating agent (N-2-(aminoethyl)-3-aminopropyl group). In the pH range of this study, there is an AATMS's major micro species change at pH 8.4 (predicted value). The coating agent molecule changes its protonation state from positively charged (+2) to non-charged major micro species. A summary of the protonation states is shown in **Table 3**.

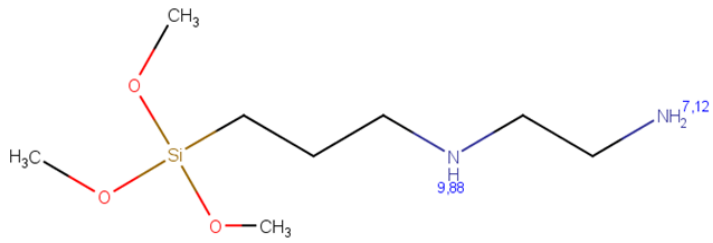


Figure 7: AATMS coating agent's chemical structure and predicted pKa values. Source: MarvinSketch 17.22

Table 3: Protonation states of glass vial, CSF and AATMS in acid, neutral and alkaline conditions. Source: Biavati et al. [38] and MarvinSketch 17.22

	Glass	AATMS	CSF
pH 4	Neutral	+2	+3
pH 7	Neutral	+2	+2
pH 10	-1	Neutral	-2

Due to the lack of studies for CSF, authors tried to hypothesize an explanation for the behavior of the studied analyte. The results obtained seemed to purpose a first rapid charge-charge interaction and after, a rearrangement or a slow adsorption occurring because of hydrophobic interactions. At pH 4 the analyte is in its more polar state and it is favored to be in aqueous solution, no effect of the vial surface treatment could be seen. At pH 7, the analyte loss one of the positive charges and it is not as polar as in the previous state so it is not so favored to be in solution. The signal could decrease as an answer of more affinity between the analyte and the surface. However, due to the positively charged AATMS state, in treated vials the signal obtained for freshly prepared samples is so much higher than in normal ones. After the first charge – charge interaction, the adsorption increases by hydrophobic interactions. In pH 10 all the protonation states of the molecules involved changed. In this condition, the analyte is in the most non-polar state and probably is not favored to be in solution. Normal vials shown better results probably due to a charge repulsion forces (- 2 of the analyte and -1 of the glass surface).

3.3 Adsorption effect in microbiology 96-well plates and application to EUCAST protocol

Resistance studies of *candida spp*. are usually carried out by means of MIC calculation and comparison with reference values. As before mentioned, no values are given for CSF in *candida spp.* due to the lack of interlaboratory reproducibility. The adsorption of CSF in 96-well plates was studied and the possibility of minimizing the adsorption by treating the material with a coating agent (AATMS, VTMS and

GPTMS) was evaluated. The study of coating agent treatment effect in the 96-well plates proved (results no shown) that the best option to minimize the CSF adsorption was the treatment with AATMS, as it was observed for glass vials too.

Results in **Figure 8** shown the differences in the normalized response obtained when comparing 96-well plates treated with AATMS and non-treated ones, using CSF real samples from an EUCAST procedure. Dilutions from 4 to 0.125 mg/L of CSF were analyzed. No chromatographic peak area was observed for dilutions below 0.5 mg/L CSF.

Results shown significant differences due to a minimization of CSF adsorption when treating the plates with AATMS. In addition, results seen for treated plates shown less variability in CSF amount. This fact could have an important impact in MIC determination, decreasing the variability in results obtained and increasing the reliability of them.

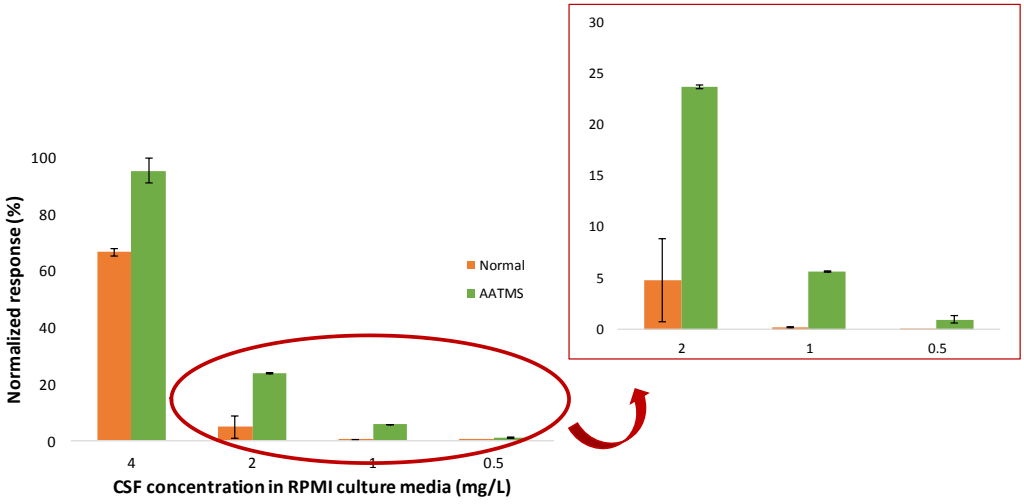


Figure 8: Normalized chromatographic peak areas of CSF concentration from real samples obtained following the EUCAST protocol for MIC determination by broth dilution procedure. Comparison of normal and AATMS's treated 96-well plates (n=3)

4. Conclusions

In this work, huge differences were found in the non-specific adsorption of CSF when using distinct laboratory materials. Dissimilarities within plastic types as low-density polyethylene, polypropylene and polyethylene terephthalate were noticed. Acidic pH 4 was found as the best condition to avoid CSF adsorption in aqueous solution and the addition of MeOH and ACN, shown the ability to decrease adsorption to HPLC glass vials since low proportion (10%). The addition of 50% of ACN to CSF aqueous solutions (pH 7), shown to avoid the adsorption of CSF in chromatographic glass vials at least for 24h. With MeOH, this occurs using 60% and 70% of solvent for treated (with AATMS) and normal vials, respectively.

Even though these approaches prevent the adsorption of CSF they are not applicable to *in vitro* or *in vivo* experiments performed in antifungal activity studies. Taking this into account, in this work we present a surface coating treatment for the sample container in order to avoid any sample manipulation or modification. The N-(2-aminoethyl)-3-aminopropyltrimethoxy silane coating agent was found to be able to minimize and slow down the adsorption process of CSF in HPLC glass vials. The treatment appears to reduce the adsorption in freshly prepared samples but is not totally effective, the adsorption still happens during time, but slower. Surface coating procedure with AATMS was found, as well, successful for minimizing adsorption of CSF in 96-well plate experiments. Important CSF concentration increase was found when treating the plates and this suggests that MIC results obtained before with CSF drug could be not reliable.

The results obtained have a vital impact on therapies and clinical microbiology tests. As it is known, CSF is a drug only administered by intra venous infusion and, obviously, in an aqueous solution. The possible loss of the drug amount during the perfusion could result in a lower dosage and the misinterpretation of the efficacy of it. From authors perspective, further studies in this field are needed to ensure reliable therapies.

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7. Kapitulua

Ondorio orokorrak eta etorkizuneko ikuspuntuak

Ondorio orokorrak

Tesi honen helburua *C. glabrata* hainbat zepen kaspofunginarekiko (CSFrekiko) erresistentzia aztertzeko ikuspegi analitiko berriak garatzea zen. Materialetan CSFren adsortzioarekin lotutako zenbait gai jorratu ziren, eta hori garrantzitsua izan da konposatu farmazeutiko honen erabilera egokia ebaluatzeko.

Matrize biologikoetan CSF determinatzeko erabilitako metodo analitikoen berrikuspena egin zen eta **2. kapituluan** deskribatu zen. Aurkitutako baldintza analitikoei buruz lortutako informazioa bereizmen handiko likido kromatografia (HPLC) metodoa garatzeko oinarri gisa erabili egin zen. **3. kapituluan** garatutako metodoa, column-switching-HPLC-FLD metodoa, azkarra, fidagarria eta sentikorra dela frogatu da CSF ekokinokandinaren kuantifikazioa egiteko kultibo zelularreko matrizean. Column-switching-ari esker, prozedura analitikoan zehar adsortzioaren ondorioz eman ahal den analito galera murrizten da, laginaren aurretratamendurik ez baitago. Baita, determinazio kuantitatiboa burutzeko metodologia horrek lagin bolumen txikia (50 µL) erabiltzen duenez, in vitro azterketetan erabili daiteke, non laginaren bolumena aldagai mugatzaile bat izaten den.

In vitro-ko eredu farmakozinetiko / farmakodinamikoa erabiltzean ikusi zen CSF galerak **4. kapituluan** proposatutako C. glabrata-ren erresistentzia azterketa egitea galarazi zuen. Horrek ere molekula honen potentzial terapeutikoaren ebaluazioa zalantzan jarri zuen. Dosifikazioari edo erresistentziari dagokionez, ezin da ondoriorik atera droga kantitatearen eta horren efektuaren arteko erlazioa egitea ezinezkoa bada.

Degradazio- eta adsortzio-prozesuak aztertu ziren in vitro ereduan ikusitako CSF kantitatearen murrizketa azaldu ahal izateko. **5. kapituluan** zehar HPLC-FLD, RMN eta LC-MS teknikak erabili ziren CSFren degradazioa aztertzeko. Estres baldintza ezberdinak frogatu ziren farmakoaren degradazioa bultzatzeko asmoarekin, hala nola, ingurune azidoa, alkalinoa eta oxidatzailea. Ikusitako degradazio produktuen

identifikazio partziala bakarrik lortu zen RMN eta MS tekniken bidez. Hala ere, baldintza horietan ikusitako farmakoaren degradazioa 4. kapituluan antzemandako CSF zenbatekoaren galerarekin bat ez zetorrela ikusi zen. Horretan oinarrituz, degradazioa analito galeraren erantzule nagusia ez zela ondorioztatu zen.

Galera horren arrazoi nagusia aurkitzeko helburuarekin, analitoaren materialekiko adsortzioaren azterketa egin zen **6. kapituluan**. Material desberdinen gainazaletan CSFk erakusten zuen adsortzioa argi ikusi zen ikerketan zehar. Beirazko eta plastikozko materialen artean desberdintasun garrantzitsuak ikusi ziren. Gainera, plastiko moten artean ere alde handiak nabarmendu ziren. pH azidoa eta disolbatzaile organikoek CSFren adsorzioa ekiditeko gai direla ikusi zen. Baldintza horiek in vitro azterketetan aplikatzea ezinezkoa denez, CSFren adsortzioa murrizteko edo saihesteko gai zen materialen tratamendu bat ikertzera eraman gintuen. Tratamendu horrek beirazko materialetan ematen den CSFren adsortzioaren zinetika moteltzen duela ikusi zen. Bere eraginkortasuna frogatu egin zen 96 mikroputzuetako plaketan ematen den adsortzioa minimizatzeko. Ikerketa horrek CSFrentzako MIC balioen ofizialen gabezia azaldu dezake eta MIC zehazteko probak modu fidagarriagoan egiteko gakoa ematen du.

CSF ekinokandinaren portaera analitikoan aurrerapen garrantzitsuak lortu dira tesi honetan, baina gaiaren inguruko ikerketa gehiago behar dira oraindik. Baldintza fisiologikoetan CSF kuantifikatzeko garatutako eta balioztatutako metodologiak etorkizunean egin beharreko ikerketetarako erabilgarritasuna erakutsi du. Tesi honek emaitza garrantzitsuak aurkezten ditu beste ikertzaile batzuek lan honen azken helburua lortu ahal izateko: antifungikoekiko erresistentziaren aurka borrokatzea.

Etorkizuneko ikuspuntuak

Tesia garatzean aurkitutako erronkek hainbat galdera sustatu zituzten inplikatutako ikertzaileengan eta atea ireki zuten etorkizuneko esperimentu batzuk proposatzeko.

Alde batetik, baldintza azido bortitzetan degradatutako lagina HPLC-FLD eta NMRren bidez analizatzean ikusitako degradazio produktuen identifikazioa ezinbestekoa da. Horretarako, esperimentu gehiago egitea beharrezkoa da, adibidez, HPLC-MS analisia ESI- ionizazio modua erabilita. Identifikazioa, kontzentrazio galera adsortzio edo degradazio prozesu baten ondorio dela ziurtatzeko lagungarria izango da.

Bestetik, adsortzioaren zinetika eta prozesuaren itzulgarritasuna aztertzea gomendagarria da. Kaspofunginaren materialekiko adsortzioaren profil zinetikoa ulertzeak horren erantzule diren mekanismoak argitzea ahalbidetuko luke eta adsortzio ekiditeko prozesua lantzeko aukera eskainiko luke. Gainera, itzulgarritasuna ikertzeak kaspofunginarekin materiala asetzeko bidea argitu dezake. Oso erabilgarria izango litzateke desortzio prozesurik ematen den ala ez jakiteko eta zein baldintzatan gertatzen da. Horrela, *in vitro* egin beharreko esperimentazioak aurrera eramateko aukera egon ahal da.

Baita ere, kaspofunginarekin *in vitro* erresistentziaren azterketa egitea komenigarria da. SIMULI-PHARM 1 bi konpartimentuko PK/PD ereduarekin egindako esperimentuan ikusitako analito galerak aurkeztutako oztopoari konponbidea bilatu behar zaio. Horretarako, ikertzaileek hodi mota desberdinak erabiltzea proposatzen dute ereduko bi konpartimentuan. Jakina denez, erabilitakoak PVCak ziren eta, beraz, merkatuan dauden infusio ekipo desberdinekin saiatzea proposatzen dute ikertzaileek. Horretarako ospitaleetan erabilitako materialei buruzko informazioa garrantzi handikoa izango litzateke.

General conclusions

The aim of this thesis was to develop new analytical approaches to study the resistance of *C. glabrata* strains to caspofungin (CSF) using *in vitro* models. Several issues related to the adsorption of caspofungin on materials were addressed, which was of primary importance to properly assess the use of this pharmaceutical compound.

A literature review of the analytical methods available for CSF determination in biological matrices was carried out and described in **Chapter 2**. The information obtained regarding the different analytical conditions found served as knowledge base for the development of the HPLC method proposed. The column-switching high performance liquid chromatography coupled to fluorescence detection (column-switching HPLC-FLD) method developed, included in **Chapter 3,** has demonstrated to be rapid, efficient and sensible for the quantification of CSF echinocandin drug in cell culture medium. It offers a rapid approach without prior sample preparation, which limits the loss of analyte amount by degradation or especially adsorption during the analytical procedure. The quantitative determination of CSF using small volume of sample (50 µL) supports this methodology for its use in *in vitro* assays, where sample volume is a limitation.

CSF loss observed during *in vitro* study by an *in vitro* Pharmacokinetic/Pharmacodynamic model, prevented the resistance study of *C. glabrata* proposed in **Chapter 4**. The results obtained questioned the evaluation of the therapeutic potential of this molecule. In terms of dosage or resistance for example, no conclusion can be taken if the relationship between the amount of drug and effect could not be done.

Degradation and adsorption processes were studied to explain the CSF concentration decrease in physiological medium solution. Along **Chapter 5** NMR and LC-MS techniques were used to study the degradation of CSF in several forced

degradation corresponding to various stress conditions: acidic, alkaline and oxidative. The obtained degradation products were not successfully fully identified either by NMR or by MS. However, it was found that the decrease of CSF concentration related to degradation process was not enough to explain the loss of CSF amount observed during experimentation presented in Chapter 4.

Consequently, in **Chapter 6** an adsorption study of the analyte was carried out. Adsorption of CSF at the surface on different materials was evidenced during the research. Important differences in adsorption were seen between glass and plastic materials. Moreover, huge differences along plastic types were also proved. pH and solvent conditions able to avoid CSF adsorption were found. The uselessness of these approaches for *in vitro* studies led us to study a material treatment for the minimization or avoidance of CSF adsorption. A surface treatment, which delays the adsorption kinetic of CSF in glass was found. It was proved the efficacy of this treatment to minimize adsorption in 96-well plates tested. This study could explain the lack of any established MIC value for CSF and gives the possible key to carry out MIC determination assays in a more reliable way.

In conclusion, significant progress in the analytical behaviour of the echinocandin caspofungin was achieved in this thesis, but further research in the topic is still needed. The methodology developed and validated for CSF determination in physiological conditions demonstrated its usefulness for the future investigations required. The important findings achieved in this thesis could help researchers to achieve the final objective of our work: fight against antifungals resistance.

Future perspectives

The challenges encountered during the development of this thesis created some inquisitiveness in the researchers involved and open the door to perform some future experiments.

First, the identification of the possible degradation products seen by HPLC-FLD and NMR analysis using stress conditions is a must. For that, HPLC-MS in ESI- ionization mode is suggested. Their identification will help to be able to ensure if the analyte concentration loss is due to an adsorption or a degradation process.

Secondly, adsorption study must be continued regarding its kinetic and the reversibility of the process. The comprehension of the adsorption kinetic profile of caspofungin with different materials would allow the elucidation of the mechanisms responsible and offer the possibility of the process elaboration of adsorption suppression. In addition, establishment of the reversibility (which conditions) could allow to carry out material saturation assays, helping the *in vitro* experimentation.

Finally, *in vitro* resistance study with caspofungin should be performed. The huge obstacle found due to the analyte loss during experimentation with SIMULI-PHARM 1 bicompartmental PK/PD model, must be solved. For this, researchers suggest to use different types of tubing for both compartments of the model. As known, the ones used were of PVC so researchers suggest to try with the different infusion equipment available in the market. The information on the materials used in hospitals for this purpose would be of great importance.

Appendix

Published articles

Review

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Bioanalysis

Chromatographic methods for echinocandin antifungal drugs determination in bioanalysis

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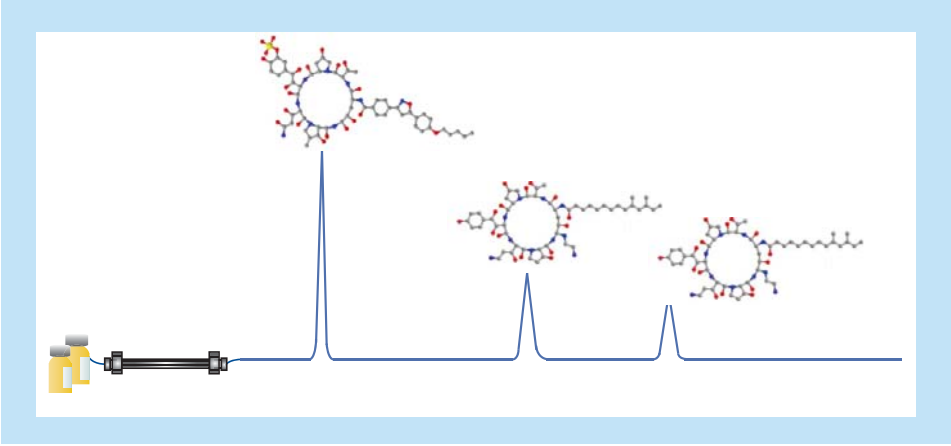
The increase of fungal resistance to drugs, such as azole family, gave rise to the development of new antifungals. In this context, echinocandins emerged as a promising alternative for antifungal therapies. Following the commercialization of caspofungin in 2001, echinocandins became the first-line therapy for invasive candidiasis in different patient populations. The quantification of these drugs has gained importance since pharmacokinetic/pharmacodynamic and resistance studies are a paramount concern. This fact has led us to exhaustively examine the methodologies used for the analysis of echinocandins in biological fluids, which are mainly based on LC coupled to different detection techniques. In this review, we summarize the analytical methods for the quantification of echinocandins focusing on sample treatment, chromatographic separation and detection methods.

Graphical abstract:



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Keywords: bioanalysis • echinocandins • LC

During the last few decades an increase in invasive infections has been observed [1–3]. In this context, fungal infections have become a major cause of human disease, particularly in hospitalized and immunocompromised patients [4–6]. The increment of this kind of diseases and the high rate of mortality related to invasive fungal infections (often >50%), together with the rising resistance of some species of *Candida* to antifungal drugs, are a

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Determination of antifungal caspofungin in RPMI-1640 cell culture medium by column-switching HPLC-FLD

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ABSTRACT

The actual scenario in the fight against fungal infections forces researchers to carry through with resistance studies to improve the therapies. These studies, which are performed in cell culture media, need accurate and sensitive analytical methodologies. That is why, in this work, an analytical method for caspofungin (CSF) concentration determination in RPMI-1640 cell culture medium with on-line sample treatment was developed and validated. CSF concentration was determined by HPLC-FLD using a column-switching procedure. The chromatographic analysis was carried out in less than 10 min using a C8 column (4 × 4 mm, 5 μm) as extraction stationary phase and a HSS T3 column (4.6 × 100 mm, 5 μm) as the analytical column. The used mobile phases were mixtures of phase A: pH 2 (adjusted with TFA) aqueous phase and phase B: ACN. For the extraction, the composition was (95:5, A:B v/v) and for the analysis (60:40, A:B v/v), both done in isocratic elution mode. These chromatographic conditions allowed reaching a limit of quantification of 10 μg/L, using 100 μL of sample with an injected volume of 40 μL. The proposed method was successfully validated in terms of selectivity, carryover, linear concentration range, accuracy and precision according to the criteria established by the Food and Drug Administration. Available amount of CSF in RPMI-1640 solution was found critical. CSF concentrations remained stable up to 2 h at room temperature. The developed method was applied for the direct analysis of CSF concentrations from *in vitro* experiments in presence of *C. glabrata* (CAGL18). The results highlight the decrease of cell proliferation even if the CSF amount decreases too, which asks question about the real value of the efficient concentration for CSF antifungal activity.

1. Introduction

Since the last decades, the incidence of fungal diseases has drastically increased. Severity of fungal diseases range from mild mucocutaneous infections to potentially life-threatening invasive infections. Serious invasive fungal diseases occur often as a consequence of underlying health problems such as AIDS, cancer, organ transplantation and corticosteroid therapies [1].

Candidiasis is one of the most frequent invasive fungal infection and an important cause of morbidity and mortality worldwide [2]. Among the 200 species described for *Candida* about 15 can cause human diseases and 5 of them are more frequent. In a retrospective study of candidemia at Nantes Hospital between 2004 and 2010, *C. albicans* was the predominant species (51.8%) followed by *C. parapsilosis* (14.5%), *C. glabrata* (9.8%), *C. tropicalis* (9.8%) and *C. krusei* (4.1%) [3]. Candidiasis are under growing interest due to the increase of incidence (particularly among immunocompromised patients), the emergence of new species (*i.e. Candida auris*) [4] and the rise of resistant and multi-resistant isolates to antifungal treatments [5].

The current armamentarium of antifungal drugs is limited to four families: azoles, polyenes, echinocandins and pyrimidines [6]. Over the years, the emergence of acquired resistance to azoles has forced the specialists to begin handling echinocandins as first-line therapy. According to Infectious Diseases Society of America (IDSA) [7,8], European congress of Clinical Microbiology & Infectious Diseases (ESCMID) [9] and European Conference on Infections in Leukaemia (ECIL) [10] guidelines, this

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molecules

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Article

Analysis of the Heterogeneous Distribution of Amiloride and Propranolol in Dried Blood Spot by UHPLC-FLD and MALDI-IMS

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Abstract: Dried blood spot (DBS) has lately experienced an increase in its use in bioanalysis due to its several advantages compared with traditional blood sampling methods. Nevertheless, the use of DBS with quantitative purposes is hindered by the heterogeneous distribution of some compounds in the supporting matrix and the dependence of the response on different factors, such as the hematocrit, blood volume, and sampling position. In this study the effect of those factors in the analytical response was investigated by ultra high performance liquid chromatography coupled to fluorescence detection, using amiloride and propranolol as model compounds. The results showed a heterogeneous and drug-dependent distribution of the compounds in the blood spot. While amiloride concentration was higher in the center, propranolol concentration was higher in the periphery of the spot. Besides, the influence of the hematocrit on the quantitative results was observed. MALDI mass spectrometry imaging (MALDI-IMS) has allowed study of the distribution of the two cardiovascular drugs when they were placed in the DBS card using water:methanol solutions, demonstrating that they followed a similar distribution pattern as in blood. This work has showed the potentiality of the MALDI-IMS technique to predict the distribution of the drugs in the DBS card.

Keywords: DBS; bioanalysis; UHPLC-FLD; MALDI-IMS

1. Introduction

Dried blood spot (DBS) sampling is a simple technique in which a drop of blood pricked from a heel, ear, or finger [1,2] is placed in a support and is left to air dry prior to analysis. The analyte extraction from the dried blood is usually carried out offline by using different solvents or, alternatively, by employing integrated procedures using online systems [3]. The DBS technique is used as a sampling method for a wide range of analytical techniques, such as DNA-based assays, enzyme activity assays, immunoassays, direct mass spectrometry, and liquid chromatography coupled to different detectors [4].

DBS sampling method was first used with human blood in 1963 by Roberth Gurthrie to detect metabolomic diseases (phenylketonuria) in newborn infants [5]. Since then, it has been employed in many different areas, such as toxicokinetics and pharmacokinetic studies, diagnostic screening, and therapeutic drug monitoring [1,4]. DBS shows many advantages compared with conventional blood, plasma or serum venipuncture collection procedures, such as the stability of the cellulose-fixed analytes, the little blood volume required, the possibility of automation of the sample processing, the easy storage and transportation, or the lower biological risk in comparison with liquid blood

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