Određivanje fizikalno-kemijske kompatibilnosti 5-aminosalicilata i folne kiseline kao preduvjet razvoja fiksne kombinacije lijekova

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PHYSICOCHEMICAL COMPATIBILITY DETERMINATION OF 5-AMINOSALICYLATES AND FOLIC ACID AS A PREREQUISITE FOR THE DEVELOPMENT OF A FIXED-DOSE COMBINATION

DOCTORAL DISSERTATION

Supervisor: Prof. Ana Mornar Turk, PhD

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Rad je predan na ocjenu Fakultetskom vijeću Farmaceutsko-biokemijskog fakulteta Sveučilišta u Zagrebu radi stjecanja akademskog stupnja doktora znanosti iz područja biomedicine i zdravstva, polje farmacija, grana farmacija.

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SAŽETAK

Crohnova bolest i ulcerozni kolitis, poznatiji kao upalne bolesti crijeva, predstavljaju kronične upalne bolesti gastrointestinalnog trakta. Zacjeljivanje sluznice i postizanje duboke remisije omogućuje se ciljanom terapijom, međutim postoje skupine pacijenata kojima koristi isključivo liječenje protuupalnim lijekovima iz skupine 5-aminosalicilata; mesalazin i njegovi prolijekovi sulfasalazin, balsalazid i olsalazin. Nedostatak folne kiseline primijećen je u više od polovine oboljelih od Crohnove bolesti i više od trećine pacijenata s ulceroznim kolitisom. Njezin nedostatak može dovesti do razvoja megaloblastične anemije, a povezan je i s povećanom prevalencijom karcinoma debelog crijeva, stoga se uz redovitu terapiju često propisuju povišene doze folne kiseline od 1 do 5 mg na dan. Razvojem fiksnih kombinacija lijekova potencijalni rizik od nepridržavanja propisane terapije nastoji se svesti na najmanju moguću mjeru, što je posebno važno kod pacijenata koji boluju od kroničnih bolesti gdje se terapija sastoji od više lijekova iz različitih farmakoloških skupina. Razvoj fiksnih kombinacija lijekova složen je postupak u kojem važnu ulogu ima ispitivanje fizikalno-kemijskih svojstava svakog pojedinog lijeka kao i njihovih smjesa. U ovom radu predložene su analitičke metode kao podrška razvoju fiksnih kombinacija 5-aminosalicilata i folne kiseline pri čemu su korištene različite analitičke tehnike, poput termalnih, spektroskopskih te kromatografskih, s ciljem ispitivanja fizikalno-kemijske kompatibilnosti 5-aminosalicilata i folne kiseline. Provedena istraživanja pokazala su da su ispitivani 5-aminosalicilati; mesalazin, sulfasalazin, balsalazid i olsalazin, fizikalno-kemijski kompatibilni s folnom kiselinom, što otvara mogućnost za daljnja ispitivanja potrebna za razvoj predloženih fiksnih kombinacija.

KLJUČNE RIJEČI: upalne bolesti crijeva, 5-aminosalicilati, folna kiselina, fizikalno-kemijska kompatibilnost, analitičke tehnike, kombinacija fiksnih doza

SUMMARY

Introduction: Crohn's disease and ulcerative colitis are chronic inflammations of the gastrointestinal tract known as inflammatory bowel diseases. Although therapeutic goals to achieve mucosal healing and deep remission require early therapy with biologics, there are groups of patients for who may benefit only by treatment with anti-inflammatory drugs from the 5-aminosalicylate group. Folic acid deficiency has been observed in more than half of adults with Crohn's disease and more than a third of patients with ulcerative colitis, and its deficiency may contribute to the development of megaloblastic anemia and is associated with a higher prevalence of colon cancer. Therefore, in addition to regular therapy, patients are often prescribed increased doses of folic acid of 1–5 mg per day. The development of fixed-dose combinations seeks to reduce the potential risk of non-adherence, which is especially important in chronic diseases where therapy involves the treatment with multiple drugs from different pharmacological groups. The development of a fixed-dose combination is demanding and complex process where the examination of the physicochemical properties of each individual drug and their mutual physicochemical compatibility plays an important role in the development of such a product.

Methods: The extent of binding of 5-aminosalicylates and folic acid to plasma proteins was determined using biomimetic columns containing immobilized endogenous structures (human serum albumin (HSA) and α1-acid glycoprotein (AGP)). Based on the retention times obtained by chromatographic analyses their binding percentage to plasma proteins was calculated. The study of potential competitive binding to the same sites on the tested proteins was performed using frontal analysis and zonal elution studies.

Perkin-Elmer Diamond differential scanning calorimeter and NETZSCH STA 409 thermal analyser were used to perform the thermal analysis. Standards of 5-aminosalicylates and folic acid as well as their mixtures prepared in different ratios were analysed.

Compatibility tests using powder X-ray diffraction were performed on a Shimadzu XRD 6000 diffractometer. Samples of 5-aminosalicylates and folic acid and their mixtures in a ratio of 1:1 were analysed. The obtained diffractograms were interpreted using Pearson correlation to determine changes in the measured mixtures.

Isothermal degradation studies were performed on prepared samples of standards, 5-aminosalicylate and folic acid dosage forms and on their mixtures by storing them in a Biosan ES-20/60 incubator for a period of 4 weeks at 50.0 °C. After heating period, samples were analyzed using Shimadzu FTIR-8400S infrared spectroscope and chromatographically on

Agilent 1100 series HPLC system using previously developed analytical method. For easier and better interpretation of the obtained FTIR spectra, statistical tools such as principal component analysis and cluster analysis were used.

Forced degradation studies were performed on prepared standard samples, 5-aminosalicylate and folic acid dosage forms and on their mixtures. Samples were degraded under acidic, alkaline, oxidative and thermal conditions. Photostability was examined as well. For the purposes of chromatographic analysis after degradation of samples, a new chromatographic method was developed with the aim of determining the extent of degradation of 5-aminosalicylates and folic acid and to monitor the formation of degradation products.

Stability studies in biologically relevant media were performed using a Labtron LDLT-A10 USP 2 apparatus to monitor drug release from dosage forms. A previously developed chromatographic method for monitoring 5-aminosalicylate and folic acid assay was used to determine the concentration of released drug. Stability of 5-aminosalicylates and folic acid was tested by placing the dosage forms in the same dissolution vessel to monitor the release extent with the aim of observing possible impact on their release and stability.

Results: With the chromatographic approach using HSA and AGP biomimetic columns it was observed that 5-aminosalicylates bind mostly to HSA protein (>61.44%) while showing lower affinity for AGP protein (>6.22%). Folic acid shows affinity and binding to HSA protein in the range of 69.40% while it is almost non-binding to AGP (3.45%). Competitive binding of 5-aminosalicylate and folic acid was tested using the HSA protein since they all have an affinity to bind to it. Frontal analysis studies examined how many specific binding sites folic acid binds to. The results of the study indicate that folic acid binds to one specific site on the HSA protein. Zonal elution studies have been used to determine whether 5-aminosalicylates tend to bind to the same specific binding site as folic acid, or whether there is a certain type of competence and positive or negative allosteric modulation because of folic acid binding to protein. The results of the study showed that there is no change in the binding of all examined 5-aminosalicylates to HSA protein, which directly indicates that there is no competition for the same binding site.

Three chromatographic methods have been developed to test the physicochemical compatibility of 5-aminosalicylates and folic acid. The first method was developed for the purpose of simultaneous determination of all four 5-aminosalicylates and folic acid. For the purposes of the forced degradation study, two methods have been developed for the simultaneous determination of folic acid, 5-aminosalicylate and their process related or degradation products. The first method for the determination of folic acid and mesalazine and

its 9 listed impurities: 4-aminosalicylic acid, 4-aminophenol, 2,5-dihydroxybenzoic acid, 3-aminobenzoic acid, 3-aminophenol, salicylic acid, 2-chloro-5-nitrobenzoic acid, 2-aminophenol and 5-nitrosalicylic acid. Another method has been developed for the determination of folic acid and sulfasalazine and its two impurities: sulfapyridine and salicylic acid. Methods were developed using reverse phase columns with gradient elution and validated according to ICH Q2 (R1) guidelines.

The compatibility of mesalazine and folic acid was determined using differential screening calorimetry (DSC), isothermal and forced degradation studies, and stability studies in a biologically relevant medium. DSC measurements showed characteristic peaks for mesalazine and folic acid corresponding to their melting points. Thermograms of mixtures in a ratio of 1:1 and 5:1 showed shifts in the peaks of mesalazine (<21.1 °C) and folic acid (<22.6 °C) indicating potential incompatibility. Forced degradation studies did not show a significant difference in the extent of degradation (<4%) or the formation of new degradation products during the degradation of mesalazine and folic acid and their mixtures. The samples showed stability in isothermal degradation and stability studies in biologically relevant media indicating the absence of chemical reactions.

Compatibility of sulfasalazine and folic acid was investigated using simultaneous thermogravimetry (TG) and differential thermal analysis (DTA), forced and isothermal degradation studies, as well as stability studies in biologically relevant media. Thermal analyses indicated a possible incompatibility due to the shift of the sulfasalazine peak in the thermogram of the mixture of 9.6 °C whilst the folic acid peak shifted insignificantly (1.6 °C). Isothermal degradation studies followed by chromatographic analysis and infrared spectroscopy showed the absence of chemical interactions. All contents were higher than 98.4% while spectroscopic measurements did not show the formation of new absorption bands. Forced degradation studies have shown a similar extent of degradation in mixtures compared to the degradation of pure constituents. Folic acid was completely degraded (100%) in the mixture by thermal stress of the prepared solution, however degradation was shown to be due to a combination of temperature and low pH (2.8, resulting from dissolution of sulfasalazine in the selected solvent) and not due to direct chemical reaction between sulfasalazine and folic acid. Stability studies in a biologically relevant medium did not show an interaction between sulfasalazine and folic acid when present in the same solution.

Physicochemical compatibility between additional two 5-aminosalicylates, balsalazide and olsalazine, and folic acid was examined as well. With the thermal analysis of balsalazide/folic acid blends as well as pure compounds, no changes were observed in

thermograms of blends. Although melting related peak of balsalazide was not observed, peak of its dehydration was used for the interpretation of compatibility. Observed balsalazide and folic acid related peaks did not change their shape and the changes of temperatures were less than 4.3 and 3 °C, respectively. In the case of olsalazine and folic acid studies, peaks related to their melting were observed and no significant changes were observed in measured blends (<2 and 0.3 °C, respectively). To support the results of thermal analyses, X-ray powder diffraction was used. Diffractograms of olsalazine, balsalazide and folic acid showed peaks at defined 2θ angles characteristic for their crystalline nature. Diffractograms of blends showed no disappearance of occurrence of new peaks which indicates that no change in the structure of compounds occurred. Chromatographic analysis of isothermally stressed samples showed high recoveries for both pure compounds and prepared blends, implying so chemical interaction between solids. Average recoveries of balsalazide and folic acid from analysed blends were 99.4 and 99.4 with the relative standard deviation values of 1.2% and 1.5%, respectively. Similarly, average recoveries of olsalazine and folic acid from their blends were 100.4 and 100.6% with the relative standard deviation values of 2.0 and 2.2 %, respectively. With the spectroscopic measurements, absence of chemical interaction was observed since there was no change in the infrared spectra between pure compounds and their blends. Stability studies in biologically relevant media did not show difference in the amount of released substance from the finished dosage form when dissolved separately and in combination.

Conclusion: Biomimetic measurements have shown that folic acid and 5-aminosalicylates predominantly bind to the HSA protein. Frontal analysis studies showed that folic acid binds to one specific site on the HSA protein while zone elution studies found that there was no competition between 5-aminosalicylate and folic acid for the same binding site on the HSA protein. Outcome of these measurements favours the development of fixed-dose combinations because the effect of the components on their pharmacokinetics is not expected. Compatibility studies have shown possible interactions in thermoanalytical measurements; however, conclusions should not be drawn solely from these measurements, but other analytical methods should be included to confirm or rule out possible interactions. Using chromatographic and spectroscopic techniques with studies of forced and isothermal degradation and stability in a biologically relevant medium, it was found that there are no physicochemical interactions between the tested 5-aminosalicylates and folic acid. This research represents one segment in the development of fixed drug combinations and further research is needed regarding the

preparation of the optimal formulation, dosage form and pharmacokinetic and pharmacodynamic studies to produce a safe and therapeutically effective product.

KEY WORDS: inflammatory bowel diseases, 5-aminosalycilates, folic acid, physicochemical compatibility, analytical techniques, fixed-dose combinations

SADRŽAJ

1. UVOD

1.1. Upalne bolesti crijeva

Prvi zapisi o bolestima koje danas nazivamo upalnim bolestima crijeva sežu još iz doba antičke Grčke i Hipokrita kada su zabilježeni slučajevi poremećaja u radu probavnog sustava kod čovjeka (1). Prvi rad objavljen na temu kronične upalne bolesti crijeva objavljen je 1859. godine, u kojemu je Sir Samuel Wilks spomenuo pojam „ulcerozni kolitis" kako bi opisao stanje najsličnije današnjem stanju ulceroznog kolitisa (2). Nagli skok u pažnji posvećenoj toj bolesti bio je nakon 1909. godine kada je Kraljevsko medicinsko društvo u Londonu (engl. *The Royal Society of Medicine*) održalo skup u kojem je opisano preko 300 kliničkih slučajeva ulceroznog kolitisa, a u narednim desetljećima, razvojem medicine i tehnološkim napretkom, bolest se mogla sve preciznije definirati i okarakterizirati te su se razvile brojne metode glede njezinog liječenja (3).

Crohnova bolest prvi puta je opisana 1932. godine u radu američkog gastroenterologa Burrilla Bernarda Crohna kao posebna vrsta upale gastrointestinalnog sustava nekarakteristična onoj uzrokovanoj ulceroznim kolitisom (4). Iako je Crohnov rad bio prvi koji je opisao bolest, postoje objave koje datiraju prije 1932. godine, koje su opisivale bolest karakterističnu Crohnovoj bolesti (3). 1960. godine britanski kirurg Hugh Evelyn Lockhart-Mummery objavio je istraživanje u kojem radi jasnu razliku između upale u debelom crijevu uzrokovane Crohnovom bolešću i ulceroznim kolitisom, međutim mnogi su znanstvenici smatrali da Crohnova bolest ne može zahvatiti debelo crijevo, pa tako i sam Crohn. Kao i kod ulceroznog kolitisa, tijekom posljednjih 50 godina, imunologija, genetika i molekularna biologija omogućili su bolje razumijevanje i učinkovitije liječenje Crohnove bolesti (3, 5).

Iako se ne zna što je glavni uzročnik pojave upalnih bolesti crijeva, kroz godine su se pojavljivale razne teorije; od tih da upale uzrokuju razne bakterije pa do onih da porast razine svijesti o osobnoj higijeni igra ulogu u pojavi upala (6). Takozvana „higijenska hipoteza" tvrdi da je povećana svijest o higijeni u svijetu rezultirala oslabljenim imunosnim sustavom kod ljudi s obziromna to da se više ne mora boriti s raznim virusima i bakterijama. U trenutku kada se organizam napokon suoči s mikroorganizmima dolazi do nepravilnog odgovora imunosnog sustava što uzrokuje dugotrajne upalne procese (7). Jedna od teorija, potaknuta većom prevalencijom upalnih bolesti crijeva u razvijenim dijelovima svijeta, govori kako uzrok može biti prehrana „zapadnog svijeta", odnosno prehrana bazirana na hrani bogatoj masnoćama, šećerima te rafiniranoj hrani, dok se izostavljaju zdrave namirnice bogate vlaknima i zdravim mastima (8). Iako se ne zna točan uzrok pojave, dokazano je kako postoji veća prevalencija bolesti kod potomaka ljudi oboljelih od Crohnove bolesti ili ulceroznog kolitisa, što je

potaknulo istraživanje veze između genske predispozicije i prevalencije upalnih bolesti crijeva (9, 10). Provedene studije su pružile uvjerljive dokaze da genetski čimbenici pridonose patogenezi upalnih bolesti crijeva, pri čemu je utvrđeno da je rizik za obolijevanje od upalnih bolesti crijeva osam do deset puta veći među rođacima (11). Pojava upalnih bolesti crijeva povezuje se s disbiozom i pušenjem, a postoje i studij koje smatraju da povećana količina aluminija u zagađenom okolišu rezultira većom prevalencijom (12).

Incidencija upalnih bolesti crijeva se naglo povećala nakon Drugog svjetskog rata, a studije koje su uslijedile pokazale su porast incidencije i u drugim zemljama svijeta (13). U 2017. godini u svijetu je zabilježeno oko 6,8 milijuna pacijenata koji boluju od upalnih bolesti crijeva, što u odnosu na 1990. godinu, kada je ta brojka iznosila 3,7 milijuna, predstavlja porast broja oboljelih od 85,1 %. Dobno standardizirana stopa prevalencije povećala se sa 79,5 na 100.000 stanovnika u 1990. godini, na 84,3 na 100.000 stanovnika u 2017. dok se stopa smrtnosti smanjila s 0,61 na 0,51 na 100.000 stanovnika u tom istom razdoblju. Prevalencija upalnih bolesti crijeva znatno se povećala u mnogim dijelovima svijeta u zadnjih trideset godina, što predstavlja značajni socijalni i ekonomski teret za pojedine države (14). U novo industrijaliziranim zemljama Azije, Afrike i Južne Amerike bilježi se rast incidencije, iako je stopa incidencije upalnih bolesti crijeva u zapadnim dijelovima svijeta stabilna ili je počela padati, globalni teret ostaje visok jer prevalencija prelazi 0,3 % (15).

1.1.1. Crohnova bolest i ulcerozni kolitis

Crohnova bolest opisana je kao transmuralna upala koja može zahvatiti bilo koji dio gastrointestinalnog sustava, od usta do perianalnog područja, a bolest karakteriziraju faze remisije i relapsa (16). Glede raspodjele upale, otprilike 25 % pacijenata ima kolitis, odnosno upalu debelog crijeva, 25 % ileitis ili upalu tankog crijeva, dok 50 % pacijenata ima ilekolitis. Kod trećine pacijenata upala zahvaća i perianalno područje dok kod 5 do 15 % pacijenata upala zahvaća oralno i gastroduodenalno područje (17). Simptomi bolesti mogu biti nespecifični te ovise o mjestu i intenzitetu upale. Bol u trbuhu i dijarea najučestaliji su simptomi Crohnove bolesti, dok drugi simptomi mogu biti umor, gubitak tjelesne težine, vrućica, anemija, pojava fistula i čireva. Fistule kod Crohnove bolesti mogu se manifestirati u penetrirajući oblik bolesti, a s obzirom na lokaciju fistula simptomi mogu varirati od krvave stolice do upale urinarnog trakta (18).

Ulcerozni kolitis je kronična upalna bolest crijeva koja zahvaća sve dijelove debelog crijeva počevši od upale rektuma te progresivnog širenja kroz debelo crijevo, a obilježena je stanjima remisije i relapsa. Karakterizira ga kontinuirana upala sluznice debelog crijeva koja se

neprekinuto širi te u konačnici može zahvatiti cijelo debelo crijevo, a bolest se najčešće razvija u drugom i trećem desetljeću života. Najčešći simptomi ulceroznog kolitisa su dijarea i krv u stolici, međutim, ovisno o jačini upale mogu se pojaviti i bolovi u trbuhu, noćna dijarea, sluzavi iscjedak i tenesmus. Simptomi se pojavljuju postepeno te se pojačavaju kroz nekoliko tjedana. U rijetkim slučajevima kod jakih i neliječenih upala može doći do gubitka težine, groznice i perforacije debelog crijeva dok dugotrajna prisutnost ulceroznog kolitisa koji je zahvatio najmanje trećinu debelog crijeva povećava rizik od dobivanja karcinoma debelog crijeva (19).

Kako bi se upalne bolesti crijeva točno dijagnosticirale te se jasno razlikovala upala uzrokovana Crohnovom bolešću i ulceroznim kolitisom, a i odredila terapija za učinkovito liječenje, potrebno je provesti laboratorijska ispitivanja poput seroloških testova, analize stolice te endoskopije. Također, koriste se i drugi pristupi poput radiografije i uzorkovanja kliničkih uzoraka iz gastrointestinalnog trakta, međutim, razlika se teže uočava što je upala jača (17, 20).

1.1.2. Liječenje

Pri liječenju upalnih bolesti crijeva u kliničkoj praksi i tijekom istraživanja naglasak je na smanjivanju učestalosti hospitalizacije pacijenata te potrebe za operativnim zahvatima, a samo liječenje je fokusirano na poticanje zacjeljivanja sluznice te induciranje duboke, odnosno, kliničke i endoskopske remisije (21–24). Liječenju se pristupa odabirom terapije s obzirom na aktivnost bolesti, mjesto upale, proširenost upale te zahvaćenost drugih organa. Procjena ovih parametara omogućuje personalizirani terapijski pristup dok je samo liječenje upalnih bolesti crijeva podijeljeno na fazu induciranja remisije, u kojoj se teži odgovoru na upalu, te postupku održavanja remisije kontinuiranom terapijom. Strategija liječenja obično uključuje upotrebu 5-aminosalicilata, kortikosteroida, imunosupresiva i na kraju bioloških lijekova, pri čemu liječenje uglavnom započinje 5-aminosalicilatima, a biološki lijekovi osobito su korisni kada pacijenti ne reagiraju na steroide te imunosupresive za postizanje remisije ili prevenciju relapsa (25).

5-aminosalicilati često su prva linija obrane u liječenju upalnih bolesti crijeva, odnosno blagih do umjerenih oblika ulceroznog kolitisa, iako je njihova primjena u liječenju Crohnove bolesti upitna i često tema brojnih rasprava (26, 27). 5-aminosalicilati su lijekovi koji se primjenjuju oralno ili rektalno za liječenje upala lokaliziranih u različitim dijelovima gastrointestinalnog trakta i to s vrlo malo sistemske apsorpcije. Oralne formulacije razlikuju se u načinu djelovanja, a noviji lijekovi omogućuju ciljano otpuštanje u određenim područjima gastrointestinalnog trakta te prikladniji način i raspored doziranja (28, 29).

Kortikosteroidi se mogu uzeti u obzir kod pacijenata koji imaju aktivne simptome unatoč terapiji 5-aminosalicilatima. Primjena prve generacije kortikosteroida (prednizon, hidrokortizon) u liječenju upalnih bolesti crijeva započela je u drugoj polovici 20. stoljeća, međutim zbog izraženih nuspojava krenulo se u razvoj druge generacije kortikosteroida (budezonid) koji su se pokazali uspješni u liječenju s manje nuspojava. Kod umjerenih ili teških oblika ulceroznog kolitisa, razumno je započeti liječenje kortikosteroidima kada je to potrebno, s obzirom na potencijalne dugoročne koristi, međutim, potreba za liječenjem kortikosteroidima indikator je virulentnijeg tijeka bolesti (30, 31).

Važnost imunosupresiva u liječenju upalnih bolesti crijeva uglavnom se odnosi na održavanje remisije kod Crohnove bolesti. S obzirom da se radi o lijekovima sa sporim početkom djelovanja, potrebno je najmanje 14 do 16 tjedana do značajnog farmakološkog učinka, često se za postizanje remisije koriste druge klase lijekova. Neželjeni učinci imunosupresiva mogu se pojaviti u 20 % slučajeva, najčešće u prva 2 do 3 tjedna, a uglavnom uključuju alergijske reakcije, leukopeniju i hepatotoksičnost (32).

Nedavni napredak u liječenju upalnih bolesti crijeva, posebice kod pacijenata koji ne reagiraju na konvencionalnu terapiju, uključuje terapiju biološkim lijekovima. Pod tim lijekovima podrazumijevamo preparate proizvedene rekombinantnom tehnologijom koji mogu blokirati upalu kod nekoliko bolesti povezanih s imunološkim sustavom. Prvi biološki lijek odobren za liječenje Crohnove bolesti, fistulirajuće Crohnove bolesti te ulceroznog kolitisa je infliksimab, imunoglobulin podklase G (IgG1) protiv faktora nekroze tumora (TNF-α) (33). Trenutno su odobrene tri vrste bioloških lijekova za liječenje jedne ili obje vrste upalnih bolesti crijeva: anti-TNF agensi (infliximab, adalimumab i golimumab), anti-integrinski lijekovi (vedolizumab) i anti-interleukin 12/23 agensi (ustekinumab) (34).

1.1.2.1. 5-aminosalicilati

5-aminosalicilati skupina su protuupalnih lijekova koji se koriste u ranoj fazi liječenja upalnih bolesti crijeva s ciljem postizanja remisije i sprječavanja relapsa bolesti (28). U skupinu 5-aminosaliclata spadaju protuupalni lijek mesalazin, odnosno 5-aminosalicilna kiselina, po kojoj je i skupina dobila ime te njezina tri prolijeka, sulfasalazin, balsalazid i olsalazin. Terapijski učinak 5-aminosalicilata je otkriven sasvim slučajno početkom 19. stoljeća kada je primijećeno da sulfasalazin, koji je tada korišten za liječenje reumatoidnog artritisa, ublažava simptome kod pacijenata koji boluju i od upalnih bolesti crijeva (3). Tek se naknadnim studijama uspostavilo kako je zapravo 5-aminosalicilna kiselina, odnosno mesalazin, iz molekule sulfasalazina ta koja posjeduje protuupalna svojstva (35). Sulfasalazin se uspješno

koristi u liječenju upalnih bolesti crijeva još i danas, međutim poznato je da sulfapiridin, antibiotik koji je vezan na mesalazin, uzrokuje pojavu izraženih neželjenih popratnih pojava kod gotovo trećine pacijenata čija je terapija zasnovana na sulfasalazinu (36). Imajući na umu činjenicu da je mesalazin taj koji djeluje protuupalno, fokus znanstvenika se usmjerio na razvoj lijekova na bazi mesalazina, iako se ni danas ne zna točan mehanizam njegovog protuupalnog djelovanja (37, 38). Glavni nedostatak mesalazina je njegova izražena apsorpcija u gornjem dijelu gastrointestinalnog trakta, ostavljajući vrlo malu količinu za liječenje daljnjih dijelova, što je potaknulo razvoj novih lijekova na bazi mesalazina, odnosno njegovih prolijekova. U potrazi za modifikacijom mesalazina, da se spriječi njegova prerana apsorpcija, razvijeni su njegovi prolijekovi; balsalazid i olsalazin (39). Mehanizam djelovanja prolijekova temelji se na oslobađanju mesalazina u debelom crijevu pod utjecajem enzima azoreduktaze (40). Naime, sulfasalazin, balsalazid i olsalazin se sastoje od mesalazina povezanog azo vezom sa sulfapiridinom, 4-aminobenzoil-β-alaninom odnosno drugom molekulom mesalazina. Takvi spojevi pokazali su slabu apsorpciju u gornjem dijelu gastrointestinalnog sustava čime je spriječena prerana apsorpcija mesalazina, a time i pojačan terapijski učinak samih lijekova (41).

Režim doziranja 5-aminosalicilata ovisi o aktivnosti bolesti, pri čemu su za postizanje remisije u akutnoj fazi bolesti potrebne znatno veće doze od onih za održavanje stanja remisije te sprječavanja relapsa. Tablete koje sadrže sam mesalazin dolaze u najviše različitih dozirnih oblika kako bi iskorištenje primijenjenog lijeka bilo što veće, dok prolijekovi dolaze isključivo u obliku kapsula i tableta. Maksimalne dnevne doze veće su kod aktivnih oblika bolesti kako bi se učinkovito ublažili simptomi i smanjila aktivnost bolesti, dok za održavanje stanja remisije i prevencije relapsa dnevne doze su znatno manje.

Iako su se 5-aminosalicilati pokazali uspješnima u liječenju upalnih bolesti crijeva nuspojave nisu rijetka pojava. Studija iz 2020. godine koja je uzela u obzir 692 pacijenta koji su primali mesalazin za liječenje upalnih bolesti crijeva, u razdoblju od 2014. do 2020. godine, pokazala je da je 4,8 % pacijenata dobilo alergijsku reakciju pri čemu je gotovo 93 % imalo vrućicu, 23 % bolove u abdomenu te 12 % krv u stolici (42). Sulfasalazin se povezuje s brojnim nuspojavama, a smatra se da je sulfapiridin, koji se oslobađa nakon metaboliziranja sulfasalazina, odgovoran za većinu nuspojava (43). Kod otprilike 11 do 45 % pacijenata čija terapija je bila zasnovana na sulfasalazinu uočene su nuspojave poput mučnine, dispepsije, glavobolje i umora, a smatra se da tolerantnost na sulfasalazin ovisi o genskoj predispoziciji, odnosno o tome je li pacijent „spori“ ili „brzi“ acetilator (44, 45).

Glavna prednost novijih 5-aminosalicilata, olsalazina i balsalazida, je značajno manji broj zabilježenih slučajeva nuspojava u odnosu na sulfasalazin (46, 47). Provedene studije o primjeni sulfasalazina i novijih 5-ASA lijekova tijekom trudnoće i dojenja pokazale su da sulfasalazin ne povećava rizik od fetalnih abnormalnosti, preranog poroda ili male porođajne težine, kao što je to moguće prilikom liječenja kortikosteroidima. Međutim, zbog rizika od nedostatka folata izazvanog sulfasalazinom, preporuča se njihova suplementacija davanjem povišenih doza folne kiseline od 5 mg/dan (48–50).

1.1.3. Folna kiselina i upalne bolesti crijeva

Neravnoteža unosa energije, proteina i drugih nutrijenata glavna je posljedica upalnih bolesti crijeva i prvenstveno je odgovorna za kroničan gubitak tjelesne težine. Postojeći podaci pokazuju da pothranjenost zahvaća veći dio bolesnika s upalnim bolestima crijeva, što se procjenjuje u 65–75 % bolesnika s Crohnovom bolešću i u 18–62 % bolesnika s ulceroznim kolitisom (51). Faktori koji uzrokuju pothranjenost kod upalnih bolesti crijeva uključuju smanjeni oralni unos hrane, malapsorpciju hranjivih sastojaka, povećane potrebe za energijom zbog sustavne upale, posljedica kirurških zahvata te utjecaj terapije. Procjena nutritivnog statusa i potrebe za nutritivnom terapijom igraju ključnu ulogu u kliničkoj njezi bolesnika s upalnim bolestima crijeva (52).

Anemija je česta komplikacija uzrokovana upalnim bolestima crijeva. Uobičajeno je da pacijenti imaju anemiju uzrokovanu nedostatkom željeza kao posljedicu kroničnog gubitka krvi te smanjene apsorpcije željeza, folata i vitamina B12 kao posljedica oštećenja tkiva (53). Smanjena tjelesna aktivnost, malapsorpcija minerala i nedostatak vitamina D, genetska predispozicija te smanjena resorpcija kostiju samo su neki od čimbenika koji rezultiraju smanjenom gustoćom kostiju, odnosno pojavom osteoporoze i osteopenije (54).

Folna kiselina, poznata i kao vitamin B9, sintetski je proizvedeni vitamin topljiv u vodi koji se nalazi u obogaćenoj hrani i dodacima prehrani. Folati se prirodno dobivaju iz hrane, odnosno voća te posebice iz tamnozelenog lisnatog povrća (55), a bitni su za sintezu i popravak DNA, interkonverziju aminokiselina, metilaciju te u konačnici normalan rast stanica (56). Ljudi nisu u stanju sintetizirati folate pa su svakodnevne potrebe zadovoljavaju konzumacijom hrane bogatom ovim vitaminom (57). Izostanak folata u svakodnevnoj prehrani povećava rizik od razvoja brojnih bolesti, uključujući kardiovaskularne bolesti, aterosklerozu, megaloblastičnu anemiju, karcinome i neurodegenerativne bolesti dok nedostatak folne kiseline kod trudnica može uzrokovati defekte na plodu (58, 59). Postoji i uska povezanost nedostatka folne kiseline i upalnih bolesti crijeva. Folna kiselina se apsorbira u tankom crijevu, koje može biti zahvaćeno

Crohnovom bolešću, a zbog oštećenja stijenki crijeva onemogućena je njezinu normalna apsorpcija. S druge strane, sulfasalazin je antagonist folne kiseline, odnosno inhibitor transportera folata, što dodatno doprinosi smanjenju koncentracije folne kiseline u organizmu pacijenta (60). Studije su pokazale kako su koncentracije folne kiseline znatno niže kod pacijenata koji boluju od upalnih bolesti crijeva u odnosu na zdrave ljude (61). Nedostatak folne kiseline kod pacijenata povezuje se s pojavom megaloblastične anemije, karcinoma debelog crijeva te defekata na plodu kod trudnica, zbog čega se pacijentima koji boluju od upalnih bolesti crijeva propisuje folna kiselina uz redovnu terapiju (62–65).

1.1.3.1. Adherencija kod upalnih bolesti crijeva

Prema Svjetskoj zdravstvenoj organizaciji adherencija predstavlja mjeru u kojoj ponašanje osobe (uzimanje lijekova, dijeta, i/ili provođenje promjena načina života), odgovara dogovorenim preporukama od strane zdravstvenog djelatnika (66). Neadherencija je značajan problem u liječenju upalnih bolesti crijeva te dovodi do neželjenih kliničkih ishoda, uključujući pojačanje simptoma bolesti, relaps, gubitak odgovora na anti-TNF agense, povećane zdravstvene troškove te veći morbiditet i smrtnost (67). Adherencija u pacijenata s kroničnim bolestima u razvijenim zemljama u prosjeku iznosi 50 % dok u zemljama u razvoju postotak može biti i niži (66).

Smanjena adherencija u liječenju upalnih bolesti crijeva u prosjeku se kreće od oko 30 – 40 % (68). Negativni stavovi o lijekovima igraju važnu ulogu u svjesnom nepridržavanju terapije kod kroničnih bolesnika, a niska percepcija osobne potrebe za liječenjem i velika zabrinutost zbog potencijalnih neželjenih popratnih pojava liječenja povezani su s nepridržavanjem propisane terapije u bolesnika s upalnim bolestima crijeva (69). Kod liječenja upalnih bolesti crijeva veće je pridržavanje uočeno kod pacijenata koji su primali anti-TNF lijekove, imunomodulatore, imunosupresive te steroide u usporedbi s onima koji su uzimali 5-aminosalicilate ili antibiotike (70). Novije studije su pokazale kako smanjena adherencija prema biološkim lijekovima postaje problem zbog pandemije virusa SARS-CoV-2. Uzrok tomu je rizik od zaraze zbog odlaska liječniku na primanje terapije, dok su zabilježeni i slučajevi zatvaranja klinika za davanje bioloških lijekova kao posljedica pandemije (71).

Postoje mnogi načini kako potaknuti adherenciju pacijenata. Pojednostavljivanje režima doziranja te prilagođavanje doze i učestalosti sa svakodnevnim aktivnostima pacijenta, uvođenje pomagala za adherenciju, poput kutija s lijekovima i alarma, zatim razgovor s liječnikom, ljekarnikom ili medicinskom sestrom te pristup informacijama o zdravstvenom odgoju na internetu i slanje podsjetnika putem pošte, e-pošte ili telefona samo su neki od načina

na koji se može poboljšati adherencija (72). Fiksne kombinacije lijekova, u usporedbi s individualnim tabletama, su se pokazale kao dobar pristup u povećanju adherencije. Studije su pokazale učinkovitost prilikom liječenja hipertenzije i dijabetesa tipa 2. Fiksne kombinacije logičan su pristup kod liječenja bolesti koje zahtijevaju uzimanje više tableta istovremeno te u konačnici smanjuju sveukupan broj farmaceutika o kojima pacijent svakodnevno mora voditi brigu (73–75).

1.2. Fiksne kombinacije lijekova

Fiksna kombinacija lijekova predstavlja jedan dozirni oblik (tabletu, kapsulu…) koji sadrži dvije ili više aktivnih farmaceutskih tvari (76). Prema Europskoj agenciji za lijekove (engl. *European Medicines Agency, EMA*) definirane su dvije potencijalne terapijske prednosti fiksnih kombinacija; prva je poboljšani odgovor kod pacijenata s neadekvatnim odgovorom na monoterapiju te veći i/ili brži ukupni učinak, dok s druge strane kombinacija poboljšava sigurnost gdje jedna djelatna tvar djeluje suprotno lijeku koji uzrokuje štetne reakcije ili kombiniranjem doza koje su subterapijske kada se koriste kao monoterapija (77).

Kombinacije više aktivnih sastavnica često su se koristile kroz povijest, a kao gotovi farmaceutski oblici imali su nepovoljan početak primjene kada su se 1950-it godina proizvodile kombinacije tiazidnih diuretika i kalijeva klorida koje su uzrokovale teška oštećenja na želucu, što je iznimno depopulariziralo korištenje fiksnih kombinacija. Danas je koncept i pogled na fiksne kombinacije drugačiji, postoje mnoge kombinacije koje se uspješno koriste u liječenju različitih bolesti, poput kaptopril/hidroklorotiazida za hipertenziju, amoksicilin/klavulanata kao antibiotik, ritonavir/lopinavira za liječenje HIV-a, rifampicin/iozoniazida za liječenje tuberkuloze i drugi (78).

1.2.1. Razvoj

Sam razvoj fiksne kombinacije lijekova može se podijeliti u nekoliko glavnih skupina: razvoj dozirnog oblika te ispitivanje farmakokinetike-farmakodinamike i kliničke učinkovitosti. Provedba farmakokinetičkih i farmakodinamičkih studija, odnosno studija kliničke učinkovitosti ovisi o svrsi primjene fiksne kombinacije u pacijenata. Tako razlikujemo primjenu fiksnih kombinacija u pacijenata koji imaju nedovoljan odgovor na monoterapiju, zamjenu lijekova u terapiji s fiksnom kombinacijom ili ako je u pitanju inicijalno liječenje fiksnom kombinacijom. Osim ako se prisutnost ili odsutnost farmakokinetičke interakcije može utvrditi drugim dokazima, potrebno je provesti studije interakcije između aktivnih sastavnica u fiksnoj kombinaciji. Također su potrebna nasumična kontrolirana klinička ispitivanja za

dokazivanje superiornosti fiksne kombinacije kod nedovoljno reagirajućih pacijenata te činjenice da fiksna kombinacija ima veću učinkovitost u odnosu na zasebno primjenjivane lijekove (77).

Iako se u literaturi mogu pronaći radovi na temu razvoja fiksnih kombinacija, ne postoji mnogo službenih dokumenata koji definiraju zahtjeve i uvjete za razvoj fiksnih kombinacija lijekova glede kvalitete samog proizvoda (79–81). Svjetska zdravstvena organizacija jedina je organizacija koja je izdala službeni dokument koji sadrži smjernice za razvoj takvog proizvoda (82). Iako se sam razvoj u suštini ne razlikuje od razvoja drugog lijeka koji sadrži jednu aktivnu sastavnicu, potrebno je napraviti niz dodatnih ispitivanja kako bi se utvrdila sigurnost i učinkovitost novog proizvoda, a sama ispitivanja se uglavnom temelje na određivanju sadržaja, stabilnosti, fizikalno-kemijskih svojstava sastavnica te studija bioekvivalencije. Ispitivanja specifična za razvoj fiksnih kombinacija su slijedeća:

- fizikalno-kemijska svojstva čistih sastavnica i smjesa,
- kemijska i fizikalno-kemijska stabilnost aktivnih sastavnica te aktivnih sastavnica u interakciji s pomoćnim tvarima u formulaciji,
- definiranje razgradnih profila aktivnih sastavnica kada su istovremeno izložene prisilnoj razgradnji,
- određivanje ujednačenosti sadržaja formulacije prije samog oblikovanja,
- provođenje studija oslobađanja lijekova iz dozirnih oblika,
- razvoj i validacija prikladnih analitičkih metoda za studije razgradnji i studije oslobađanja lijekova iz dozirnih oblika,
- definiranje proizvodnog procesa te kritičnih parametara i
- stabilitetne studije na pilot formulacijama.

Obuhvaćanjem ovih ispitivanja u postupku razvoja fiksne kombinacije osigurava se proizvod koji je u skladu sa specifikacijama, ujednačene kvalitete neovisno o proizvodnoj šarži, zadovoljavajuće fizikalno-kemijske stabilnosti te proizvod prikladan za stabilitetne studije te studije bioekvivalencije (82).

1.2.2. Ispitivanje fizikalno-kemijske kompatibilnosti sastavnica

Ispitivanje fizikalno-kemijske kompatibilnosti sastavnica igra važnu ulogu u razvoju lijekova, bilo u pitanju ispitivanje kompatibilnosti lijek-pomoćna tvar u razvoju formulacije koja sadrži jednu aktivnu tvar ili ispitivanje kompatibilnosti lijek-lijek za potrebe razvoja fiksne kombinacije. Ispitivanje fizikalno-kemijske kompatibilnosti u razvoju lijeka provodi se s ciljem razvoja stabilnog, sigurnog i terapijski učinkovitog lijeka (83). Stabilnost lijeka dolazi u obliku

kemijske i fizikalne stabilnosti. Ispitivanjem kemijske stabilnosti dobivaju se podaci o potencijalnim kemijskim promjenama u lijeku koje mogu rezultirati smanjenjem terapijske učinkovitosti lijeka, ali i nastajanjem toksičnih produkata. Nadalje, ispitivanjem fizikalne stabilnosti uočavaju se promjene fizikalnih karakteristika, poput boje, okusa i mirisa, ali i promjene u strukturi samog lijeka, poput promjene polimorfa, hidracije/dehidracije, solvatacije i sličnih, koji u konačnici mogu utjecati na farmakokinetiku samog lijeka i bioraspoloživost (84, 85). U svrhu ispitivanja fizikalno-kemijske stabilnosti aktivnih sastavnica u procesu razvoja lijeka već dugi niz godina uspješno se koriste razne kromatografske, termalne i spektroskopske tehnike (86–92).

Termalne tehnike, diferencijalna pretražna kalorimetrija (engl. *Differential Scanning Calorimetry*, DSC) i diferencijalna termalna analiza (engl. *Differential Thermal Analysis*, DTA) s ili bez istovremene termogravimetrije (engl. *Thermogravimetry*, TG), jedne su od prvih tehnika koja se koristi u ispitivanju kompatibilnosti, s obzirom da su to jednostavne i brze tehnike za dobivanje uvida u moguće promjene u pripremljenim smjesama ispitivanih spojeva (93,94). Binarne smjese su najispitivanije te se pripremaju u omjeru 1:1, kako bi se pojačao intenzitet mogućih reakcija te ostvarila bolja vizualizacija promjena (95), iako se smjese mogu pripremati i u drugim omjerima, u korist jedne i druge sastavnice (93). Moguće interakcije u ovakvim mjerenjima mogu se pratiti kao promjene u masi uzorka, pomaci u specifičnim pikovima, poput endotermnih pikova tališta, te promjene u entalpijama u dobivenim termogramima smjesa u odnosu na termograme čistih sastavnica (88, 96–98).

Infracrvena spektroskopija i rendgenska difrakcija na prahu nedestruktivne su spektroskopske tehnike koje se koriste u fizikalno-kemijskoj karakterizaciji lijekova kao i u ispitivanjima kompatibilnosti sastavnica lijeka (94, 99–103). Infracrvena spektroskopija, uglavnom s Fourierovim transformacijama (engl. *Fourier-Transform Infrared Spectroscopy*, FTIR), tehnika je koja se koristi za karakterizaciju aktivnih tvari, međutim često se koristi u studijama kompatibilnosti s ciljem uočavanja promjena u specifičnim apsorpcijskim vrpcama kao posljedica reakcije sastavnica smjese. Međutim, prisutnost preklapajući vrpci u spektrima može uvelike ometati analizu i interpretaciju dobivenih spektara (86, 102, 104). Jedan od načina interpretacije FTIR spektara je primjena statističkih metoda poput analize glavnih komponenata (*engl. Principal Component Analysis*, PCA) i klaster analize (*engl. Cluster Analysis*), koji se posebno pokazao učinkovit kod spektara koji sadrže puno vrpci koje se preklapaju, a sam princip primjenjivan je i u ispitivanju fizikalno-kemijske kompatibilnosti sastavnica lijeka (100, 105, 106). S druge strane, rendgenska difrakcija na prahu (*engl. x-Ray Powder Diffraction*,

XRPD) često je korištena tehnika karakterizacije kristaliničnih sastavnica lijekova pri čemu se mjeri intenzitet izlaznih zraka u ovisnosti o difrakcijskom kutu (107–109). Sastavnice lijeka koje su u kristalnom obliku imaju svoj karakteristični difraktogram, a promjene u kristalnoj strukturi (prijelaz u drugi polimorfni oblik ili u amorfno stanje) jasno se očituju kao nastajanje ili nestajanje karakterističnih pikova te pomak i promjena njihova intenziteta (86). Upravo se na tom principu temelji ispitivanje kompatibilnosti sastavnica lijeka, odnosno prate se promjene u strukturi spojeva kada su prisutni u istoj smjesi (87, 101, 107, 110–112).

Kromatografske tehnike u kombinaciji sa studijama poput prisilne razgradnje i izotermalnog stresiranja te studijama oslobađanja lijekova iz dozirnog oblika jedan su od najčešćih analitičkih pristupa u ispitivanju kompatibilnosti sastavnica lijekova. Razgradni produkti nastali uslijed interakcije lijek-pomoćna tvar ili interakcije lijek-lijek u razvoju fiksnih kombinacija mogu se jasno utvrditi provođenjem prisilne razgradnje uzoraka pojedinih sastavnica i njihovih smjesa te usporedbom profila razgradnji (113).

Prisilna razgradnja se temelji na sresiranju uzoraka u raznim uvjetima; kiselim, lužnatim, oksidativnim, termalnim i svjetlosnim, a informacija o stabilnosti u određenim uvjetima služe kao vodilja u fazi razvoja stabilitetno-indikativnih analitičkih metoda i formulacije lijeka te je preporučeno njezino provođenje prema smjernicama Međunarodnog vijeća za usklađivanje tehničkih zahtjeva za lijekove za ljudsku uporabu (*engl. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use*, ICH) (114, 115). Ne postoji jasno definiran protokol po kojem se moraju provoditi studije prisilne razgradnje. Iako nije službeno definirano, postoje literaturni zapisi u kojima se definiraju uvjeti razgradnje, međutim ključna je činjenica da se razgradnja mora provoditi u rasponu od 10–30 % kako bi se izbjeglo nastajanje nereprezentativnih razgradnih profila koji bi mogli istraživanje usmjeriti u pogrešnom smjeru (82, 116, 117). Stabilitetno-indikativna analitička metoda, metoda s kojom je moguće uočiti sve razgradne produkte koji mogu nastati razgradnjom lijeka prilikom stabilitetnih studija, konačni je rezultat prisilne razgradnje lijekova (117–119). Razvoj takve metode izazovan je poduhvat pogotovo u razvoju fiksne kombinacije lijekova, a tu činjenicu opravdava ograničen broj publikacija na temu razvoja takvih analitičkih metoda (120–123).

Izotermalna studija razgradnje (engl. *Isothermal Stress Testing*, IST) je često korištena metoda u studijama ispitivanja kompatibilnosti sastavnica lijeka. Temelji se na skladištenju uzoraka u uvjetima definirane povišene temperature s ili bez prisutnosti vlage nakon čega slijedi analiza prikladnom analitičkom tehnikom (99). Najčešće primjenjivane tehnike analize uzoraka

nakon IST su kromatografske poput tekućinske kromatografije visoke djelotvornosti (engl. *High Performace Liquid Chromatography*, HPLC) i/ili spektroskopske poput FTIR-a (99, 103, 119, 124–127).

Studije oslobađanja lijekova iz dozirnih oblika je *in-vitro* test koji se izvodi kako bi se ispitalo koliko se učinkovito lijek oslobađa iz dozirnog oblika, međutim ova tehnika se primjenjuje i u svrhu ispitivanja interakcija sastavnica lijeka i njihove stabilnosti kada se istovremeno nalaze u istom biološki relevantnim mediju (128–131).

2. **Rad 1** - A chromatographic approach to development of 5-aminosalicylate/folic acid fixed-dose combinations for treatment of Crohn’s disease and ulcerative colitis

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A chromatographic approach to development of 5-aminosalicylate/folic acid fixed-dose combinations for treatment of Crohn's disease and ulcerative colitis

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Medication adherence is an important factor in inflammatory bowel disease therapy, which includes regular supplementation of malabsorbed vitamins. Absorption of folic acid is limited due to the damaging of the gastrointestinal tract, which can increase the chances to develop megaloblastic anaemia and colorectal cancer. In this work, 5-aminosalicylates (mesalazine, balsalazide, sulfasalazine and olsalazine) and folic acid were characterized regarding their pharmacokinetic related properties (hydrophobicity, phospholipid and plasma protein binding) using the biomimetic chromatographic approach. Despite the high binding percentage of 5-aminosalicylates for human serum albumin (> 61.44%), results have shown that folic acid binding to human serum albumin protein is far greater (69.40%) compared to α1-acid-glycoprotein (3.45%). Frontal analysis and zonal elution studies were conducted to provide an insight into the binding of folic acid to human serum albumin and potential competition with 5-aminosalicylates. The analytical method for the simultaneous determination of assay in proposed fixed-dose combinations was developed and validated according to ICH Q2 (R1) and FDA method validation guidelines. Separation of all compounds was achieved within 16 min with satisfactory resolution (R_s > 3.67) using the XBridge Phenyl column (150 × 4.6 mm, 3.5 µm). High linearity (r > 0.9997) and precision (RSD < 2.29%) was obtained, whilst all recoveries were within the regulatory defined range by British (100.0 ± 5.0%) and United States Pharmacopeia (100.0 ± 10.0%).

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are chronic inflammations of the gastrointestinal tract. The incidence and prevalence of IBD are increasing worldwide, however, the etiology is not precisely defined[1]. Although therapeutic goals to achieve mucosal healing and deep remission require early therapy with novel biologics and small molecules, there are subgroups of patients that can benefit only from 5-aminosalicylate treatment[2]. 5-aminosalicylates are a group of anti-inflammatory drugs including mesalazine (MSZ) and its prodrugs balsalazide (BSZ), sulfasalazine (SASP) and olsalazine (OSZ) (Fig. 1).

Daily intake of multiple drugs during the therapy of IBD is mandatory due to the malabsorption of vitamins, mostly as a consequence of damaged tissue in the gastrointestinal tract. Deficiency of important vitamins, such as folic acid (FA) (Fig. 1), which is necessary for the normal functioning of the human body, can lead to the development of megaloblastic anaemia and colorectal cancer[3,4]. With the rise of fixed-dose combinations (FDCs) the potential risk to fail the adherence should be minimized[5]. FDC presents a single formulation containing two or more different active pharmaceutical ingredients, where the safety and efficacy of the novel combination is not compromised compared to every product separately[6].

To improve the quality of IBD patients' lives, FDCs are proposed in collaboration with Clinical Hospital Centre Zagreb. The four formulations would consist of each 5-aminosalicylate combined with FA (MSZ + FA,

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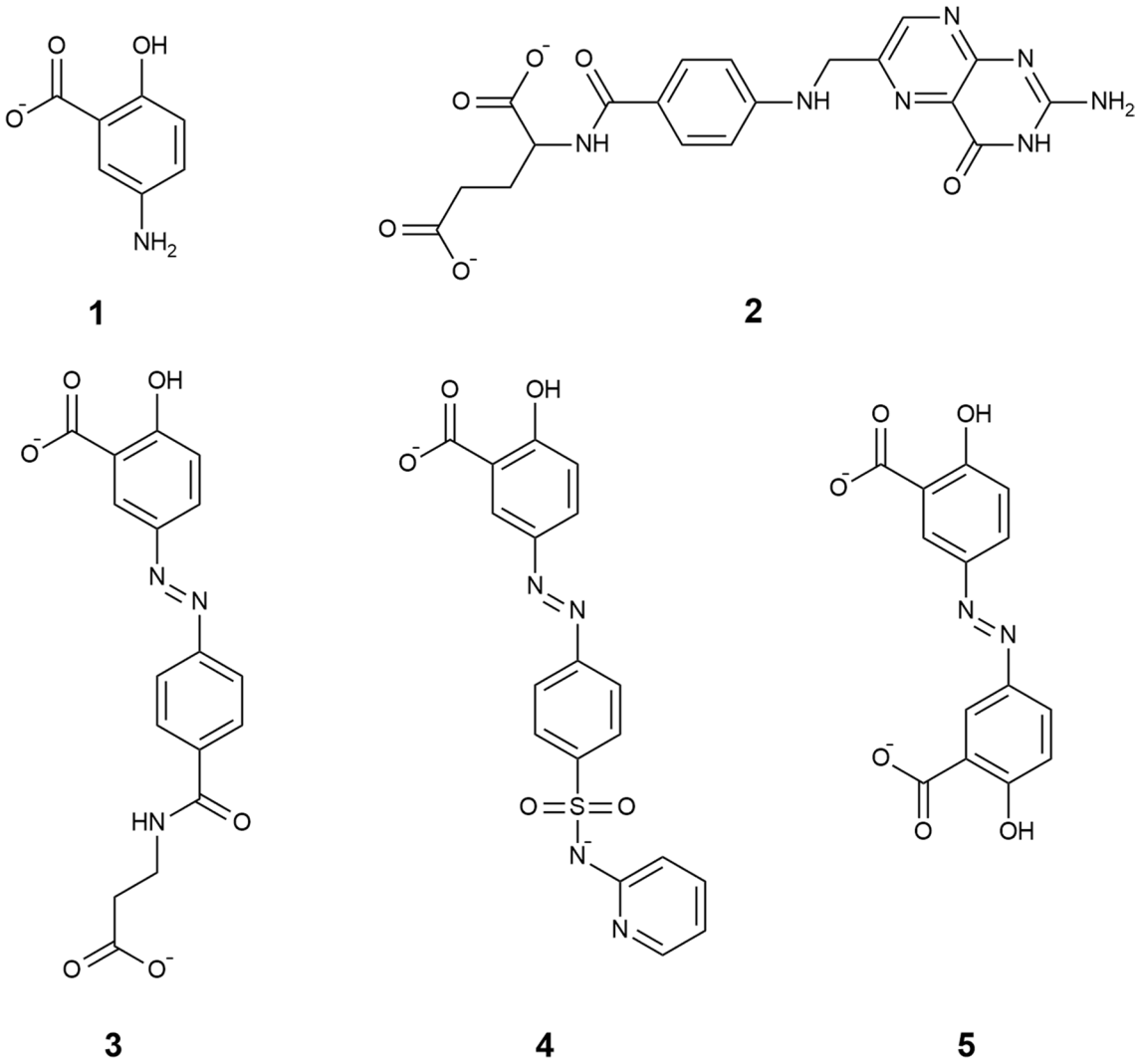

Figure 1. Structures of: MSZ (1), FA (2), BSZ (3), SASP (4) and OSZ (5) at physiological pH (pH = 7.4).

BSZ + FA, SASP + FA, OSZ + FA). Proposed FDCs would contain therapeutic doses of each drug in order to ensure the maintenance of the remission state and to supplement the malabsorbed FA.

The development of FDCs is driven by several factors, among which is the investigation of the physicochemical properties of each drug. Reversed-phase columns can offer a great alternative to the conventional methods for hydrophobicity determination, where on the other hand, biomimetic columns with immobilized endogenous structures, such as artificial membrane (IAM), human serum albumin (HSA) and α1-acid-glycoprotein (AGP) can give additional information regarding the drug behaviour once it enters the human body, such as cell membrane permeability and plasma protein binding (PPB)[7]. The pharmacokinetics of drugs is mainly influenced by the interaction of drugs with the plasma proteins. HSA, being the most abundant protein in plasma (35–50 g/L), and AGP (0.6–1.2 g/L) are major binding agents for most of the drugs[8,9]. Frontal analysis and zonal elution studies are well-known approaches in characterization of drug binding to plasma proteins which include the application of high-performance affinity chromatography (HPAC) with the use of biomimetic columns to monitor binding behaviour of drugs[10]. So far, it has shown to be a successful method for getting an insight into drug binding and possible competition on both HSA and AGP[8,9]. The ability of high throughput biomimetic measurements reduces the required time and resources in gaining important information about active pharmaceutical ingredients and their interactions early on in the drug development process.

From the analytical point of view, each developed formulation requires sufficient quality control methods. Various techniques such as spectrophotometry[11], HPLC[12–15], differential pulse voltammetry[16] resonance light scattering method[17] and high-performance thin-layer chromatography[18] were reported through the years for assay determination of 5-aminosalicylates and FA in their formulations. However, there is only one published paper related to the simultaneous determination of MSZ and FA using the electroanalytical approach[19]. Up till now, no developed methods were reported for the simultaneous determination of all four 5-aminosalicylates and FA in a single analytical method.

The aim of this work is to apply different chromatographic techniques and biomimetic HPLC measurements to provide an insight into pharmacokinetic related drug properties. On the other hand, the development and

validation of a single assay method for simultaneous determination of 5-aminosalicylates and FA in four in-house prepared FDCs will be approached according to the current guidelines defined by the pharmaceutical regulatory authorities. In the end, multiple 5-aminosalicylate and FA related drug products that are marketed in Europe for treatment of IBD will be tested using the developed method.

Methods

Reagents and chemicals. MSZ (100.0%), FA (90.7%) and OSZ (100.0%) sodium European Pharmacopoeia certified reference standards, BSZ disodium US Pharmacopoeia reference standard (100.0%), SASP British Pharmacopoeia Chemical Reference Substance (99.6%) and 5-acetylmesalazine internal standard (≥ 98.0%) were all provided by Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, ethanol, methanol and 2-propanol (all HPLC grade solvents), sodium hydroxide pellets (≥ 98.0%), formic acid for HPLC (98 – 100%), phosphate-buffered saline tablets (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at 25 °C), potassium phosphate monobasic and potassium phosphate dibasic (HPLC grade) were also obtained by Sigma-Aldrich. For preparation of *in-house* FDCs and quality control following products were purchased: Pentasa by Ferring Pharmaceuticals (Saint-Prex, Switzerland), Salofalk by Dr. Falk Pharma GmbH (Freiburg, Germany), Mesalazin-Kohlpharma by Kohlpharma GmbH (Merzig, Germany), Folacin by JGL (Rijeka, Croatia), Premid by Almirall S.A. (Barcelona, Spain), Salazopyrin by Recipharm Uppsala AB (Uppsala, Sweden) and Dipentum by Waymade PLC (Basildon, Essex, UK). Excipients for preparation of placebo used in method validation as well as its composition is presented in Supplementary Table S1. Placebo blend was prepared according to the information available in literature regarding the use of excipients in preparation of pharmaceutical products[20].

Stock and working solutions. Standard solutions of 5-aminosalicylates and FA for drug hydrophobicity and phospholipid affinity determination, frontal analysis and zonal elution studies were prepared in a concentration of 50 µM by dissolving the standards in 20 mM phosphate buffer (pH 7), as well as series of mobile phases containing FA in concentrations of 1, 5, 10, 15 and 20 µM. For the development of assay method, stock solutions of MSZ (250 µg/mL), FA (10 µg/mL) and internal standard (IS) (250 µg/mL) were prepared by dissolving the standards in ultrapure water (40 °C). Stock solutions of SASP (250 µg/mL), OSZ (125 µg/mL) and BSZ (375 µg/mL) were prepared by dissolving the standards in 5 mM NaOH. For method validation prepared placebo blend was dissolved in both water and 5 mM NaOH in the concentration of 1.0 mg/mL. Working solutions (mixtures of each 5-aminosalicylate with FA in the proposed ratio) were prepared by spiking the placebo solution with the standard solutions. The final working solutions contained 100 µg/mL of MSZ, 100 µg/mL of SASP, 50 µg/mL of OSZ, 150 µg/mL of BSZ, 100 µg/mL of IS and 0.2 µg/mL of FA. All solutions were freshly prepared before analysis, filtered through 0.20 µm polyethersulfone filters and stored in amber glassware.

Preparation of in-house FDCs and samples for quality control of commercially available drugs. In-house FDCs used for method development were prepared by using commercially available 5-ASA drugs for treatment of IBD. Ten randomly chosen MSZ and SASP tablets and OSZ and BSZ capsules content were weighed and separately ground to a fine powder. For preparation of FDCs, an amount of powdered tablet or capsule equal to 500 mg of MSZ, 500 mg of SASP, 250 mg of OSZ and 750 mg of BSZ was mixed with the 1 mg of FA standard to achieve the desired ratio (Supplementary Table S2). The amount of each prepared FDC powder was weighed and transferred into an individual 10 mL amber volumetric flask and dissolved in water for MSZ/FA mixture and in 5 mM NaOH for other mixtures. An appropriate amount of IS solution was added, flasks were filled to the mark and sonicated for 15 min to provide complete solubilization. Solutions were centrifuged, filtered through a 0.2 µm polyethersulfone injection filter and diluted to achieve the concentrations equal to those of working solution described in previous section.

For quality control of commercially available 5-ASA and FA drugs, an amount of powdered tablets or capsules were weighed and transferred in 10 mL volumetric flask with the addition of IS and properly diluted with water for drugs containing MSZ and in 5 mM NaOH for other drugs to achieve the final concentrations of 100 µg/mL of MSZ, 100 µg/mL of SASP, 50 µg/mL of OSZ, 150 µg/mL of BSZ, 100 µg/mL of IS and 0.2 µg/mL of FA, which represents the 100% concentration level of calibration curve.

RP-HPLC hydrophobicity method. All HPLC measurements were carried out on an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with a diode array detector (DAD). System control, data collection and data processing were accomplished using ChemStation for LC 3D software by Agilent Technologies. RP-HPLC hydrophobicity determination was carried on Symmetry C18 reversed-phase column (150 × 4.6 mm, 3.5 µm particle size) obtained by Waters (Milford, MA, USA), with phosphate-buffered saline and methanol as mobile phase. The flow rate was set at 1.0 mL/min with the injection volume of 10.0 µL whilst the column temperature was maintained at 25.0 ± 0.1 °C. To obtain hydrophobicity, the log k values were calculated using retention times of investigated compounds (t_R) and retention time of sodium nitrate as unretained compound (t_0). All compounds were eluted starting with higher fractions of methanol in the mobile phase (60%) followed by measurements with 5% methanol reduction after each following run. At least five different concentrations of methanol in the mobile phase were applied for each standard substance and all measurements at each methanol fraction were performed in triplicates. For highly lipophilic compounds such as SASP, OSZ and BSZ measurement were performed until 25% of methanol in mobile phase due to the long retention times at lower methanol fractions in mobile phase, whilst measurements including MSZ and FA were carried until 5% and 10%, respectively. Column equilibration time of 20 min was necessary after every change of mobile

phase. Finally, log $k_{w\ C18}$ value, describing hydrophobicity, was obtained by calculating the *y*-intercept of the regression line in a plot of log *k* vs. methanol fraction in the mobile phase.

Biomimetic studies. The affinity of analytes for immobilized phospholipids was measured using the immobilized phosphatidylcholine column (IAM.PC.DD2, 100 × 4.6 mm, 300 Å pore size, 10 µm particle size) which was obtained from Regis Technologies (Morton Grove, IL, USA). The same HPLC conditions, mobile phases and calculation approaches were used for evaluation of IAM affinity, as in determining hydrophobicity with the RP-HPLC column.

Binding percentage of analytes to plasma proteins (PPB_{HSA} and PPB_{AGP}) was determined using the procedure described in our previously published paper[21].

Frontal and zonal elution studies were carried out on HSA (Chiralpak-HSA, 50 × 4.6 mm, 5 µm particle size) biomimetic column obtained by ChromTech (Cedex, France). Elution of 5-aminosalicylates was achieved with a series of mobile phases consisting of 20 mM potassium phosphate buffer (pH 7.0) (A) and iso-propanol (B) with the FA addition of 0, 1, 5, 10, 15 and 20 µM. Isocratic elution with the flow rate of 1.8 mL/min for HSA was applied for all compounds, where for the elution of MSZ organic modifier was excluded due to the low retention of MSZ, whilst 30% (*v*/*v*) of iso-propanol in mobile phase was mandatory due to the unacceptably high retention times of 5-aminosalicylate prodrugs. Injection volume was set at 10.0 µL and the measurements were carried out at 25.0 ± 0.1 °C.

Simultaneous determination of 5-aminosalicylates and FA. Same chromatographic system and software for data processing was used as in previous sections. Simultaneous chromatographic separation of 5-aminosalicylates and FA was achieved on an XBridge Phenyl column (150 × 4.6 mm, particle size 3.5 µm) obtained by Waters (Milford, MA, USA) maintained at 25.0 ± 0.1 °C with the flow rate of 1.0 mL/min. The mobile phase consisted of ultrapure water as mobile phase A and methanol as mobile phase B, both acidified with formic acid up to 0.2% (*v*/*v*). A sample volume of 10.0 µL was injected into the system and eluted using the following gradient program: 0–2 min, isocratic 5% B; 2–3 min, linear gradient 5–25% B; 3–7 min, isocratic 25% B; 7–9 min, linear gradient 25–70% B; 9–11 min linear gradient 70% B; 11–12 min, linear gradient 70–95% B; 12–16 min isocratic 95% B. Analysis time was 16 min while total run time was 20 min to allow re-equilibration of the stationary phase. MSZ and FA were detected at 300 and 285 nm respectively, whilst OSZ, SASP and BSZ were detected at 320 nm.

Method was validated according to ICH Q2 (R1) "Validation of analytical procedures: text and methodology"[22] and Food and Drug Administration (FDA) "Analytical Procedures and Methods Validation for Drugs and Biologics"[23]. Solutions of placebo spiked with the standard solutions were used to replicate the matrix of the real sample. Visual examination of obtained chromatograms was performed and the peak purity factors were calculated. The linearity of the method was examined in the range of 50% up to 150% of nominal concentration on five concentration levels (50%, 75%, 100%, 125% and 150%). IS was used for calculating the surface ratio between analysed compound and IS and same principle was used through all calculations concerning peak area. Limit of detection (LOD) and limit of quantification (LOQ) were calculated as a signal to noise ratio 3:1 and 10:1, respectively. The precision of the method was studied as repeatability by analysing six individually prepared samples within the one day and intra-day precision by individually preparing and analyzing three samples each day for three consecutive days. All samples were analysed at 100% concentration level and results are expressed as RSD (%). The accuracy of the method was tested as a recovered amount of the analyte compared to the known concentration in the sample. Recoveries were tested on three levels; the lowest point is equal to 50%, medium to 100% and highest to 150% concentration level. Finally, small changes in temperature (± 1 °C), flow rate (± 5%), mobile phase composition (± 1%) and formic acid addition to mobile phase (± 0.05%) were applied to the method. Changes in retention times and peak areas were calculated to determine the robustness of the developed method and all changes were expressed as RSD (%).

All calculations regarding the method validation such as linearity, recovery and all RSD values were done using Microsoft Excel software. All data generated and analysed during this study are included in the article and in supplementary information file.

Results and discussion

RP-HPLC hydrophobicity and phospholipid-binding determination. All analytes, except MSZ, have shown strong interaction for the RP-C18 column. Linear relationships ($r \geq 0.99$) between log *k* values and the volume fractions of methanol were found for all compounds. All log *k* values are the average of three measurements, with RSD (%) values lower than 0.99% (Table 1). Positive hydrophobicity parameters were obtained for all analytes with MSZ being highly hydrophilic ($\log k_{w\ C18} = 0.37$) compared to all three prodrugs which have high hydrophobicity ($\log k_{w\ C18} \geq 3.01$), whilst FA has shown moderate hydrophobicity ($\log k_{w\ C18} = 0.99$).

On the other hand, the development of IAM chromatography added new perspectives for the use of chromatographic techniques in the profiling of new drugs, combining simulation of the cell membrane environment with rapid and reliable measurements. Our previous investigations encouraged us to apply this chromatographic system on 5-aminosalicylates and FA. Linear relationships ($r \geq 0.94$) between log k_{IAM} values and the volume fraction of methanol were found for all compounds. The presented finding showed lower degree of interaction with phosphatidylcholine than with octadecyl group for both 5-aminosalicylates ($\log k_{w\ IAM} \leq 2.77$) and FA ($\log k_{w\ IAM} = -0.37$). This phenomenon might be due to the presence of sterically exposed charged moieties on the phospholipid chains. Furthermore, a moderate linear correlation between hydrophobicity ($\log k_{w\ C18}$) and lipophilicity data ($\log k_{w\ IAM}$), obtained by HPLC, was observed ($r = 0.91$). It is possible that the unexpectedly high affinity of MSZ for the IAM column ($\log k_{w\ IAM} = 0.17$) was due to its small size and consequently ability to

Analyte	log $k_{w\,C18}$[a]	log $k_{w\,IAM}$[a]	PPB_{HSA} (%)[b]	PPB_{AGP} (%)[b]
MSZ	0.37 ± 0.19	0.17 ± 0.02	61.44 ± 0.51	6.22 ± 1.08
FA	0.99 ± 0.31	− 0.37 ± 0.14	69.40 ± 0.53	3.45 ± 0.61
BSZ	3.01 ± 0.09	1.20 ± 0.11	91.64 ± 0.04	77.60 ± 0.58
SASP	3.71 ± 0.24	2.77 ± 0.23	93.62 ± 0.05	96.36 ± 0.13
OSZ	3.25 ± 0.09	1.69 ± 0.17	96.00 ± 0.03	90.33 ± 0.20

Table 1. Experimental parameters obtained by chromatographic techniques. [a]log $k_{w\,C18}$ and log $k_{w\,IAM}$ values with the accompanied 95% confidence interval. [b]PPB_{HSA} (%) and PPB_{AGP} (%) values with accompanied RSD (%).

penetrate within the phospholipid monolayer. All log *k* values are the average of three measurements with RSD (%) values lower than 3.55% (Table 1).

Plasma protein binding studies. PPB has a noteworthy role in modulating the effective drug concentration at the pharmacological target. The mentioned concentration as well as consequential pharmacological activity of drugs may be influenced by co-administered drugs due to potential competition for the same binding site on plasma proteins.

HSA, the most abundant plasma protein, has binding sites capable of binding xenobiotics with a preference for acidic and neutral compounds. All obtained values are the average of three measurements (RSD values lower than 0.53%). High HSA binding affinity for all compounds (more than 61%) due to the presence of fully ionized carboxyl groups in the structure of investigated compounds (pK_a values of all compounds are not higher than 3.37) is very important finding (Table 1). On the other hand, AGP shows the preference for basic and neutral compounds. All obtained values are the average of three measurements (RSD values lower than 1.08%). According to obtained results, a high affinity for AGP, similar to that of HSA, was observed for SASP and OSZ (higher than 90%). Somewhat lower affinity for AGP than for HSA was found for BSZ (14% lower), while MSZ and FA show quite low affinity for AGP (Table 1). Furthermore, it is worth pointing out that the linear correlation in a plot of PPB_{HSA} vs. log $k_{w\,C18}$ ($r = 0.99$), as well as in the plot of PPB_{AGP} vs. log $k_{w\,C18}$ and ($r = 0.98$) was observed for 5-aminosalicylates and FA. The obtained results imply that hydrophobic forces are generally predominant in the binding mechanism. Drug binding to the HSA usually occurs in Sudlow site I or II, which is a hydrophobic cavity that is capable of holding multiple drug molecules. As well as HSA, AGP also has a wide central hydrophobic pocket that occurs as a main binding site for the ligand molecules[24,25].

Observed high affinity of 5-aminosalicylates and FA for HSA protein led to further studies using frontal analysis and zonal elution methods to get an insight into FA binding on HSA and possible binding competition between FA and 5-aminosalicylates for the same place on HSA protein.

Based on considerations above, frontal analysis was approached using FA which was run through the HSA column to monitor the saturation of FA specific binding sites. As the mobile phase containing FA is applied through the column, the detector signal increases due to the saturation of HSA binding sites, which results in a specific breakthrough curve. Breakthrough curves were obtained for each applied concentration (1, 5, 10, 15 and 20 µM) and were used to create a double-reciprocal plot of apparent FA moles required to reach the equilibrium ($1/m_{Lapp}$), vs. concentration of FA that was applied (1/[A]) as illustrated in Fig. 2a. Double-reciprocal plot (Fig. 2b) shows great linearity ($r > 0.9999$), even with high applied concentrations, implying that the FA has a single type of binding site on the HSA.

The slope and intercept of the obtained curve were used for calculation of the equilibrium constant (K_a) and moles of binding sites (m_L) for FA-HSA specific interaction, using the Eq. (1):

$$\frac{1}{m_{\mathrm{Lapp}}} = \frac{1}{m_{\mathrm{L}} \times K_a \times [\mathrm{A}]} + \frac{1}{m_{\mathrm{L}}} \quad (1)$$

The obtained value of moles of binding sites ($m_L = 2.34\ (\pm 1.16) \times 10^{-7}$ mol) was further used for calculation of binding constant (K_a) which equals $1.64\ (\pm 0.03) \times 10^4\ M^{-1}$; the values in brackets represent 95% confidence intervals. Hence, the obtained binding constant is specific for the conditions used and is determined using the HPAC method for the FA-HSA system for the first time. Previous studies using different techniques have shown comparable binding constants ranging from 0.53 to $9.7 \times 10^4\ M^{-1}$[26–30].

Having determined that FA has site-specific binding on HSA protein, a zonal competition study was conducted to examine the possible competitive binding between 5-aminosalicylates and FA. A mobile phase with FA in different concentrations (ranging from 0 to 20 µM, with 5 µM increments in each mobile phase) was run through the biomimetic column to saturate the FA specific binding sites. Upon saturation of all FA specific binding sites, a small fraction of examined compound, in this case each 5-aminosalicylate, was injected into the system at each applied FA concentration and the retention times were monitored. Obtained retention times were used to calculate the retention factor *k*, whose reciprocal value was used in a plot of 1/*k* vs. FA concentration in the mobile phase. Change in retention time with the increments of FA would imply that there is a competition for the same binding site on HSA between FA and 5-aminosalicylates. Linear dependency ($r > 0.991$) was obtained with a positive slope for each 5-aminosalicylate. Linear correlation implies that there is no positive or negative allosteric modulation on HSA protein due to the binding of FA. Furthermore, low values of the slopes

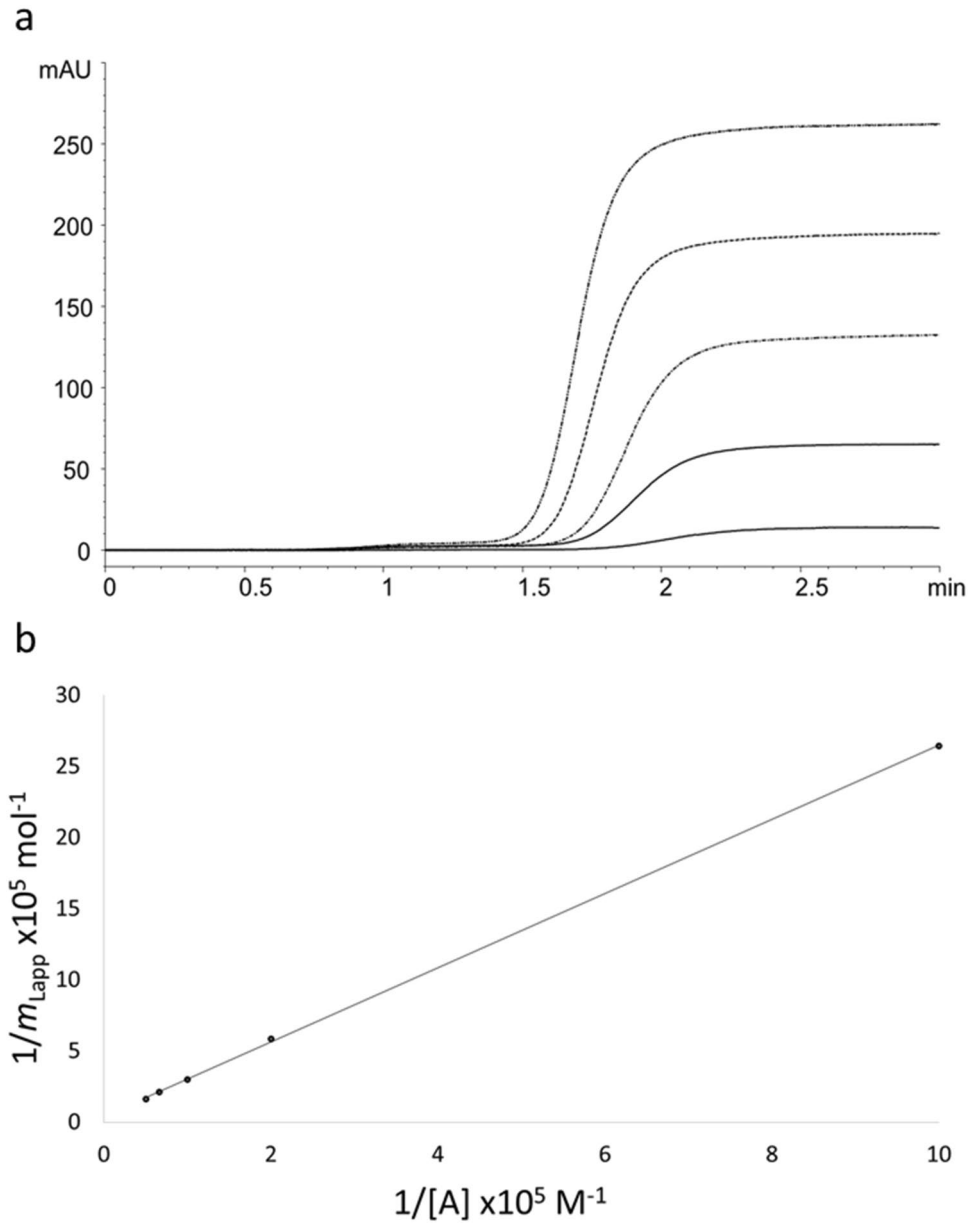

Figure 2. (**a**) Breakout curves obtained with frontal analysis, (**b**) double-reciprocal plot of apparent FA moles ($1/m_{Lapp}$), versus applied concentration of FA (1/[A]).

(0.0004–0.0019) imply that the competition of the 5-aminosalicylates for the same binding site is very low. With the statistical analysis of the slope of the regression line, whether it is equal or different from zero we can confirm the above stated. With the regression analysis (95% confidence interval) all obtained *P*-values of slopes were higher than 0.05, ranging from 0.09 to 0.13. With *P*-values > 0.05 we can accept the null hypothesis, stating that the slope is not different from 0. This could be explained by the fact that FA binds to a different site on HSA compared to the rest of 5-aminosalicylates. According to the findings in the literature, FA binds to a site located in Domain I on HSA protein whilst SSZ binds to Domain IIb and BSZ and MSZ bind to Domain IIIa[31–33]. To the best of our knowledge, this is the first study on possible competitive binding between 5-aminosalicylates and FA using frontal analysis approach. Altogether these results indicate that the obtained results are in favour of further development of fixed-dose combination implying that there will be no interactions of FA with any of 5-aminosalicylates if taken simultaneously in a single formulation.

Simultaneous assay method development. Our preliminary study has shown that column chemistry and mobile phase composition will have a major impact on method performance, such as peak resolution, retention time and tailing factor, due to the considerable difference in analyte hydrophobicity. Reversed-phase columns, XBridge C18, 150 × 4.6 mm, particle size 5 µm column by Waters and Zorbax SB C8, 150 × 4.6 mm, particle size 5 µm by Agilent Technologies, were tested. Due to the high polarity of MSZ, poor retention was

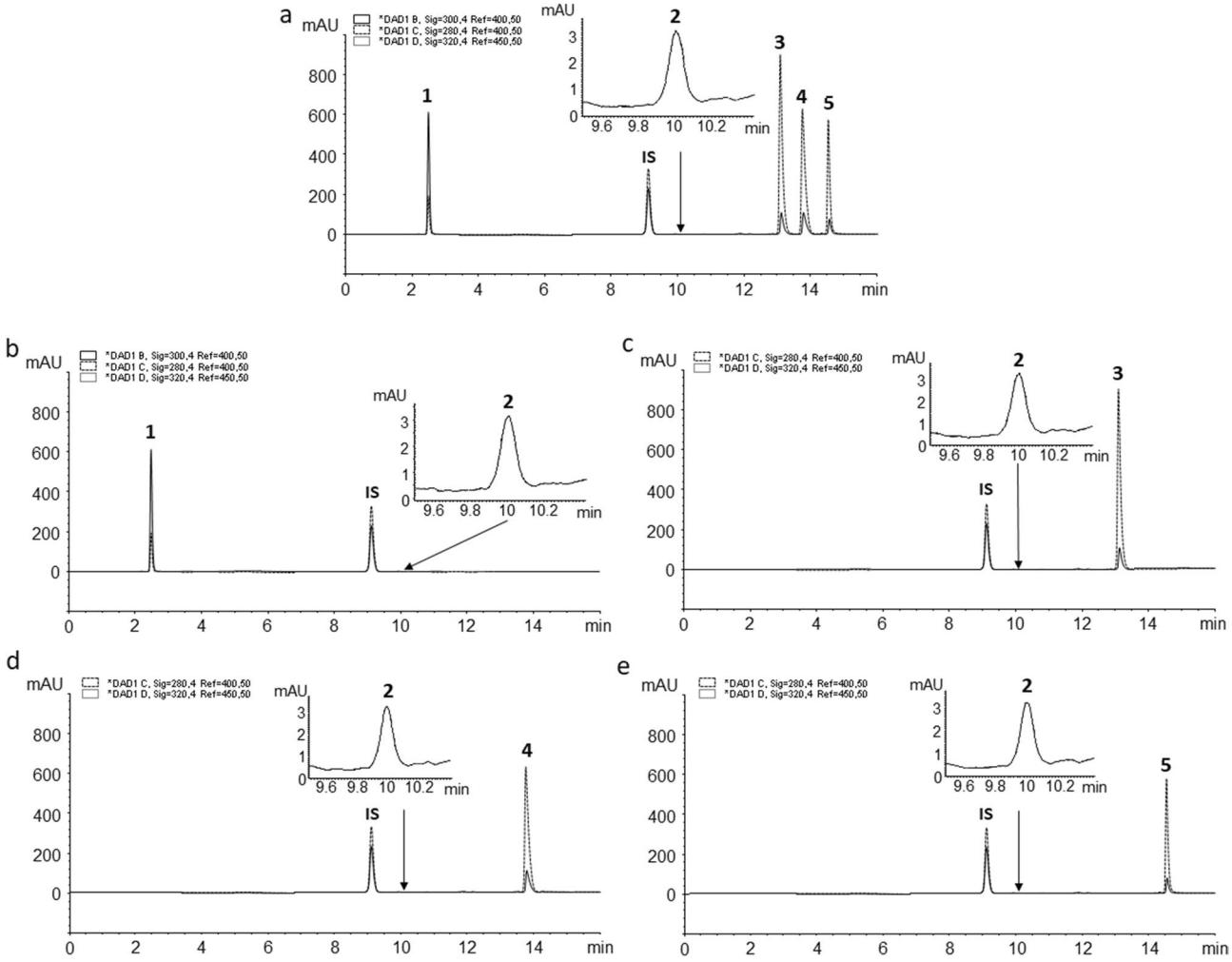

Figure 3. Chromatograms of: (**a**) mixed standard solution including MSZ (1), FA (2), BSZ (3), SASP (4), OSZ (5) and IS; and in-house FDCs (**b**) MSZ + FA; (**c**) BSZ + FA; (**d**) SASP + FA and (**e**) OSZ + FA at 100% concentration level (Chromatographic conditions: XBridge Phenyl column (150 × 4.6 mm, particle size 3.5 µm) and the mobile phase consisted of ultrapure water (A) and methanol (B), both acidified with formic acid up to 0.2% (*v*/*v*)).

observed ($k = 0.08$) with gradient elution starting with low levels of organic solvent (less than 5% of methanol) in the mobile phase, whilst high hydrophobicity of prodrugs resulted in a wide peak with prominent peak tailing (tailing factor, TF > 3.87). Best results in terms of retention factor, reproducibility, complete separation and peak shape were obtained with XBridge Phenyl (150 × 4.6 mm, particle size 5 µm) by Waters. The π–π interaction between phenyl groups bonded on silica and aromatic rings present in the analytes resulted in higher retention of MSZ. Therefore, this column was chosen as the most suitable for future research.

Gradient elution, starting with a low percentage of organic modifier (5%), was necessary to achieve satisfactory retention of MSZ ($k = 0.248$) but also to elute prodrugs in acceptable time ($k \geq 6.078$). As it was expected, acidified mobile phase (0.2% (*v*/*v*) formic acid, pH = 2.34), compared to neutral or alkali, had the advantage of creating an environment in which retention of polar acidic analyte such as MSZ ($k = 0.337$) was more easily achieved. Peak tailing of all peaks, including prodrugs, was minimized (TF < 2.26) as well as resolution maximized ($Rs > 3.67$). Therefore, a 0.2% (*v*/*v*) addition of formic acid was chosen as an additive in both mobile phases. Optimal wavelengths were chosen for the detection of each analyte: MSZ was detected at 300 nm, IS, BSZ, SASP and OSZ at 320 nm and FA at 285 nm. Injection volume of 10.0 µL was chosen as optimal to achieve the best sensitivity without overloading the column or detector. To minimise the error of the injector IS was introduced. The chromatograms of mix standard solutions and each 5-aminosalicylate/FA FDC are presented in Fig. 3. System suitability test, as proof of method quality and applicability, was used by analysing seven replicate injections of standard solutions at 100% concentration level to determine the parameters such as retention and relative retention time, number of theoretical plates, retention factor and tailing factor (Supplementary Table S3).

Method validation. As mentioned above the method was validated according to the current ICH Q2(R1) and FDA guidelines[22,23]. Solutions of placebo spiked with the standard solutions were used for method validation. Satisfactory resolution between adjacent peaks was achieved as resolution factor was higher than 1.5 All

Analyte	Linearity range (μg/mL)	Equation	r^a	$s_E{}^b$	LOD (μg/mL)[c]	LOQ (μg/mL)[d]
MSZ	50–150	$y = 0.0120\,x + 0.0079$	0.9999	0.0063	0.10	0.33
FA	0.1–0.3	$y = 0.0533\,x + 0.0012$	0.9997	0.0001	0.01	0.03
BSZ	75–225	$y = 0.0196\,x + 0.0024$	0.9997	0.0246	0.05	0.16
SASP	50–150	$y = 0.0261\,x + 0.0210$	0.9998	0.0181	0.04	0.14
OSZ	25–75	$y = 0.0261\,x + 0.0130$	0.9999	0.0037	0.02	0.08

Table 2. Method calibration data. [a]*r*—Pearson correlation coefficient. [b]s_E—standard error of estimate. [c]LOD calculated as a signal to noise ratio (3:1). [d]LOQ calculated as a signal to noise ratio (10:1).

Commercial formulation	Manufacturer	Active substance	Labelled amount (mg)	Found amount (mg)	Found/labelled (%)	RSD (%)
Pentasa	Ferring Pharmaceuticals, Saint-Prex, Switzerland	MSZ	500	499.50	99.9	1.6
Salofalk	Dr. Falk Pharma GmbH, Freiburg, Germany	MSZ	500	493.70	98.7	1.5
Mesalazin-Kohlpharma	Kohlpharma GmbH, Merzig, Germany	MSZ	500	511.60	102.3	0.2
Folacin	JGL, Rijeka, Croatia	FA	5	4.99	99.7	1.6
Premid	Almirall S.A., Barcelona, Spain	BSZ	750	756.75	100.9	1.9
Salazopyrin	Recipharm Uppsala AB, Uppsala, Sweden	SASP	500	510.55	102.1	0.6
Dipentum	Waymade PLC, Basildon, Essex, UK	OSZ	500	512.30	102.5	1.9

Table 3. Results of analyses of marketed formulations ($n = 3$).

peak purity factor values were greater than 999.7 and it was observed that there are no interferences between excipients in placebo solution and analyte peaks. Correlation coefficients obtained by linear regression analysis were all above 0.9997 indicating satisfactory linearity of method. Low LOD (0.01 μg/mL) and LOQ (0.03 μg/mL) values indicate that method is suitable for quantifying low concentrations of FA (Table 2).

All samples for precision evaluation were prepared as described in section "Stock and working solutions" and analysed at 100% concentration level (results are expressed as RSD (%) (Supplementary Table S4)). Obtained RSD (%) values were lower than 0.70 for 5-aminosalicylates and lower than 2.29 for FA, implying that the method satisfies the repeatability test but also remains precise during the longer period. Slightly higher RSD (%) values related to FA are due to its low concentration in prepared samples. Accuracy of the method was expressed by calculating the recovery percentage on three levels as described above. Consistent analytical recovery (98.9–101.8%; RSD ≤ 2.6%) was obtained over the investigated concentration range implying that the method is accurate in the whole range of the calibration curve (Supplementary Table S4).

The analytical method robustness was expressed as RSD (%) values of changes in retention times and peak areas. Put together, all obtained values of RSD (%) were below 4.87% implying that selected analytical parameters did not affect precision and accuracy of the method.

Analysis of marketed drugs. Finally, the developed method was successfully applied to determine the assay of MSZ, BSZ, SASP, OSZ and FA in tablet and capsule formulations available in Europe that are used in the treatment of IBD. Recoveries were expressed as a percentage of the recovered sample compared to the labelled claim. All obtained results are presented in Table 3. Thus, the results of analysis of market drugs (recoveries range from 98.7 to 102.5% with the RSD (%) ≤ 1.9) evidenced high extraction efficiency, reproducibility and reliability of the novel method. It is evident from these results that assay of investigated formulations of MSZ, SASP and FA lie within the limits specified in British Pharmacopeia (BP) requirements (95.0–105.0% of the stated amount)[34]. It is important to emphasize that assay results obtained from the analysis of BSZ capsules were well within the limits specified in United States Pharmacopoeia (USP) (90.0–110.0%)[35]. On the other side, OSZ does not have a finished product monography in either BP or USP.

The developed and applied method provides a simple solution for analysis of multiple 5-aminosalicylates and folic acid within a single run, thus eliminating the exceptionally time-consuming process of mobile phase change and system preparation as it would be necessary if different methods for each analyte were used, which is also in accordance with the principles of green chemistry. The simplicity of the method lays in the usage of non-buffered mobile phases that are easily prepared, which makes this method usable with mass spectrometry, as well as in its capability of analysing multiple samples with complex matrices consisting of various excipients without compromising method selectivity.

Conclusions

With the use of conventional and biomimetic columns an insight into pharmacokinetic related drug properties was achieved. High correlations between measured hydrophobicity values and drug permeability and PPB were obtained. The obtained drug properties provided useful information for upcoming development stages such as

formulation optimization and in vitro pharmacokinetic studies. FA was successfully characterized regarding the HSA binding properties using frontal analysis and no interactions between FA and any of the 5-aminosalicylates were observed with the zonal elution studies. Furthermore, the analytical method for assay determination in proposed FDCs was successfully developed, including easy and simple sample and mobile phase preparation. The developed method was successfully validated and applied to in-house prepared FDCs as well as to commercially available products marketed in the European Union for IBD treatment. With further studies, proposed 5-aminosalycilates and FA based FDCs could find potential application in treatment of inflammatory bowel diseases.

Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Author contributions

M.-L.J. did the experimental work and with A.M. wrote the main manuscript. E.B., D.A.K., B.N. and N.T. reviewed the manuscript and provided positive and constructive opinions.

Competing interests

The authors declare no competing interests.

Additional information

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3. **Rad 2 -** Physicochemical Compatibility Investigation of Mesalazine and Folic Acid Using Chromatographic and Thermoanalytical Techniques

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Physicochemical Compatibility Investigation of Mesalazine and Folic Acid Using Chromatographic and Thermoanalytical Techniques

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Abstract: Inflammatory bowel disease is a common name for Crohn's disease and ulcerative colitis. These inflammatory states cause damage in the sidewalls of the gastrointestinal tract, resulting in malabsorption of food and vitamins. Folic acid (Vitamin B9) is often associated with inflammatory bowel diseases since reduced overall folate concentration in the human body may lead to the development of colorectal cancer and megaloblastic anaemia. However, its deficiency is easily compensated by taking an additional folic acid pill during regular therapy. At the moment, there are no studies that have examined the compatibility of folic acid with 5-aminosalicylate drugs used in the treatment of inflammatory bowel diseases. In this work, differential scanning calorimetry, forced degradation studies, isothermal stress testing and dissolution stability testing were used to determine the stability of folic acid and one of the most commonly used 5-aminosalicylates, mesalazine, when present in the same solution or blend. To monitor the assay of folic acid, mesalazine and nine of its related impurities, a single HPLC method was developed. Results of compatibility studies showed that no physicochemical interaction between mesalazine and folic acid occurs when combined, opening the path to the development of new formulations, such as a mesalazine/folic acid fixed-dose combination.

Keywords: mesalazine; folic acid; inflammatory bowel disease; drug compatibility; fixed-dose combination

1. Introduction

Incompatibility of two or more drugs is defined as a change in a product which leads to unacceptable stability, safety and efficacy, and comes in a form of physical, chemical or therapeutic incompatibility. Physical incompatibility can emerge when two or more substances are combined, forming an unwanted product, which can be observed as a change in colour, taste, odour or morphology. Chemical incompatibility changes the effectiveness of a drug by forming inactive or toxic products as a result of chemical change in the drug itself. Therapeutic incompatibility of two drugs can emerge when two or more drugs are administered simultaneously, affecting each other's pharmacokinetics by changing the absorption, distribution, metabolism and excretion, or by having an impact on pharmacodynamics, subsequently resulting in synergistic or antagonistic effects [1,2]. The process of combining drugs has to be approached with care in order to prevent the occurrence of drug incompatibility, hence increasing the therapy effectiveness and safety.

Mesalazine (MSZ) is one of the most prescribed 5-aminosalicylate drugs in the treatment of inflammatory bowel diseases (IBD). It is an anti-inflammatory drug that acts locally on the site of

inflammation in the gastrointestinal tract. MSZ has been used for the treatment of IBD for over four decades and was successful in the induction and maintenance of remission [3]. On the other hand, folic acid (FA) is often taken simultaneously with 5-aminosalicylates in order to reduce the risk of birth defects in pregnant women, colorectal cancer and megaloblastic anaemia development in patients with IBD [4–8]. As IBD damages parts of the gastrointestinal tract, the overall surface for absorption of FA is reduced, resulting in its malabsorption. Therefore, IBD patients are often prescribed elevated doses of FA (1–5 mg/day) compared to non-IBD patients (0.4 mg/day) [9,10]. Therapies in which multiple drugs have to be taken simultaneously, as in IBD, often raise the question of medication adherence, as it is important to take FA regularly.

Currently, there is no reported study on the physicochemical compatibility of MSZ and FA. Drug-drug incompatibility testing is approached using various studies to gather as much information as possible about the potential drug-drug interaction. Differential scanning calorimetry (DSC), a thermoanalytical technique, is commonly used in the screening of drug-excipient blends, solely based on shift or disappearance/occurrence of endothermic or exothermic peaks, however, the same principle can be applied to drug-drug combinations [11–13]. Forced degradation studies are applied to induce the formation of representative degradation products as much as possible in various conditions in order to develop a stability-indicating method for further studies. This approach is useful for drug-drug compatibility tests, as it allows one to obtain insight into the formation of new degradation products by comparing drugs stressed in combination vs. separately [14,15]. Isothermal stress testing in combination with the developed stability-indicating method is used for the monitoring of drug-drug and drug-excipient compatibility by storing the blends in conditions of elevated temperatures and controlled humidity for longer periods to monitor physicochemical interactions [16]. The dissolution method represents the approach in which the drug is dissolved in different biorelevant media in order to monitor drug release and drug stability. This approach was successfully applied in drug-drug and drug-excipients compatibility studies [17]. Considering the results obtained by all these methods, a wider picture can be obtained and conclusions can be made regarding the compatibility of the two drugs.

The aim of this work was to examine the possible interactions between MSZ and FA when present together in a homogenous blend or solution to open the path to the development of MSZ/FA fixed-dose combinations. MSZ and FA blends contained the amount of each drug in a ratio which would ensure the maintenance of the remission state and supplement the malabsorbed FA during the therapy of IBD. Preliminary thermoanalytical measurements using DSC were conducted followed by forced degradation studies and isothermal stress testing. In the end, dissolution tests using simulated gastric and intestinal fluids were carried out to examine the possible interactions and their stability in such media. In order to monitor the degradation rate of MSZ and FA, an analytical method was developed for the detection and quantification of both active pharmaceutical ingredients (APIs) as well as nine MSZ-related impurities: 4-aminosalicylic acid (4-ASA), 4-aminophenol (4-AP), 2,5-dihydroxybenzoic acid (2,5-DHB), 3-aminobenzoic acid (3-AB), 3-aminophenol (3-AP), salicylic acid (SA), 2-chloro-5-nitrobenzoic acid (2Cl5-NBA), 2-aminophenol (2-AP) and 5-nitrosalicylic acid (5-NSA) (Figure 1).

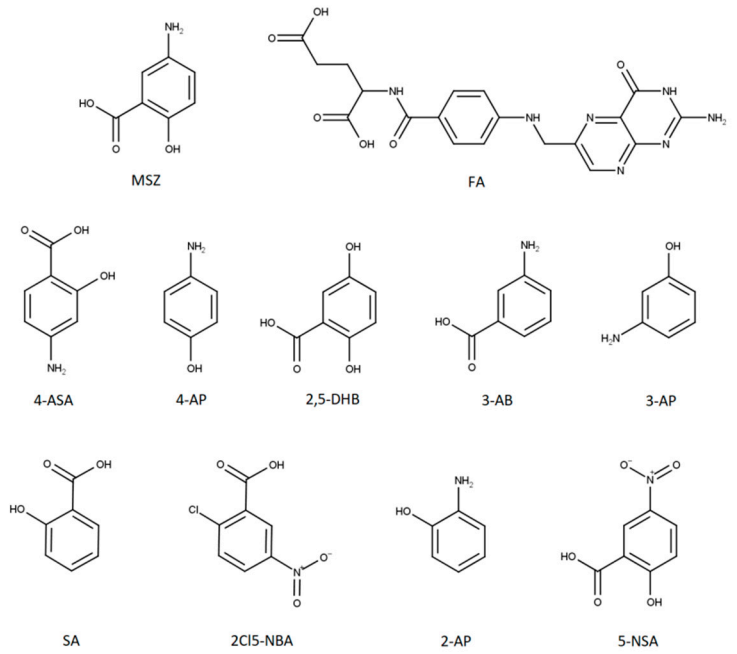

Figure 1. Structures of mesalazine (MSZ), folic acid (FA) and MSZ-related impurities.

2. Results and Discussion

2.1. Differential Scanning Calorimetry

The results of DSC scans of MSZ and FA as well as MSZ/FA standard blends in a ratio of 1:1 and 5:1 are shown in Figure 2. The endothermic peak of the MSZ standard occurs at 287.58 °C, which matches the MSZ melting point in the literature [18]. On the other hand, in the literature [19,20], FA only has a reported decomposition temperature at approximately 250 °C, at which it begins to darken/char and a significant weight loss is observed. However, it does not have a reported melting temperature.

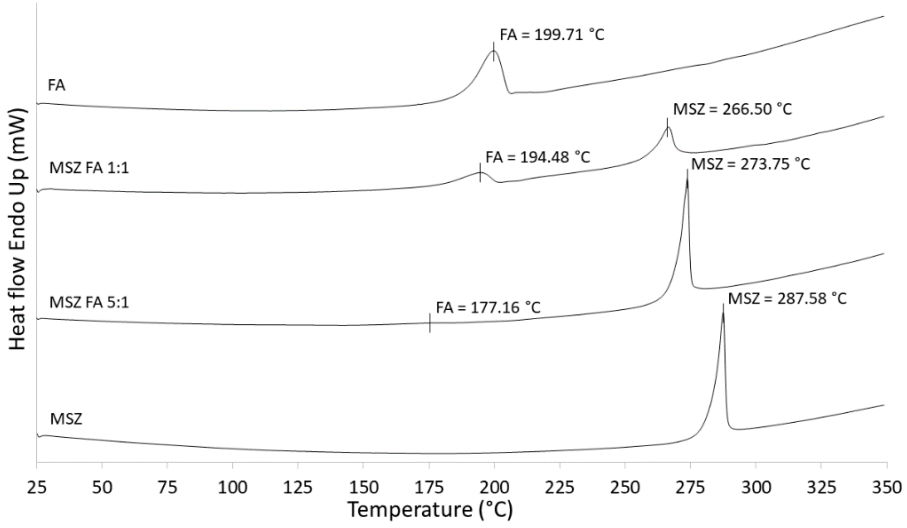

Figure 2. Differential scanning calorimetry (DSC) curves of MSZ, FA and the prepared blends.

In our measurements, a clear endothermic peak at 199.71 °C is visible and it is possibly due to the melting. In DSC, when screening MSZ/FA 5:1 and 1:1 blends, a shift in the MSZ peak to lower temperatures was observed, resulting in temperatures of 273.75 and 266.50 °C, respectively. In the case of FA, a significant shift in peak was observed (177.16 °C) in the 5:1 blend, while in the 1:1 blend,

the temperature was less shifted (194.48 °C). In all measurements, in spite of the obvious shift in temperature, peak shapes remained similar without noticeable broadening or disappearance. If the obtained DSC curve of a blend shows a shift in temperature to a lower or higher value (more than 5 °C) with absence of existing or occurrence of new endothermic or exothermic peaks when compared to the same compound alone, then there is a possibility that incompatibility might occur. Considering that the observed shifts in temperatures were higher than 5.23 °C, there was a doubt that MSZ and FA are incompatible; however, conclusions cannot be made solely on DSC measurements and there was a need for the use of other complementary methods [21,22].

2.2. HPLC Method Development

MSZ is a hydrophilic, polar molecule with a poor retention time on reversed-phase columns. Moreover, its impurities are structurally very similar and also polar; therefore, the goal was to develop a single method to separate MSZ, nine of its related impurities and FA up to an acceptable resolution. Column performance plays an important role in peak separation, especially when close-eluting peaks are expected. The column with smaller particles (2.7 µm), such as the Cortecs Phenyl column, was shown to be superior over standard columns (5 µm) due to narrower and higher eluting peaks that increased the separation efficiency.

Different compositions of mobile phases were tested in order to achieve the best chromatographic performance. Methanol, being a weaker eluent than acetonitrile, has shown to be a better choice since the components are more retained by the stationary phase of the column. Examining the various mobile phase additives, the best separation and peak performance was obtained using the ammonium acetate buffer adjusted to pH 5.0 ± 0.1 with glacial acetic acid.

The temperature was shown to have a strong impact on column performance. With the increment of temperature from 25.0 up to 50.0 °C, the peak characteristics were greatly improved. At 50.0 ± 0.1 °C column temperature, the peaks were noticeably narrower without shortening their retention time. Furthermore, different flow rates were tested in a range of from 0.4 mL/min up to 1.0 mL/min. No significant improvement in performance was observed with lowering the flow rate, therefore, it was kept constant at 1 mL/min. MSZ and FA were detected at 240 nm and 285 nm, while MSZ-related impurities were detected at 220 nm. The chromatogram obtained with the final chromatographic conditions showing all peaks with accompanied relative retention times (RRT) in relation to MSZ is shown in Figure 3.

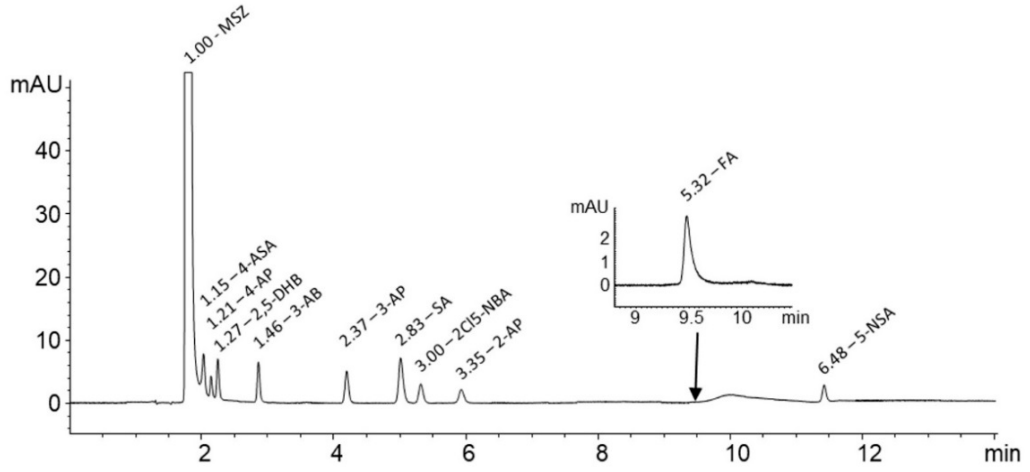

Figure 3. Chromatogram of MSZ and its related impurities (100% concentration level) at 240 nm. Insert shows FA peak at 285 nm. Hump at the 10th minute is due to the gradient.

2.3. Method Validation and Application

The method was validated as per the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline Q2 (R1) for selectivity, linearity, precision, accuracy, limit of detection, limit of quantification and robustness [23].

The selectivity of the method was determined by the visual examination of chromatograms obtained with the analysis of excipient solutions held in the same conditions as in each conducted degradation. No peaks were observed at the times of the elution of standards. Resolution between all peaks was higher than 1.5 and the peak purity of each peak was calculated where all values were higher than 998.

The linearity of the method was determined on five concentration levels in the range of from 25% to 120% of the maximum allowed concentration according to British Pharmacopoeia (BP). Linearity ranges, calibration curve equations and accompanying correlation coefficients are shown in Table 1. High values of correlation coefficients ($R \geq 0.998$) imply that satisfactory linearity was obtained in the given range.

Table 1. Method calibration data.

Analyte [1]	RRT [2]	Linearity Range (μg/mL)	Equation	R [3]	Limit of Detection (μg/mL)	Limit of Quantification (μg/mL)
Active Pharmaceutical Ingredients						
MSZ	1.00	500–1500	$y = 2.3629x + 273.1$	0.999	-	-
FA	5.32	1.00–3.00	$y = 22.987x - 0.845$	0.999	0.30	1.00
Impurities						
4-ASA	1.15	0.375–1.875	$y = 10.406x + 0.005$	0.998	0.02	0.07
4-AP	1.21	0.25–1.25	$y = 11.882x + 0.145$	0.999	0.03	0.09
2,5-DHB	1.27	0.375–1.875	$y = 17.735x + 2.553$	0.999	0.02	0.07
3-AB	1.46	0.25–1.25	$y = 36.573x + 0.129$	0.999	0.01	0.05
3-AP	2.37	0.25–1.25	$y = 25.545x + 0.389$	0.999	0.02	0.06
SA	2.83	0.75–3.75	$y = 13.102x + 0.041$	0.999	0.04	0.13
2Cl5-NBA	3.00	0.375–1.875	$y = 13.185x + 0.101$	0.999	0.04	0.15
2-AP	3.35	0.25–1.25	$y = 14.757x + 0.439$	0.999	0.04	0.14
5-NSA	6.48	0.25–1.25	$y = 15.720x - 0.571$	0.999	0.03	0.11

[1] Analyte: mesalazine (MSZ), folic acid (FA), 4-aminosalicylic acid (4-ASA), 4-aminophenol (4-AP), 2,5-dihydroxybenzoic acid (2,5-DHB), 3-aminobenzoic acid (3-AB), 3-aminophenol (3-AP), salicylic acid (SA), 2-chloro-5-nitrobenzoic acid (2Cl5-NBA), 2-aminophenol (2-AP) and 5-nitrosalicylic acid (5-NSA); [2] relative retention time; [3] correlation coefficient.

Method precision was determined as inter- and intra-day precision at a level of 100%. Intra-day precision was studied with the analysis of six separately prepared samples, where all samples were analysed during the same day. Inter-day precision was carried out with the preparation and analysis of three samples each day for three consecutive days. Obtained results are expressed as relative standard deviation (% RSD) and are shown in Table 2. As can be seen from provided data, all RSD values never exceeded 3.80% and 3.83% for intra- and inter-day precision, respectively, which implies that the method is precise.

Accuracy of the method was expressed as recovery and was tested in triplicates on three concentration levels (25%, 100% and 120%) in order to cover the whole range of the calibration curve. Results are shown in Table 2 as a percentage of recovery with an accompanied RSD value. RSD values never exceeded 3.13%.

The method was shown to be robust regarding the retention times (RSD < 5%) on changes in flow rate (1.00 ± 0.02 mL/min), column temperature (50.0 ± 1.0 °C), pH of the buffered mobile phase (5.0 ± 0.2) and changes in gradient (±1% of methanol). The resolution between the close-eluting peaks remained above 1.5 with all changes applied.

The MSZ standard and tablets used in further studies were analysed to determine the amount of impurities using the developed method. In the MSZ standard, 2,5-DHB impurity was found, albeit below the limit of quantification. Analysis of the MSZ tablet showed that 2,5-DHB and SA

impurities were present. Moreover, 2,5-DHB was present in a concentration of 0.08 µg/mL, while SA was below the limit of quantification. The concentrations of impurities found in the standard and tablet samples are below or near the limit of quantification and will not impact the results of forced degradation studies.

Table 2. Precision and accuracy data.

Analyte [1]	Precision as Relative Standard Deviation (RSD) (%)		Accuracy as Recovery ± RSD (%)		
	Intra-Day Precision (n = 6)	Inter-Day Precision (n = 9)	Low (n = 3)	Medium (n = 3)	High (n = 3)
Active Pharmaceutical Ingredients					
MSZ	0.77	0.78	97.88 ± 0.99	99.23 ± 0.61	99.44 ± 0.05
FA	1.31	1.56	103.60 ± 0.83	99.24 ± 2.11	100.23 ± 0.59
Impurities					
4-ASA	0.65	1.29	102.02 ± 1.02	99.36 ± 2.05	99.90 ± 0.66
4-AP	1.49	1.71	101.02 ± 0.47	97.61 ± 1.58	99.44 ± 0.82
2,5-DHB	1.56	1.64	100.27 ± 0.54	99.36 ± 1.18	99.90 ± 1.04
3-AB	1.03	1.05	100.02 ± 0.34	99.14 ± 0.65	98.97 ± 1.50
3-AP	1.14	1.64	102.03 ± 1.38	98.48 ± 1.96	98.60 ± 1.99
SA	0.89	0.99	98.83 ± 1.67	100.50 ± 0.67	99.18 ± 0.87
2Cl5-NBA	1.51	2.57	100.15 ± 2.11	100.10 ± 3.09	98.72 ± 1.36
2-AP	3.80	3.83	101.08 ± 3.13	98.01 ± 2.99	101.65 ± 2.94
5-NSA	2.36	2.45	97.77 ± 1.90	96.40 ± 2.41	99.64 ± 1.29

[1] Analyte: mesalazine (MSZ), folic acid (FA), 4-aminosalicylic acid (4-ASA), 4-aminophenol (4-AP), 2,5-dihydroxybenzoic acid (2,5-DHB), 3-aminobenzoic acid (3-AB), 3-aminophenol (3-AP), salicylic acid (SA), 2-chloro-5-nitrobenzoic acid (2Cl5-NBA), 2-aminophenol (2-AP) and 5-nitrosalicylic acid (5-NSA).

2.4. Forced Degradation Study

The goal was to achieve optimal degradation from 5% up to a maximum of 20%. Degradations higher than 20% were avoided due to the fact that formed degradation products could also degrade in the same conditions, giving additional peaks that could mislead the course of method development [24]. Furthermore, extremely harsh conditions were avoided to eliminate the rapid degradation of the examined API to eliminate the creation of non-representative degradation products. Each API was subjected to degradation separately in order to examine the best degradation conditions. The time needed for the first API to degrade (in between 5% and 20%) was used as the optimal time for degradation, even if the other one did not degrade significantly in that period. The same principle was applied for experiments with standard and tablet blends.

Chromatograms obtained from the analysis of the samples that were subjected to the stress study were examined in order to determine the presence of new major unknown peaks (Figure 4).

In this manner the impact of MSZ on FA and vice versa could be determined as a change in the degradation rate and through the appearance of new unidentified peaks in the mixed samples. The optimal degradation times and percentage of sample degradation are presented in Table 3.

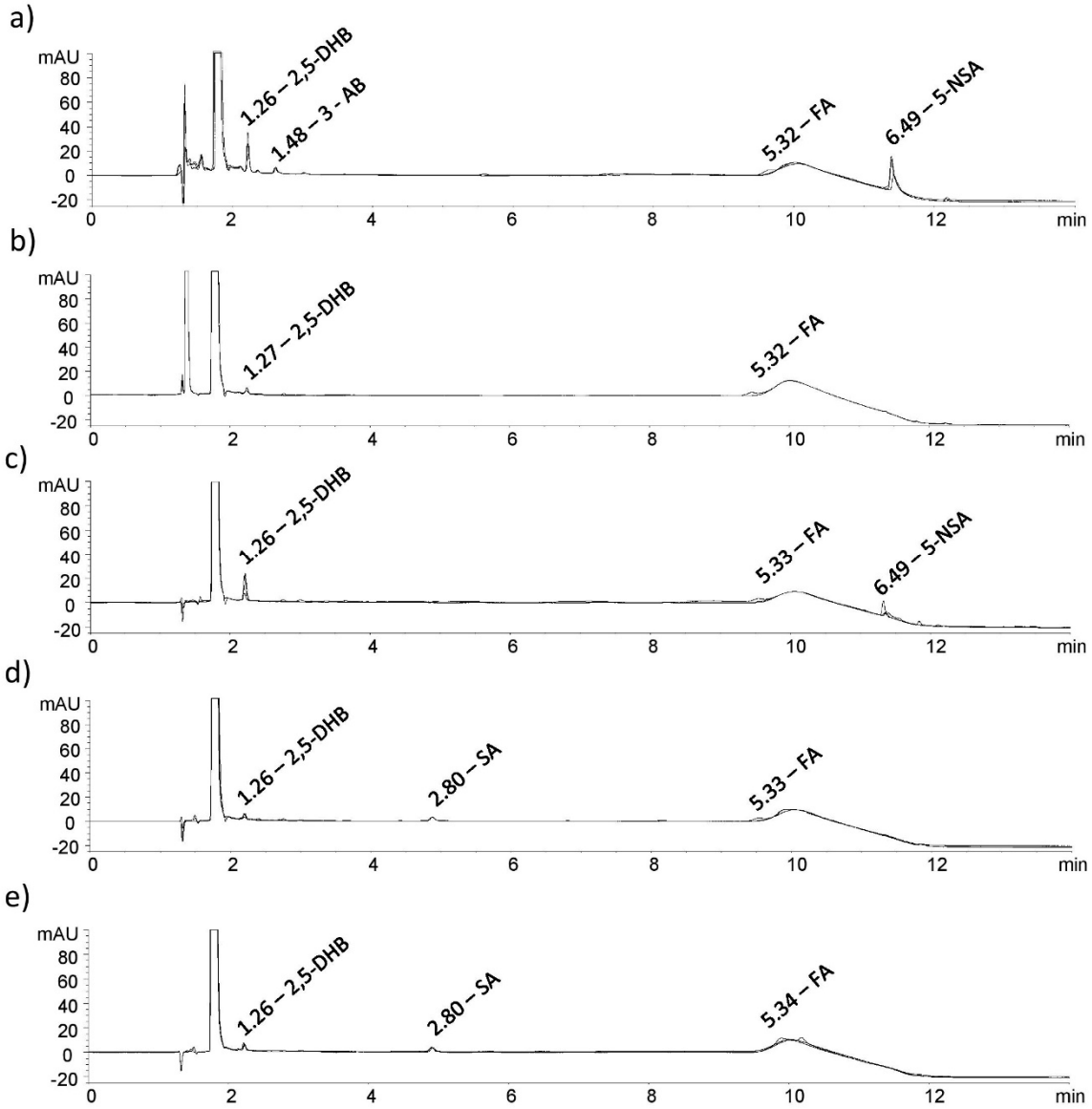

Figure 4. Overlapped chromatograms of stressed MSZ, FA and their combinations in: (**a**) alkali, (**b**) oxidative, (**c**) photo (solution), (**d**) photo (solid) and (**e**) thermal (solution) conditions.

Table 3. Forced degradation study results.

Degradation Condition	*t*/*h*	Standards		Standard Blend		Tablet Blend	
		% MSZ	% FA	% MSZ	% FA	% MSZ	% FA
0.1 M NaOH	1	9.4	2.1	11.6	1.4	10.4	1.6
0.1 M HCl	0.5	0.8	10.6	0.5	8.7	0.1	6.0
3% H_2O_2	1	9.1	0.7	9.8	0.7	10.3	1.0
Photo (solution)	4 (12 [1])	2.9	18.3	13.4	4.6	11.1	2.3
Photo (solid) [2]	168	4.4	2.9	6.4	4.1	4.2	6.5
Thermal (solution)	18	15.4	n.d. [3]	11.1	0.8	13.0	2.8
Thermal (solid) [2]	168	9.0	n.d. [3]	7.4	n.d. [3]	8.6	n.d. [3]

[1] 12 h reaction time refers to the standard and tablet blends; [2] 168 h reaction time equals 7 days; [3] n.d. = no degradation observed.

2.4.1. Alkali Degradation

The prepared samples were kept in alkali conditions (0.1 M NaOH) for one hour and it was observed that MSZ was unstable in such conditions. A drop in the MSZ assay of 9.4% and in the

FA assay of 2.1% was measured for alkali degradation of each API separately. The standard blend and tablet blend, also kept in the same conditions, showed similar degradations. Reductions in the MSZ assays of 11.6% and 10.4% and FA assays of 1.4% and 1.6% were observed. Considering the concentration of the FA in solution (2 µg/mL) and the related assay drops, it can be assumed that FA is stable in alkali conditions alone and in the presence of MSZ. The overlapped chromatograms showing MSZ impurities caused by alkali degradation are shown in Figure 4a. Three degradant products emerged: the peak at RRT of 1.26 corresponds to 2,5-DHB, the peak at 1.48 corresponds to 3-AB and that at 6.49 RRT corresponds to 5-NSA. Furthermore, by visually examining the obtained chromatograms, no new major peaks evolved as a consequence of FA being mixed with MSZ.

2.4.2. Acid Degradation

Keeping the standards of MSZ and FA separately in acidic conditions (0.1 M HCl) the assay of FA in the solution was reduced by 10.6% after 30 min of reaction time. MSZ showed greater stability during the same period with only 0.8% of assay reduction. The standard blend and tablet blend were also kept under the same conditions for 30 min; the FA assay was reduced by 8.7% and 6.0% while MSZ assay by 0.5% and 0.1%, respectively. Comparing the given results, FA was shown to be slightly more stable in the blend than when separated. This could be due to the fact that when combined, MSZ is present in a far greater concentration, reducing the chance of FA to be hydrolysed. Considering the low degradation percentage of MSZ, no new major peaks were observed.

2.4.3. Oxidative Stress

Samples were exposed to oxidative stress using a 3% hydrogen peroxide solution. The reaction time of 1 h was required for the MSZ assay to drop by 9.1%. The FA sample showed to be stable for that period with only a 0.7% assay reduction. The standard blend showed almost identical behaviour with a 9.8% reduction in the MSZ assay and a 0.7% reduction in the FA assay. The MSZ and FA assays in the tablet blend were similarly reduced, with a decrease of 10.3% and 1.0%, respectively. Overlapped chromatograms of the stressed samples are shown in Figure 4b. The only peak that emerged after the reactions was that at 1.27 RRT which corresponds to 2,5-DHB.

2.4.4. Photodegradation

Solutions and solids of the MSZ and FA standards, their blend and the tablet blend were continuously exposed to indirect daylight. After seven days of maintaining exposure of the solid samples, degradations in the range of 6.4% to 4.2% for MSZ were measured, while for the FA assay, reductions were in range of 6.5% to 2.9%. Considering the conditions to which the samples were exposed and the observed degradations, it can be assumed that MSZ and the FA solid samples are relatively stable. When examining the overlapped chromatograms, two peaks emerged. RRT of 1.26 and 2.80 corresponds to 2,5-DHB and SA, respectively (Figure 4c). Considering the results obtained above, the prepared MSZ and FA formulations should be kept protected from light to reduce photolytic degradation over a longer period.

After four hours of exposing the standard solutions to light, a drop in the MSZ assay of 2.9% was observed. On the other hand, as expected, the FA solution was shown to be very sensitive to light, displaying a reduction of 18.3% during the same period [25]. By keeping the standard blend solution exposed to the light, interesting results were obtained. After exposing the solution for 4 h, FA concentration changed insignificantly. However, by exposing the solution for an additional 8 h to the light, FA concentration dropped by only 4.6%, while MSZ degraded by 13.4%. It can be concluded that FA is shielded by MSZ molecules, notably reducing the chance of FA exposure to UV light. Similar results were obtained with the solution of the prepared tablet blend as with the standard blend solution. A reduction in the assay of 11.1% and 2.3% for MSZ and FA, respectively, shows that MSZ presence in greater concentration than FA has a major impact on FA stability in

solutions. When examining the overlapped chromatograms, two peaks emerged. RRT of 1.26 and 6.49 corresponds to 2,5-DHB and 5-NSA, respectively (Figure 4d).

2.4.5. Thermal Degradation

As in the photolytic stress study, both the solution and solid samples were subjected to thermal degradation. The MSZ solution appeared to degrade at higher temperatures during the period of 18 h. Assay reductions of 15.4%, 11.1% and 13.0% were measured for the MSZ standard, the standard blend and the tablet blend, respectively. On the other hand, FA is shown to be stable at a set temperature with maximum degradation, as in the case of the tablet blend (2.8%). Inspecting the obtained chromatograms, only two peaks emerged as a result of MSZ degradation, at RRT of 1.26 and 2.80, corresponding to 2,5-DHB and SA, respectively (Figure 4e).

Solid samples were kept in the same conditions, and assay reductions of 9.0%, 7.4% and 8.6% were measured for the MSZ standard, the standard blend and the tablet blend, respectively. On the other hand, FA showed no degradation whatsoever. No significant degradation peaks were observed in this study.

2.5. Isothermal Stress Testing

Isothermal stress testing is a common technique used in compatibility studies which involves keeping the samples at higher temperatures with or without moisture in order to induce the possible interactions between the components of the sample [26]. It is followed by HPLC measurements to determine the assay of the components after their storage, or other techniques to observe any other changes. Any noticeable drops in the assay of any component may indicate that there is possible interaction/incompatibility. Results of the conducted stress testing on both MSZ and FA standards, their combination as well as on each tablet and the prepared tablet blends are presented in Table 4.

Table 4. Isothermal stress testing results (after 4 weeks).

Sample	Appearance	Physical Change	Recovery (Mean ± % RSD) (n = 3)
MSZ tablets	Light grey powder	No significant visual changes	103.56 ± 0.21
FA tablets	Yellow powder		101.2 ± 1.56
MSZ (tablet blend)	Light yellow powder		101.73 ± 0.18
FA (tablet blend)			101.98 ± 0.62
MSZ standard	Light grey powder		99.50 ± 2.23
FA standard	Yellow powder		99.47 ± 0.69
MSZ (standard blend)	Light yellow powder		98.80 ± 0.79
FA (standard blend)			101.40 ± 1.91

All samples were visually inspected before and after the storage period of 4 weeks, and no changes in the appearance of the samples were observed. Recoveries of all samples are in the range of 98.80% to 103.56% with a maximum RSD of 2.23%. These results indicate that there was no chemical reaction between MSZ and FA. Any notable incompatibility between MSZ and FA could result in a significant drop in the FA assay, considering that MSZ is present in s far greater share in the prepared blends.

2.6. In Vitro Dissolution Testing

Dissolution studies were reported to be used for the determination of the stability of two drugs when present in media that simulates the gastric and intestinal fluids [27,28]. The stability of MSZ and FA tablets and their combination was examined in United States Pharmacopoeia (USP)-simulated gastric and intestinal fluids (SGF, 0.1 N HCl and SIF, pH 6.0 and 7.2). Due to local acting of MSZ and the fact that it can be easily absorbed before reaching inflamed parts of the gastrointestinal tract, it comes in the form of a gastro-resistant tablet; therefore, it is not expected to see any interaction between MSZ

and FA in gastric conditions. Results of dissolution testing of each tablet and their combination are shown in Figure 5.

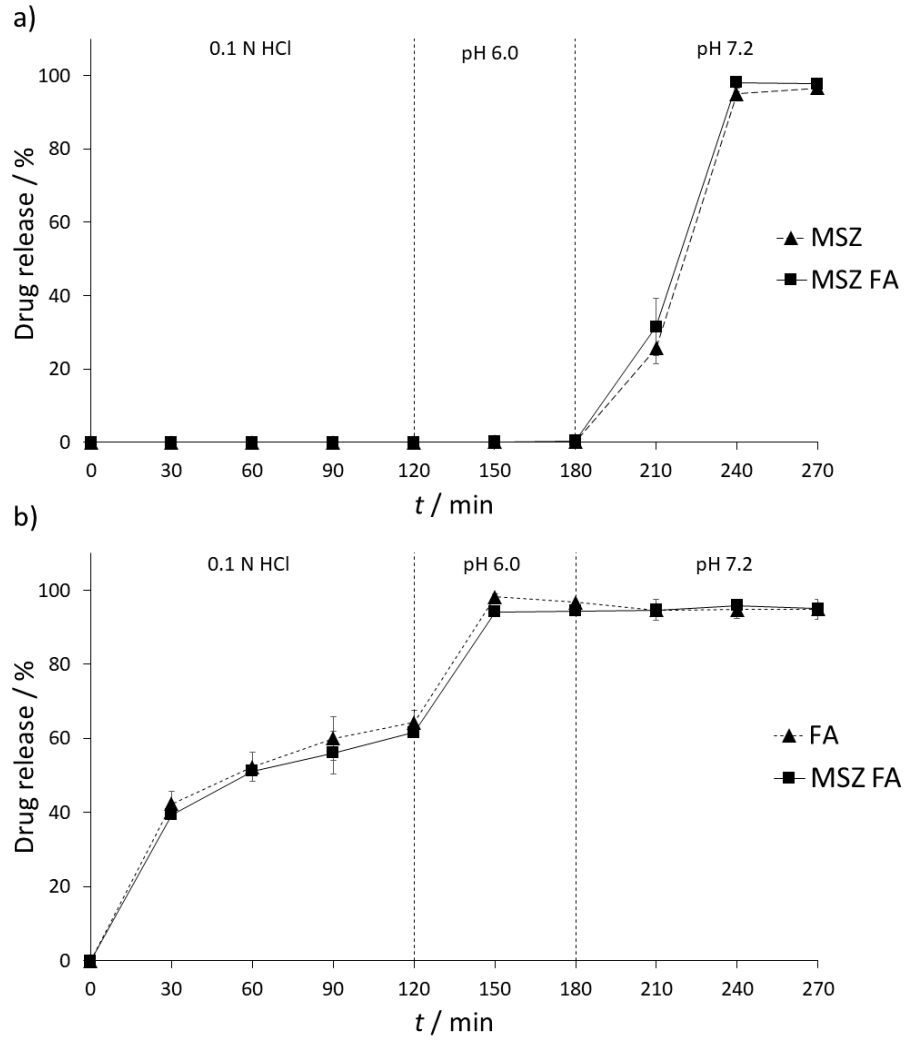

Figure 5. Drug release profiles of: (**a**) MSZ and (**b**) FA alone and in combination in different dissolution media (n = 3).

As expected, there was no release of MSZ from the formulation in both SGF and SIF (pH 6.0) dissolution media. Changing the SIF pH to 7.2 resulted in a gradual release of MSZ. Over the period of one and a half hours, up to 96.69% ± 1.49% of MSZ was released from the formulation (Figure 5a). On the other hand, a slow release of FA was detected in SGF, with a maximum release of 64.29% ± 3.18% over a period of two hours. In SIF (pH 6.0), complete release of FA was observed (96.75% ± 0.08%). FA remained stable over the whole period of SIF (7.2) conditions (96.76% ± 0.06%) (Figure 5b).

Possible interferences between MSZ and FA were tested by placing both tablets in the same dissolution vessel. No interaction between MSZ and FA was expected during the SGF and SIF (6.0) stage since MSZ is not released at this stage; however, since FA is released in both stages, possible interaction between FA and the MSZ tablet coating could occur, resulting in premature release of MSZ from the formulation. In both stages, FA did not have any impact on the release of MSZ and the amount of released FA was similar to that when not combined (94.35% ± 0.31%). Furthermore, no interaction between MSZ and FA was observed upon increasing the dissolution media pH to 7.2, which resulted in the release and dissolution of MSZ. Upon the end of experiment, the recovered FA

assay was 94.97% ± 0.89%, implying that FA remained stable in the dissolution media in the presence of MSZ and that no chemical or physical interaction occurred.

In order to confirm the similarity of the obtained dissolution curves, difference (f_1) and similarity factors (f_2) were calculated. Difference factor is a measure of relative error between two curves and is expressed as a difference percentage, with values between 0 and 15% as acceptance criteria. The difference factor for MSZ-MSZ/FA dissolution profiles is 4.51%, while for FA-MSZ/FA profiles it is 3.16%. These values indicate that there is no significant difference between the two curves. On the other hand, similarity factor (f_2) is a measure of similarity of two curves; values from 100% to 50% are considered acceptable where curves are identical if f_2 value is 100%. In the case of MSZ-MSZ/FA and FA-MSZ/FA, f_2 values were 82.09% and 77.01%, respectively, implying that the two obtained dissolution profiles are similar.

3. Materials and Methods

3.1. Reagents and Chemicals

MSZ and FA certified reference standards were provided by European Pharmacopoeia and MSZ-related impurities, namely, 4-ASA (99%), 4-AP (>98.0%), 2,5-DHB (>99.0%), 3-AB (>99.0%), 3-AP (>98.5%), SA (≥99.0%), 2Cl5-NBA (97.0%), 2-AP (>98.0%), and 5-NSA (>98%), were provided by Sigma Aldrich (St. Louis, MO, USA). The following chemicals were used for pH adjustment, the mobile phase and buffer creation: hydrochloric acid 37% (Carlo Erba, Val-de-Reuil, France), sodium hydroxide pellets ≥98.0% (Sigma Aldrich), hydrogen peroxide 30% (T.T.T. d.o.o., Sveta Nedelja, Croatia), glacial acetic acid (Panreac Química S.L.U., Barcelona, Spain), potassium phosphate and dipotassium phosphate (Kemika, Zagreb, Croatia) and acetonitrile and methanol HPLC-grade solvents by J.T. Baker (Avantor, Deventer, Netherlands). For selectivity tests and method validation, the following excipients were used: hydroxypropyl methylcellulose Methocel K100M Premium CR (Colorcon, Harleysville, PA, USA), magnesium stearate (Acros Organics, Princeton, NJ, USA), lactose monohydrate, stearic acid, wheat, rice and corn starch (Kemig, Zagreb, Croatia).

For experiments which required finished product, Salofalk® 500 mg gastro-resistant tablets, (Dr. Falk Pharma GmbH, Freiburg, Germany) and folacin 5 mg tablets (Jadran-Galenski Laboratorij d.d., Rijeka, Croatia) were used.

3.2. Preparation of Blends of Standards and Tablets

The blends of standards in the proposed ratio was prepared by carefully weighing and mixing 1000 mg of the MSZ standard with 2 mg of the FA standard. Powders were thoroughly mixed over a period of 20 min to achieve complete homogenisation. Five Salofalk® tablets containing 500 mg of MSZ were weighed and ground to a fine powder. The amount of powder equal to 2000 mg of MSZ was carefully weighed and mixed with the folacin powder equal to 4 mg of FA in order to achieve the desired ratio. Tablet powders were thoroughly mixed over a period of 20 min in order to achieve complete homogenisation.

3.3. Preparation of Standard and Working Solutions

All MSZ impurity standards used in the development of the HPLC method were carefully weighed and dissolved in water:methanol (90:10 *v*/*v*), and sonicated for 10 min in an ultrasonic bath (Elmsonic XtraTT, Biosan, Riga, Latvia) maintained at 40.0 °C. The final working solution containing 1000 µg/mL MSZ, 2 µg/mL FA, 0.02 µg /mL 4-AP and 2-AP, 0.10 µg/mL 3-AP, 5-NSA and 3-AB, 0.15 µg/mL 2,5-DHB, 4-ASA and 2Cl5-NBA, and 0.30 µg/mL SA represents the maximum allowed concentration of impurities to be present in the formulation as per the British Pharmacopoeia (BP) monograph for the MSZ finished product [29]. All samples were prepared with the addition of a mixture of commonly used excipients to the standard samples in a ratio that is expected in commercially available products in order to replicate the matrix of the real sample.

3.4. Chromatographic Conditions

The Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with a diode array detector with Chemstation for data processing was used for all chromatographic analyses. A Cortecs Phenyl column (150 × 4.6 mm, particle size 2.7 µm) with a suitable guard column, both obtained by Waters (Milford, MA, USA), was used for chromatographic separation. The column was maintained at 50.0 ± 0.1 °C during the analysis, while the flow rate was kept constant at 1.0 mL/min. Mobile phase A consisted of 5.0 mM ammonium acetate buffer (adjusted to pH 5.0 ± 0.1 with glacial acetic acid using the FiveEasy pH meter by Mettler Toledo, Columbus, OH, USA), while methanol was used as mobile phase B. Gradient elution was applied as follows: 0–7 min, isocratic 5% B; 7–9 min, linear gradient 5–20% B; 9–16 min, isocratic 20% B; 16–20 min, isocratic 5% B. The analysis time was 16 min and the total run time was 20 min to allow re-equilibration of the stationary phase for the following analysis. A sample volume of 5.0 µL was injected into the chromatographic system. MSZ and its related impurities were detected at 240 nm and 220 nm, respectively, whilst FA was monitored at 285 nm.

3.5. Differential Scanning Calorimetry

DSC curves were obtained using the Perkin-Elmer Diamond differential scanning calorimeter (Perkin Elmer, Inc., Waltham, MA, USA), calibrated with indium (99.98% purity; melting point 156.61 °C and fusion enthalpy of 28.71 J/g). Samples were carefully weighed (3–5 mg) directly into 50 µL aluminium pans and closed with a pierced aluminium lid. Measurements were carried under the atmosphere of pure nitrogen in a flow rate of 25 mL/min at a heating rate of 10 °C/min. The heating temperature for all samples was in a range from 25 to 350 °C. All samples were measured in duplicates.

3.6. Forced Degradation Study Conditions

All stress studies were conducted on samples containing 1000 µg/mL of MSZ and 2 µg/mL of FA when stressed separately or in combination. Samples were kept in stress conditions until optimal degradation of 5–20%. For each stress study, control samples were prepared and kept in the dark at room temperature for the same period. All samples, after the reaction time, were prepared and analysed using the chromatographic procedure described in Section 3.4.

Acid, alkali and oxidative degradations were conducted on solutions prepared by carefully weighing the standards and the prepared blends and dissolving them in water:methanol (90:10 *v*/*v*). Samples were sonicated for 15 min to achieve complete dissolution, and aliquot of 1 M NaOH, 1 M HCl or 30% H_2O_2 was added to each vial depending on the conducted stress study. The final conditions were: 0.1 M NaOH, 0.1 M HCl and 3% H_2O_2. The vials were sealed and stored in the dark at room temperature during the reaction time. The reaction time of 30 min was needed for optimal degradation in acidic conditions and 60 min in alkaline and oxidative conditions.

For photodegradation and heat degradations, the standards and the prepared blends were carefully weighed directly into a transparent HPLC vial separately and dissolved in 1 mL of water:methanol (90:10 *v*/*v*). Powders of the MSZ and FA standards, as well as the standard and tablet blend, were spread in a thin layer on a Petri dish. The prepared solutions and solid samples were exposed to indirect daylight. The solid samples were kept exposed to indirect daylight for 7 days, while the solutions were exposed for 4 h in the case of the FA solution and 12 h in the case of solutions containing MSZ. The effect of thermal stress was determined by placing the prepared solutions and solids in an ES-20/60 Orbital Shaker-Incubator by Biosan (Riga, Latvia) and thermostated at 70 °C. The solutions were heated for 18 h, while the solid samples were heated up to 7 days.

3.7. Isothermal Stress Testing

The MSZ and FA standards and their blends as well as the tablet powders and their blends were visually inspected and placed in the ES-20/60 Orbital Shaker-Incubator and thermostated at 50 °C for

4 weeks. Samples were examined to identify any visual changes and dissolved in water:methanol (90:10 *v*/*v*) to achieve a concentration of 1000 μg/mL MSZ and 2 μg/mL FA. Samples were filtered and injected into the HPLC system. Control samples were prepared and stored in the dark at room temperature for the same period.

3.8. In Vitro Dissolution Studies

Dissolution of MSZ, FA and their combination was examined using the USP method for dissolution of MSZ delayed-release tablets [30]. Testing was performed on USP 2 apparatus LDLT-A10 (Labtron Equipment Ltd., Fleet, UK) in three different dissolution media: simulated gastric fluid (SGF, 0.1 N HCl) and phosphate buffer solutions of pH 6.0 (stage 1) and 7.2 (stage 2), both also known as simulated intestinal fluids (SIF). The dissolution media volume was kept constant at 500 mL, while the paddle rotation speed was 100 rpm for acid and buffer stage 1 and 50 for buffer stage 2. Samples were taken every 30 min from the dissolution vessel, filtered and injected into the HPLC system for assay determination. The drawn sample volume was replenished with an equal amount of fresh dissolution media. All measurements were conducted in triplicate.

4. Conclusions

Based on the obtained results, it can be considered that FA and MSZ have no chemical nor physical interaction. Although DSC measurements showed possible incompatibility, forced degradation studies and isothermal stress testing showed that there is no significant interaction when the drugs are combined as a blend or in a solution. Moreover, if MSZ and FA had any chemical interaction, it would be observed unambiguously, as the FA assay would be significantly reduced since it is 500 times less present in the sample. This research can open the path to possible development of an MSZ and FA fixed-dose combination, considering the importance of FA compensation since medication adherence is one of the problems in polypharmacy therapy of chronic diseases.

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4. **Rad 3** - Drug-drug compatibility evaluation of sulfasalazine and folic acid for fixed-dose combination development using various analytical tools

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Article

Drug–Drug Compatibility Evaluation of Sulfasalazine and Folic Acid for Fixed-Dose Combination Development Using Various Analytical Tools

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Abstract: The simultaneous administration of sulfasalazine and folic acid is regular practice in the therapy of inflammatory bowel diseases in order to maintain sufficient folate concentration in patients. Having multiple drugs in the therapy increases the possibility of patients failing adherence, thus unintentionally endangering their health. A fixed-dose combination of sulfasalazine and folic would simplify the classical polytherapeutic approach; however, the physicochemical compatibility investigation of two active pharmaceutical ingredients plays an important role in the development of such a product. In this work, various analytical tools were used to determine the physicochemical compatibility of sulfasalazine and folic acid. For the evaluation of chemical compatibility, infrared spectroscopy in combination with advanced statistical methods, such as the principal component analysis and cluster analysis, were used, whilst a simultaneous thermogravimetry/differential thermal analysis gave us an insight into the physical compatibility of two drugs. Isothermal stress testing, forced degradation and dissolution studies, followed by the analysis with a developed chromatographic method for the monitoring of folic acid, sulfasalazine and two of its related impurities, sulfapyridine and salicylic acid, gave us an insight into its chemical compatibility. The combination of the results obtained from the used techniques implies a satisfactory physicochemical compatibility between sulfasalazine and folic acid, which opens the path to the development of the proposed fixed-dose combination.

Keywords: sulfasalazine; folic acid; fixed-dose combination; physicochemical compatibility; thermal techniques; spectroscopy; chromatography

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1. Introduction

Sulfasalazine (SASP) is an anti-inflammatory drug that has been mainly used for the treatment of rheumatoid arthritis since its discovery in the 1930s. Nevertheless, patients with ulcerative colitis given the SASP therapy have noticed a significant reduction of symptoms, which ultimately led to its use in the treatment of inflammatory bowel diseases (IBD) [1]. The SASP molecule consists of the anti-inflammatory 5-aminosalicylic acid and an antibiotic sulfapyridine (SP) connected via an azo bond. Once administered, SASP is absorbed up to 25% in the small intestine, while the rest of the drug reaches the colon mostly unchanged, where enzyme azo-reductase breaks the azo bond, releasing 5-aminosalicylic acid at the site of inflammation [2]. The main downsides of SASP are the toxicity of its metabolite SP, which is responsible for its side effects in nearly 30% of patients, and the SASP-induced inhibition of the reduced folate carrier at clinically relevant plasma concentrations. This inhibition leads to a folate deficiency in patients that consequently

increases the chances of developing colorectal cancer and megaloblastic anemia, as well as defects, in newborns [3–6]. To maintain a sufficient folate concentration in IBD patients, a supplementation of folic acid (FA) during the regular therapy of IBD is mandatory, usually in elevated doses of 1–5 mg/day compared to the intake of 0.4 mg/day in healthy people [7].

A fixed-dose combination (FDC), a drug formulation comprising of multiple active pharmaceutical ingredients (APIs), is a great alternative to the classical therapy approach. For diseases that require polytherapy, such as IBD, multiple drugs must be administered simultaneously, increasing the risk that some of the medications will be omitted. SASP/FA FDC would increase the medication adherence in IBD patients by ensuring the regular intake of FA, where the proposed FDC would contain enough SASP and FA to maintain the remission state of the disease and to supplement malabsorbed FA during the therapy. The process of FDC development requires thorough drug–drug compatibility testing to ensure the physical and chemical compatibility of APIs. Physical and chemical incompatibility can result in unwanted interactions between two drugs leading to the questionable safety, efficacy and stability of the final product. Physical incompatibility can result in the change of odor, color and morphology whilst chemical incompatibility can lead to the creation of new inactive or toxic products [8]. Various techniques and methods, such as Fourier-transform infrared spectroscopy (FTIR), simultaneous thermogravimetry, differential thermal analysis (TG/DTA) and isothermal stress testing (IST), have been used so far for examining drug–drug and drug–excipient physicochemical compatibility [9–12]. Forced degradation studies, comprising of a series of degradation conditions applied to APIs and finished products, as well as their blends, are recommended during the FDC development process by The World Health Organisation guidelines for the registration of FDCs [13].

Up until now, there has been no published study concerning the physicochemical compatibility investigation of SASP and FA when present in the same solution or blend. In this research, the physicochemical compatibility of SASP and FA was examined using the FTIR, TG/DTA and IST techniques, as well as forced degradation and dissolution studies. For the assay determination after the IST, forced degradations and dissolution studies, a single high-performance liquid chromatography (HPLC) assay method was developed for the monitoring of SASP, FA and two SASP-related impurities, SP and salicylic acid (SA) (Figure 1).

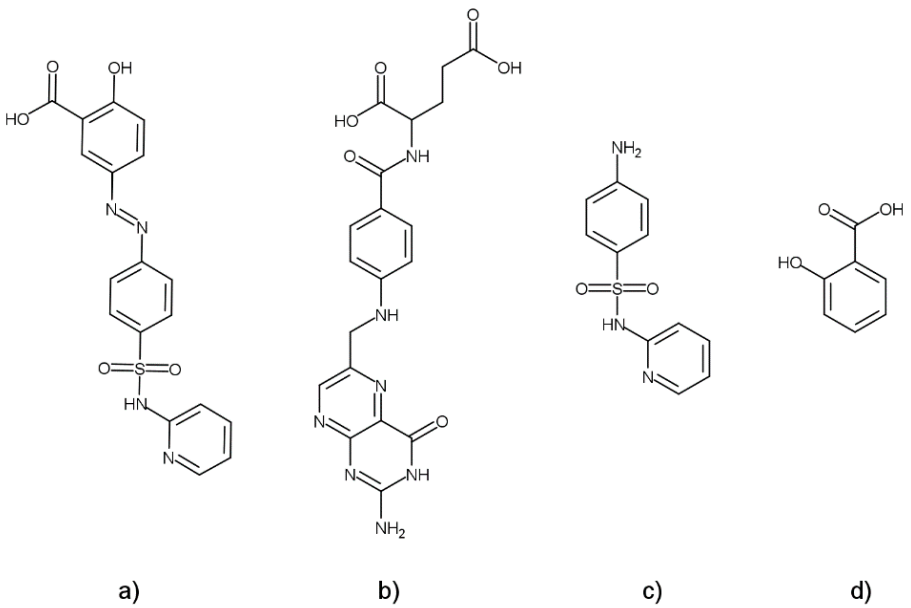

Figure 1. Structures of (**a**) sulfasalazine (SASP), (**b**) folic acid (FA), (**c**) sulfapyridine (SP) and (**d**) salicylic acid (SA).

2. Materials and Methods

2.1. Reagents and Chemicals

The SASP (as per the British Pharmacopoeia (BP), 99.6%), FA (meets the United States Pharmacopoeia (USP) specifications, 100.6%), SP (98.0%) and SA (≥99.0%) standards were provided by Sigma-Aldrich (St. Louis, MO, USA). Chemicals used for the preparation of samples, buffers, mobile phase, pH adjustment and degradation studies were the following: ultrapure water (<0.1 μS/cm) purified with a Series Ultra Clear and Integra Ultra-pure water system (SG Water, Barsbuttel, Germany), methanol and acetonitrile HPLC grade solvents (≥99.9%) (Avantor, Deventer, The Netherlands), *N*,*N*-dimethylformamide (DMF) (99.5%) (VWR Chemicals, Radnor, PA, USA), formic acid for HPLC (98–100%) (Merck, Kenilworth, NJ, USA), glacial acetic acid (99.5–100.5%) (Panreac Química S.L.U., Barcelona, Spain), ammonium acetate (≥98.0%) (Lach-Ner s.r.o., Neratovice, Czech Republic), sodium hydroxide pellets (≥98.0%) (Sigma-Aldrich), hydrochloric acid (37.0%) (Carlo Erba, Val-de-Reuil, France), hydrogen peroxide (≥30.0%) (T.T.T. d.o.o., Sveta Nedelja, Croatia), monobasic potassium phosphate (≥99.0%) (Kemika, Zagreb, Croatia) and α-alumina (Sigma-Aldrich).

Finished drug products Sulfasalazin Krka EN 500-mg gastro-resistant tablets (KRKA, d.d., Novo Mesto, Slovenia) and Folacin® 5-mg tablets (Jadran-Galenski Laboratorij d.d., Rijeka, Croatia) used in experiments were obtained from the local pharmacy. For selectivity tests and method validation, the following excipients (present in the above-stated drug products) were used for the preparation of the placebo blend: lactose monohydrate, cellulose (microcrystalline), povidone, crospovidone, starch, magnesium stearate and silicon dioxide (colloidal anhydrous). All excipients were obtained from Fagron Hrvatska d.o.o. (Donja Zelina, Croatia).

2.2. Preparation of Working Solutions and Blends

Stock solution containing 1000-μg/mL SASP, 2-μg/mL FA, 5-μg/mL SP and 5-μg/mL SA was prepared by carefully weighing and dissolving each standard in 30% DMF. Working solution, containing 250-μg/mL SASP, 0.50-μg/mL FA, 1.25-μg/mL SP and 1.25-μg/mL SA, was prepared by diluting the stock solution with 30% DMF and the addition of the placebo blend followed by 10-min sonication in an ultrasonic bath (Elmasonic XtraTT, Biosan, Riga, Latvia). Working solution contained the maximum allowed concentration of impurities (in relation to SASP), as per the BP monograph for the SASP finished product [14]. Prior to HPLC analysis, the working solution was centrifuged and filtered through a polyethersulfone (PES) syringe filter (25 mm, 0.22 μm) (Obrnuta faza d.o.o., Pazin, Croatia) to remove the residues of undissolved excipients.

Standard and tablet blends for IST tests and forced degradation studies were prepared in the proposed 500:1 ratio by weighing (using a MX5 microbalance scale by Mettler Toledo, Columbus, OH, USA) and mixing 2000 mg of SASP and 4 mg of FA standard or, in the case of tablet blend preparation, by weighing the amount of previously powdered tablets equivalent to 2000 mg of SASP and 4 mg of FA. Standard blends for TG/DTA measurements were prepared in a 1:1 ratio by mixing equal amounts of each standard, whilst, for the FTIR measurements, 500:1, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5 and 1:10 blends were prepared. Placebo blend was prepared by mixing the commonly used excipients in a ratio that represented their maximum allowed concentration in the formulation [15]. All powders were thoroughly mixed using mortar and pestle for 20 min to secure complete homogenization.

2.3. Chromatographic Conditions

Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with a diode array detector with Chemstation software for data processing was used for all chromatographic analyses. Separation was performed on XBridge C18 (150 × 4.6 mm, 3.5-μm particle size) reverse-phase column with suitable guard column, both obtained by Waters (Milford, MA, USA). The column was thermostated at 25.0 ± 0.1 °C during the analysis, with a constant flow rate of 1.0 mL/min. The mobile phase consisted of 5.0-mM

ammonium acetate buffer (pH set to 5.00 ± 0.01 using a FiveEasy pH meter by Mettler Toledo) as mobile phase A and methanol as mobile phase B. Gradient elution program was as follows: 0–8 min, linear gradient 5–40% B; 8–10 min, linear gradient 40–80% B; 10–15 min, isocratic 80% B and 15–18 min, linear gradient 80–100% B. Analysis time was 18 min, with an additional 7 min to re-equilibrate the stationary phase for the following analysis. Injection volume of 5.0 µL and detection wavelength of 360 nm was chosen for the monitoring of SASP, whilst FA, SA and SP were monitored at 285 nm with a 20.0-µL injection volume.

2.4. TG/DTA Analyses, IST Conditions and FTIR Settings

TG/DTA analyses were carried out using the NETZSCH thermal analyser STA 409 (NETZSCH-Gerätebau GmbH, Selb, Germany). Approximately 10.0 ± 0.1 mg of the sample was carefully weighed directly into the priorly cleaned platinum pan. Measurements were performed under the atmosphere of synthetic air in a flow rate of 30 mL/min from 25 °C up to 400 °C, with the heating rate of 10 °C/min and the usage of α-alumina as a reference.

IST was performed on SASP and FA standards, tablets, standard blends and tablet blends. Samples were spread in a thin layer on a Petri dish, visually inspected and placed in the thermostat (ES-20/60 Orbital Shaker-Incubator by Biosan) for 4 weeks at a temperature of 50 °C. After the heating period, samples were examined to identify any visual changes and analyzed using the developed HPLC method and FTIR.

For the chromatographic analysis, 5 mg of stressed sample was weighed and dissolved in 20 mL of 30% DMF, centrifuged, filtered and analyzed using the developed method.

Infrared spectra of SASP and FA, as well as all prepared blends, were obtained in the range from 500–4000 cm^{-1} using a Fourier-transform infrared spectrophotometer FTIR-8400S by Shimadzu (Kyoto, Japan) with PIKE MIRacle™ universal ATR by PIKE Technologies (Madison, WI, USA). All spectra were obtained as the average of 45 scans with a resolution of 2 cm^{-1}.

2.5. Forced Degradation Study Conditions

Forced degradation studies were performed on SASP and FA standards, standards blend and tablets blend. Acid, alkali and oxidative degradation studies were conducted on solutions in 0.1-M HCl, 0.1-M NaOH and 3% H_2O_2. Thermal stability of the solutions and solid samples was examined at a temperature of 70 °C, whilst photostability studies were performed by keeping the samples in indirect daylight. Degradation studies were conducted on solutions containing 250 µg/mL of SASP and 0.5 µg/mL of FA, whilst degradations conducted on solids were performed on accurately weighed amounts of prepared samples that were, after the reaction period, dissolved in 30% DMF. Samples were kept in described conditions until the optimal degradation of 10–30% [13]. All samples were analyzed using the developed HPLC method.

2.6. In Vitro Dissolution Studies

Dissolution studies of the SASP and FA finished products, as well as their simoultaenous dissolution, were performed as per the USP method for dissolution of SASP delayed-release tablets [16]. The USP 2 dissolution apparatus LDLT-A10 (Labtron Equipment Ltd., Fleet, UK) was used with the paddle rotation speed fixed at 100 rpm and bath thermostated at 37.0 °C. Simulated gastric fluid (SGF; 0.1-N HCl) and simulated intestinal fluid (SIF; 0.50-mM phosphate buffer (PB) solution, pH 7.5) were used as dissolution media at a constant volume of 900 mL for a two-stage dissolution procedure. The first stage involved the dissolution of tablets in SGF for 120 min, followed by 60-min dissolution in SIF. During the test, an aliquot of 5 mL was taken from the dissolution vessel every 30 min for assay determination using the developed HPLC method. An equal amount of fresh dissolution media was added after each sampling to keep the dissolution media volume constant. Dissolution experiments were conducted in triplicate for each tablet and their combination.

2.7. Statistical Methods

Statistical software XLSTAT by Addinsoft (New York, NY, USA) was used for interpretation of the obtained FTIR spectra and comparison of the results obtained from the chromatographic analysis of samples conducted for IST.

Statistical data processing was performed using the principal component analysis (PCA) with Pearson's correlation and a cluster analysis (CA) using Euclidean distance measures and Ward's agglomerative clustering on the spectral region from 550–1800 cm^{-1}, where most of the information was present. A 10 × 650 matrix was created where the number of rows represented SASP, FA and eight of their blends, whilst the columns represented the spectral data obtained with the 2-cm^{-1} resolution.

Two sample *t*-test was used for the comparison of recoveries obtained from the chromatographic analysis of the samples conducted for IST. The first group represented the recoveries of the samples stressed alone, whilst the second were recoveries from the stressed blends.

3. Results and Discussion

3.1. HPLC Method Development and Validation

The development of the stability-indicating assay method started with the selection of the optimal HPLC column. An XBridge C18 reverse-phase column (150 × 4.6 mm, 3.5-µm particle size) with a suitable guard column was used as a starting point for the method development. SASP was expected to be well-retained on the C18 column due to its high logK_{ow} value (3.7–4.8) [17]. Using isocratic elution with 50% methanol acidified with 0.1% formic acid as the mobile phase resulted in poor resolution between FA, SP and SA (<1.3), whilst SASP was well-retained, however, with prominent peak tailing (3.8). Considering the poor chromatographic performance obtained with the isocratic elution in acidified conditions (pH 2.87), the mobile phase was replaced with 5.0-mM ammonium acetate buffer with the pH set to 5.00 ± 0.01 with acetic acid. A gradient elution was necessary to get acceptable SASP retention and, thus, overall method run time. Usage of the above-defined conditions resulted in the satisfactory separation of all compounds (resolution > 3.98), peak symmetry (<1.26, as per USP) and purity factor (>998) (Figure 2).

The column temperature and flow rate had a minor impact on the separation and were set to be constant at 25.0 °C and 1.0 mL/min. To increase the sensitivity of the method, FA, SA and SP were monitored at 285 nm with a 20.0-µL injection volume, whilst SASP was monitored at 360 nm with a 5.0-µL injection volume to avoid a column and detector overload.

The method was validated regarding selectivity, linearity, accuracy, precision, limit of quantification, limit of detection and robustness as per the ICH guideline Q2 (R1) [18]. The selectivity of the method was determined using Chemstation software to calculate the peak purity of each peak with the purity factor threshold set at 995. No interferences were observed at the elution time of the analytes, whilst all peaks were shown to be pure, with the purity factor values greater than 998. The resolution factor between all the peaks was higher than 3.98, which exceeds the regulatory proposed minimal value of 1.5.

Five-point calibration curves of high linearity ($r \geq 0.999$) were obtained in the range from 80% up to 120% of the working solution concentration for SASP and FA and from the limit of quantification up to 180% of the maximum allowed impurity concentration for SA and SP as per the BP [14]. The limits of detection and quantification were determined as 3:1 and 10:1 signal-to-noise ratios, respectively (Table 1).

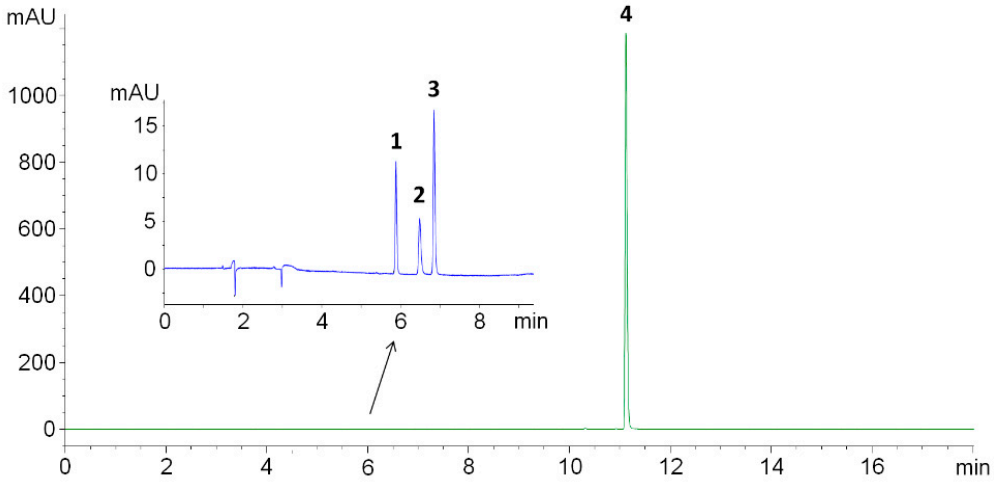

Figure 2. Chromatogram of a working solution showing FA (1), SA (2) and SP (3) at 285 nm (blue line) and SASP (4) at 360 nm (green line).

Table 1. Method validation data.

Analyte	Relative Retention Time	Linearity Range (μg/mL)	Regression Equation	*r*	Limit of Detection (μg/mL)	Limit of Quantification (μg/mL)
SASP	1.00	200–300	$y = 15.12\,x + 373.40$	0.9999	/	/
FA	0.53	0.4–0.6	$y = 64.79\,x - 0.04$	0.9999	0.03	0.10
SA	0.58	0.13–2.25	$y = 19.02\,x + 0.14$	0.9997	0.04	0.13
SP	0.62	0.13–2.25	$y = 45.09\,x - 0.30$	0.9999	0.04	0.13

Analyte	Precision as RSD (%)		Accuracy as Recovery ± RSD (%)		
	Intra-Day Precision (*n* = 6)	Inter-Day Precision (*n* = 9)	Low (*n* = 3)	Medium (*n* = 3)	High (*n* = 3)
SASP	0.08	0.10	99.14 ± 0.16	99.74 ± 0.09	99.27 ± 0.16
FA	0.77	0.78	101.71 ± 2.05	100.45 ± 0.96	101.54 ± 0.76
SA	0.35	0.35	103.84 ± 0.76	102.50 ± 0.37	102.25 ± 0.46
SP	0.67	0.67	101.15 ± 1.40	101.01 ± 0.34	99.59 ± 1.01

Six individually prepared samples were analyzed within one day for determination of the intra-day precision, whilst for the inter-day precision, samples in triplicate were prepared and analyzed each day for three consecutive days. The low relative standard deviation (RSD) values of the intra-day (≥0.77%) and inter-day (≥0.78%) precisions implied that the sample preparation process and method remained precise within and between the days. The method accuracy at the three concentration levels was tested in triplicate and expressed as the recovery with accompanying RSD values. The accuracy for SASP and FA was tested in triplicate at 80%, 100% and 120% concentration levels of the working solution, whilst for SA and SP, the accuracy was tested at the limit of quantification as the lowest point, as well as at 100% and 180% of the working solution concentration. Satisfactory results were obtained for all analytes, ranging from 99.14% ± 0.16% up to 103.84% ± 0.76%. The accuracy and precision data are shown in Table 1.

The method was shown to be robust regarding the recovery (assays remained within the regulatory limit of 100.0% ± 5.0%), retention times (RSD < 5%) and resolution (R_s > 1.5) on the changes in the flow rate (1.00 ± 0.05 mL/min), column temperature (25.0 ± 1.0 °C), mobile phase pH (5.0 ± 0.1) and changes in the gradient (±1% of methanol).

3.2. TG/DTA Measurements

The TG/DTA measurements of SASP and FA were done on a 1:1 binary mixture to increase the intensity of the possible reactions and for a better visualization of the incompatibility [19]. The TG/DTA curves of SASP, FA and the SASP/FA blend are shown in Figure 3. The DTA measurements of SASP showed an endothermic peak at 259.0 °C, which corresponded to its melting point, followed by degradation, which was observed as an exothermic peak. The TG measurement of SASP showed a weight loss of 2.0% in the temperature range of 0–100 °C due to the water evaporation from the sample. The weight remained constant until the beginning of the melting of SASP, followed by its degradation, resulting in a notable loss of 31.9% in the range of 240–300 °C. With the further heating of the sample, a constant weight loss was observed in the temperature range of 300–400 °C, with the final weight of the sample being 55.3% of the starting value (Figure 3a).

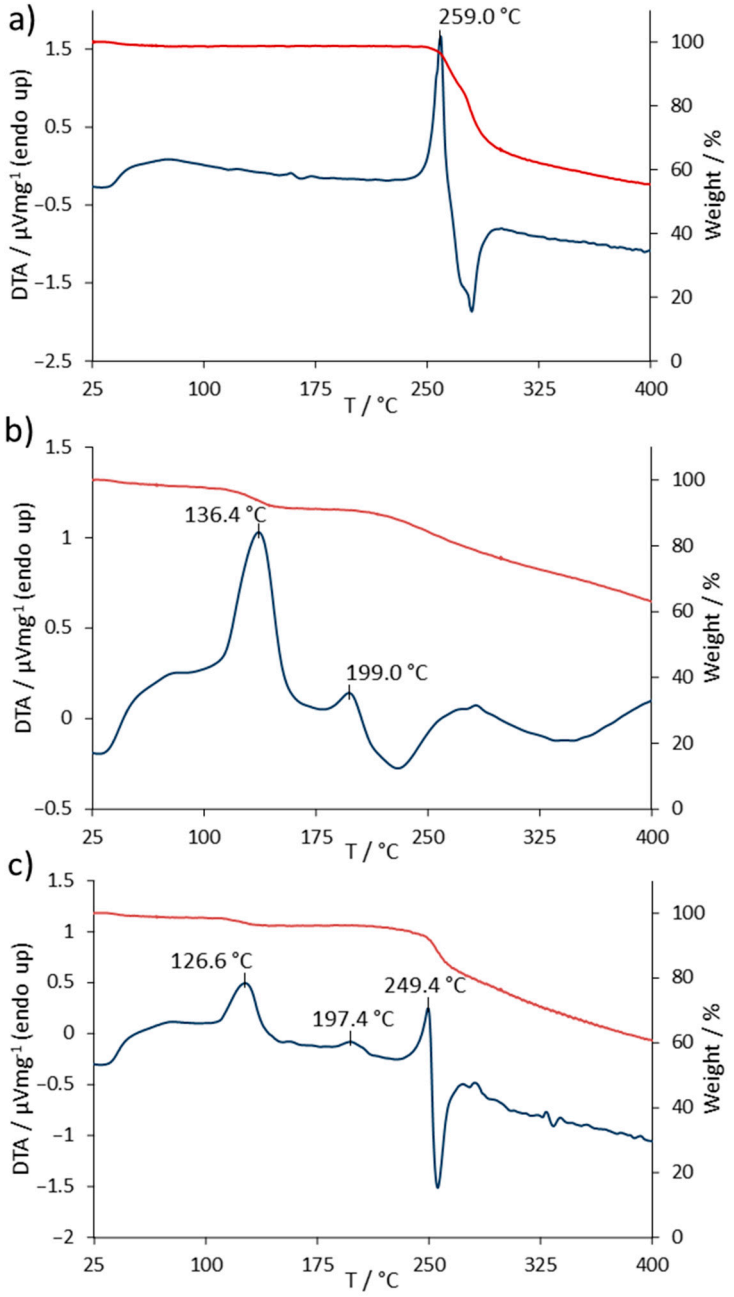

Figure 3. Thermogravimetry (TG) (red line) and differential thermal analysis (DTA) (blue line) curves of (**a**) SASP, (**b**) FA and (**c**) SASP/FA 1:1 blend.

The DTA measurements of FA showed two endothermic peaks at 136.4 and 199.0 °C. The peak at 136.4 °C corresponded to the endothermic nature of the water evaporation process. The TG measurements in the range of 0–175 °C showed a weight loss of 8.4%, which suited the declared value of the water content in the used FA standard of 8.0% [20]. The endothermic peak at 199.0 °C corresponded to the FA melting temperature, followed by a slow degradation as the heating continued, which could be seen as a constant loss of weight in the range of 200–400 °C [12]. The weight of the FA sample upon the end of measurements was reduced to 63% of its starting weight (Figure 3b).

The TG/DTA measurements of the SASP/FA blend showed both SASP and FA previously observed characteristic peaks. The endothermic peak at 126 °C corresponded to the loss of water present mainly in the FA standard. A weight loss of 3.9% was observed, which was equivalent to that of 8.4% observed in the FA standard alone, since the SASP/FA blend was prepared in a 1:1 ratio. The FA characteristic melting temperature was observed at 197.4 °C, whilst a slight shift in the SASP endothermic peak occurred, resulting in a temperature of 249.4 °C. The final weight of the sample was 60.8% of its starting weight, which fell in between the observed final weights of the SASP and FA standards, implying that no additional loss in combination was observed (Figure 3c).

The incompatibility of two drugs can result in the change of their physical characteristics, which can be observed as a shift in the characteristic peak temperature (±5 °C) or if the peak notably changes its shape, disappears or a new peak occurs [12,19,21,22]. In the provided measurements, a shift of the peak temperature was observed for SASP (9.6 °C); on the other hand, the FA characteristic melting peak did not shift notably (1.6 °C), which implies that the two drugs might be compatible. Even though the peak shapes remained intact and there was no occurrence of new peaks, the shift in the SASP peak was above the tolerance described in the literature, which might indicate possible changes in the system. However, it is well-known that conclusions cannot be made based only on TG/DTA measurements, since they provide limited information, and the use of other complementary analytical methods is mandatory to get a better understanding of the possible interactions [11,23].

3.3. IST Followed by FTIR and HPLC Measurements

In isothermal stress testing, the samples are subjected to isothermal conditions for a longer period, usually two–six weeks, to get an insight into the possible interactions between two or more components in the blend. FTIR is commonly used for getting an insight into the possible chemical changes in the sample, indicating the creation of new bonds, which ultimately results in different FTIR spectra. The FTIR spectra of SASP, FA and their blends are shown in Figure 4.

The obtained spectra show most of the SASP and FA characteristic absorption bands; however, since SASP and FA are molecules with numerous bonds, the FTIR spectra of the physical mixtures are hard to interpret. The SASP characteristic bands (C=O stretching from the carboxyl group at 1674 cm^{-1}, S=O from sulphonamide at 1356 cm^{-1} and C–O stretching at 1078 cm^{-1}) are more pronounced in the 500:1, 10:1, 5:1 and 2:1 blends; however, as the ratio of SASP and FA equalized (1:1), the FA characteristic bands (C=O stretching at 1687 cm^{-1} and N–H bending at 1602 cm^{-1}) appeared and became more dominant as the FA ratio in the blends increased (1:2, 1:5 and 1:10).

The interpretation of the FTIR spectra of the physical mixtures may lead to false conclusions due to the presence of the overlapping bands. Therefore, without going into a deeper visual analysis of the obtained spectra, where, as said above, we cannot gain an unambiguous conclusion, we decided to introduce a statistical approach with the aim of a better interpretation of the obtained spectra. Multivariate statistical analysis tools, such as PCA and CA, were already used and are well-described in the literature for the interpretation of obtained data [24,25].

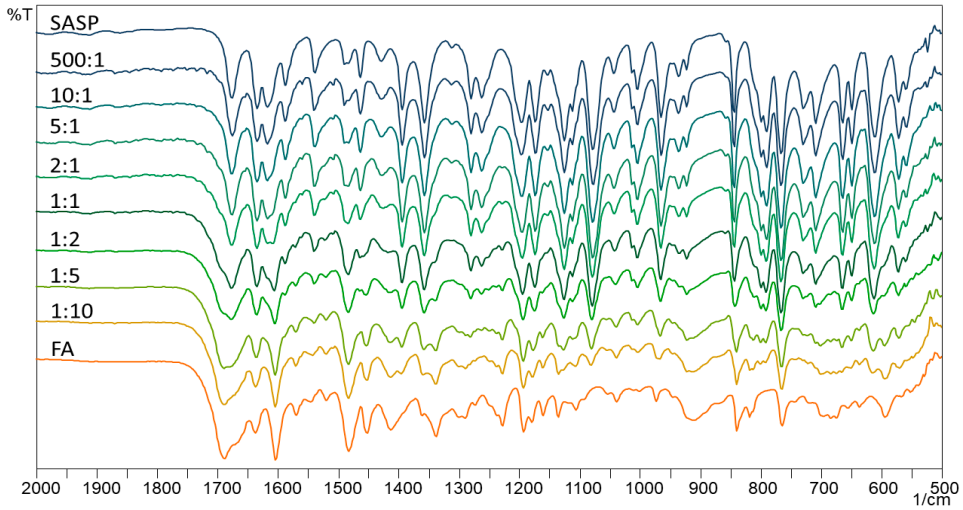

Figure 4. Fourier-transform infrared (FTIR) spectra of SASP, FA and their blends.

The results of the PCA analysis showed that eigenvalues of the nine obtained eigenvectors ranged from 494.6 to 0.2, whilst the data variability was in range from 76.21% to 0.03% (Table 2).

Table 2. Eigenvalues and variances of the nine obtained principal components.

Eigenvector	1	2	3	4	5	6	7	8	9
Eigenvalue	494.6	134.8	7.6	6.2	2.4	1.9	1.0	0.4	0.2
Variability (%)	76.21	20.77	1.17	0.95	0.37	0.29	0.15	0.07	0.03
Cumulative (%)	76.21	96.98	98.15	99.10	99.47	99.76	99.91	99.97	100.00

The eigenvector with the greatest variability and eigenvalue was the most impactful principal component, implying that the eigenvector with the eigenvalue of 494.6 and a variability of 76.21% was the first principal component (PC1). Since 96.98% of the total variance was covered by the first two eigenvectors (PC1 and PC2), the eigenvalues and variabilities of the last seven eigenvectors (three–nine) were insignificant and, therefore, were excluded from further analyses.

For a better visualization of the obtained principal components and data variability, the PCA results were best visualized using a PC1 vs. PC2 bidimensional scatterplot (Figure 5).

FA is located far to the right with the most positive PC1 value (41.33), whilst SASP is located far to the left with the most negative PC1 value (−27.49), where the blends of SASP and FA are placed in an increasing PC1 order as the SASP ratio in the blends reduces and the FA ratio increases (Figure 5a). These results are in favor of the SASP and FA chemical compatibility, showing the similarity between the spectra of the two adjacent blends. Random placement of the samples along the PC1 axis would indicate that there is no similarity between the obtained spectra due to the possible chemical reaction between the components in the blends.

The cluster analysis (Figure 5b) precisely reflected the results present in the scatterplot. Two main clusters were formed; the first connected SASP and the blends with a higher SASP share (500:1, 10:1, 5:1 and 2:1), whilst the other connected FA and its blends with a higher FA share (1:10, 1:5 and 1:2). The SASP/FA 1:1 blend was placed in a cluster with stronger linkage to the SASP-dominant blends, since the SASP spectrum overlapped most of the FA characteristic bands. The dissimilarity values between the formed clusters were below the strong Sokai and Sneath distance criteria (33% of the maximum distance, dashed line), which indicated that there was a statistically significant similarity between the obtained spectra, therefore implying that no chemical reaction occurred in the blends.

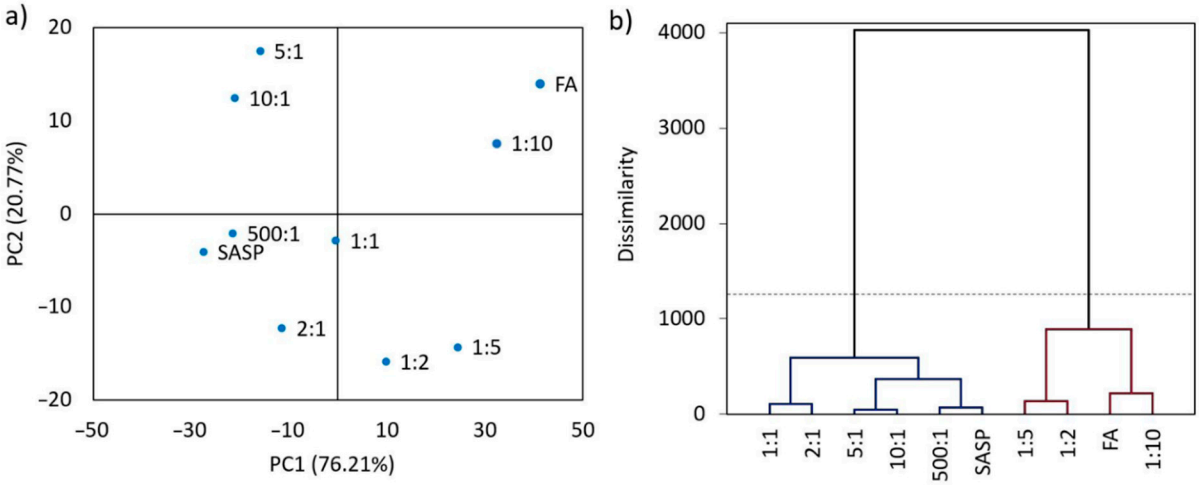

Figure 5. Results of the statistical analysis showing (**a**) the principle component analysis (PCA) bidimensional scatterplot and (**b**) cluster analysis (CA) dendrogram, indicating similarities between SASP, FA and their blends.

The IST is usually followed by HPLC measurements to determine any assay drops as a result of drug–drug interactions. Table 3 shows the results of the chromatographic analysis, as well as the visual inspection observations.

Table 3. Results of the chromatographic analysis after isothermal stress testing (IST).

API	Sample Type	Appearance	Physical Change	Recovery ± RSD (%) (n = 3)
SASP	standard	brownish-yellow powder	no significant visual changes	99.0 ± 0.5
	tablet			102.5 ± 1.3
	standards blend			99.9 ± 2.1
	tablets blend			101.0 ± 1.2
FA	standard	yellow powder	no significant visual changes	98.4 ± 0.6
	tablet	pale yellow powder		99.3 ± 1.0
	standards blend	brownish-yellow powder		98.6 ± 1.6
	tablets blend	brownish-yellow powder		± 2.7

The HPLC analysis results showed a great stability under the imposed conditions, with little to no change in the determined assays. With the statistical analysis, using the two-sample t-test, it was determined that the recovered assays were not significantly different (t-value = 0.72, t-critical = 3.18, p-value = 0.52, $\alpha < 0.05$) in the stressed blends from those when stressed alone.

3.4. Forced Degradation Study

Forced degradation studies were applied on SASP and FA standards, standards blend and tablets blend to get an insight into the degradation behavior of each component when stressed alone and in the presence of each other. The goal of this approach was to examine the chemical stability of SASP and FA and to determine whether they impacted each other's stability in the applied conditions. Samples were exposed to alkali, acid, oxidative, thermal and photolytic conditions and kept in the same until the optimal degradation of 10–30%. Keeping the samples too long in defined conditions would result in extensive degradation and the formation of unwanted secondary or tertiary degradation products, which could misslead the experiment; however, if the analyte shows little to no degradation in the seven-day period, it can be stated that the substance is stable in the applied conditions. The optimal degradation time used for stressing of the blends was the time needed for the first standard to degrade in the desired range when stressed individually; therefore, using that same reaction time for both blends' comparable results can be obtained regarding

the degradation of that component. Stressed samples were analyzed using the developed HPLC method to determine the assay drops, as well as occurrence of SASP-related impurities of SA and SP. Degradation times and assay losses of stressed samples are presented in Table 4.

Table 4. Forced degradation study results.

Degradation Condition	Degradation Times/day [1]	Observed Drops of Assay/%					
		Standards		Standards Blend		Tablets Blend	
		SASP	FA	SASP	FA	SASP	FA
0.1 M NaOH (solution)	7	1.8	2.7	0.8	2.7	2.3	2.4
0.1 M HCl (solution)	7 (1)	0.8	21.6	0.4	17.4	1.7	19.0
3% H_2O_2 (solution)	7 (1)	1.7	11.7	n.d. [2]	11.8	2.2	10.4
Photo (solution)	7 (1)	1.5	10.0	0.3	14.6	1.6	16.7
Photo (solid)	7	1.7	2.9	1.3	0.6	1.6	3.7
Thermal (solution)	7	1.9	1.1	n.d.	100.0	n.d.	100.0
Thermal (solid)	7	1.9	1.0	2.4	1.8	0.2	2.2

[1] Values in the parentheses represent the optimal degradation time for the FA standard and FA in the prepared blends. [2] n.d. = no degradation observed.

As can be seen from the results, the SASP standard remained stable in all conditions for 7 days, with the maximum assay drop of 1.9% observed in thermally stressed solution. FA solution was shown to be stable in alkali and thermal conditions for 7 days with the assay drop not exceeding 2.7%. Solid samples of FA exposed to thermal and photolytic conditions for 7 days measured assay drop of not more than 2.9%, implying stability in those conditions. FA solution was shown to undergo degradation in acid, peroxide and photolytic conditions, after 1 day, resulting in assay drops of 21.6%, 11.7% and 10.0%, respectively. SASP remained stable when combined with FA in prepared standard and tablet blends through 7 (or 1) day exposure to all conditions, with the maximum degradation of 2.4%. FA in blends has shown similar stability in all stress conditions except when heat-stressed as standard and tablet blend solution, where severe degradation occurred with FA assay drop of 100% after 1 day, compared to 1.1% degradation after 7 days as standard solution. Considering that FA is highly unstable in low pH environment, especially at elevated temperatures [26], as it was observed in our study (17.4–21.6% assay drops at room temperature and 100% at 70 °C), this phenomena can be justified with the fact that prepared 250 µg/mL SASP solution in 30% DMF measures pH of 2.85 (compared to pure 30% DMF solution that measures pH of 6.35), creating the acidic environment in which FA undergoes degradation, whilst heating of the sample significantly increases reaction kinetics, which ultimately resulted in complete FA degradation in a short period. Considering the absence of SASP/FA interactions observed with the previous methods, it can be said that the observed drop in FA assay is strictly pH & temperature related, rather than as a result of chemical interaction between SASP and FA. The impact of pH on instability of FA as well as the impact of pH in combination with elevated temperatures is well described in the literature [27–30]. However, given that SASP is responsible for creating a low pH environment when dissolved, this must be taken into consideration during the formulation process. In all degradations conducted SASP related impurities SP and SA were not detected. Purity of SASP and FA chromatographic peaks remained higher than 998 after every degradation conducted (purity threshold set to 995 whilst purity value of 1000 represents the identical spectra).

3.5. Drug Dissolution Testing

The impact of drugs on their release and stability can be examined with in-vitro dissolution studies using simulated gastric and intestinal fluids [10,12,31,32]. Drug release and stability were tested according to USP procedure using the two-stage dissolution process. The first stage concerned 120-min dissolution of tablets in 0.1 N HCl solution,

also known as USP simulated gastric fluid. The second stage involved 60-min dissolution in 0.05 mM PB with pH set to 7.5, also known as USP simulated intestinal fluid. A two-stage dissolution process is necessary since the tested SASP product comes in a form of gastro-resistant tablets, securing that most of the drug is released in the colon, rather than in the stomach.

As expected, SASP was not released from the formulation during the first two hours of the first dissolution stage, followed by the second stage with the gradual release of SASP over one hour resulting in a total release of 100.7% ± 0.8%. The effect of FA on the SASP release was tested by placing the SASP and FA tablets in the dissolution vessel at the beginning of the experiment to test the impact of dissolved FA formulation on the gastro-resistant film of the SASP tablet, which could result in premature release of SASP from the formulation. In the two-hour acid stage, no SASP was released from the formulation whilst in the following stage release of 98.8% ± 0.4% was observed. Both SASP alone and SASP in the presence of FA gave similar release curves (Figure 6a), which can be confirmed by calculating the difference (f_1) and similarity factors (f_2) [33]. The calculated difference factor of 1.6% is well below the maximum limit of 15.0%, whilst a high similarity factor value of 92.4% implies great similarity, considering that the 100.0% similarity value corresponds to two identical curves.

On the other hand, FA showed a gradual release in simulated gastric fluid, resulting in the recovery of 77.1% ± 4.9% during the first two hours, followed by the release of 109.7% ± 0.6% in the second dissolution stage. The possible occurrence of incompatibility between FA and SASP, considering the previously observed degradation in the forced degradation study, was tested by placing both tablets in the dissolution vessel at the beginning of the experiment. In both the acid and buffer stages, FA showed similar release as when tested alone, with the recovery values of 72.3% ± 4.0% and 107.2% ± 2.2%, respectively (Figure 6b). Difference (f_1) and similarity factors (f_2) were calculated as well with obtained values of 3.01 and 77.91, respectively, implying that the obtained curves were similar, and no significant interaction between two drugs occurred.

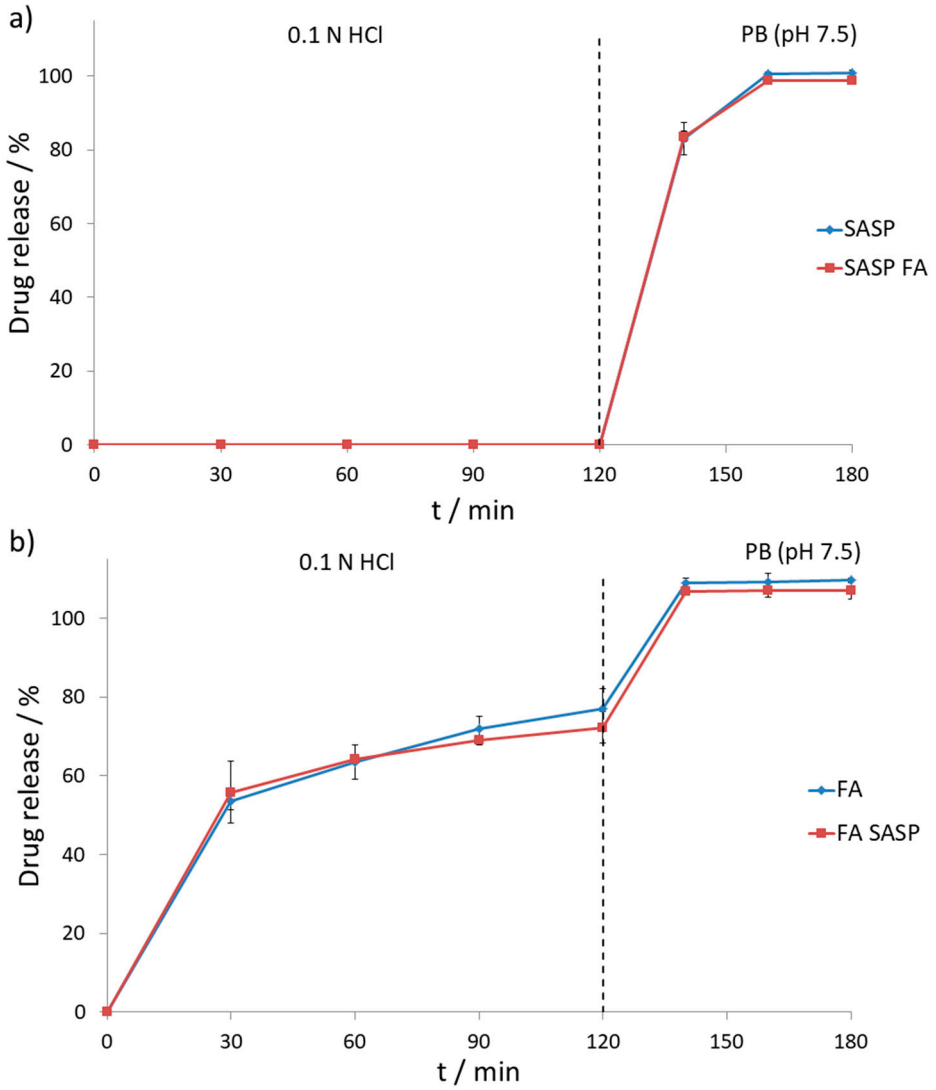

Figure 6. Drug release profiles of (**a**) SASP and (**b**) FA alone (blue line) and in combination (red line) in different dissolution media (n = 3) (Dashed line represents the change of dissolution media).

4. Conclusions

SASP and FA physicochemical compatibility was successfully examined using various analytical techniques. DTA measurements showed a shift of the SASP peak when present in a blend with FA; however, no changes of the FA peak and no occurrence of new peaks nor the disappearance of existing peaks were observed. The TG measurements showed no significant changes in weight loss, which indicated a lack of interactions; however, conclusions cannot be made solely on the TG/DTA measurements. The FTIR measurements in combination with the PCA/CA analysis implied that no interaction occurred between SASP and FA, which was also confirmed with the chromatographic analysis of the samples after IST, forced degradation and dissolution studies. Considering the ratio in which the SASP and FA samples were prepared (500:1), a significant loss of the assay of FA could be observed if any chemical interaction occurred. The thermal stress study of the SASP/FA standard and tablet solution resulted in the complete degradation of FA; however, it was explained as a pH- and temperature-related process and not a result of the SASP and FA interaction. Although the TG/DTA measurements raised concerns, with the wider picture obtained using various analytical tools, this work implies that SASP and FA are physicochemically compatible. This work presents one segment of the FDC development process; however, further studies, such as selecting optimal excipients, drug–excipient

compatibility studies and formulation stability studies, as well as dissolution studies of prepared dosage units, should be conducted to create an acceptable product.

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5. **Rad 4** - Thermoanalytical, spectroscopic and chromatographic approach to physicochemical compatibility investigation of 5-aminosalicylates and folic acid

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Thermoanalytical, Spectroscopic and Chromatographic Approach to Physicochemical Compatibility Investigation of 5-Aminosalicylates and Folic Acid

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Abstract: Fixed-dose combinations have shown to be a great alternative to traditional polytherapy; however, development of such formulation requires thorough physicochemical compatibility investigation of active pharmaceutical ingredients to provide a stable, safe and therapeutically effective product. In this work, differential scanning calorimetry, X-ray powder diffraction, isothermal stress testing followed by Fourier-transform infrared spectroscopy and chromatographic analysis as well as dissolution studies were used for physicochemical compatibility investigation of folic acid and balsalazide or olsalazine. Balsalazide and olsalazine as well as their blend were successfully characterised regarding their physicochemical properties using the mentioned techniques. Differential scanning calorimetry gave ambiguous results due to premature degradation of balsalazide. On the contrary, other techniques have implied the absence of any chemical reactions or physical changes in prepared blends. Obtained result imply that folic acid is compatible with both balsalazide and olsalazine which goes in favour of developing proposed fixed-dose combinations.

Keywords: balsalazide, olsalazine, folic acid, fixed-dose combination, physicochemical compatibility, FTIR, DSC, XRPD, chromatography.

INTRODUCTION

BALSALAZIDE (BSZ) and olsalazine (OSZ), anti-inflammatory drugs belonging to the class called 5-aminosalicylates (5-ASA), have shown to be great alternatives to mesalazine and sulfasalazine-based therapies of inflammatory bowel diseases (IBD).[1,2] Mesalazine is mostly absorbed in the upper parts of the gastrointestinal tract, leaving little to heal the inflammation in the colon, whilst mesalazine carrier sulfapyridine causes adverse reactions in patients whose therapy is based on sulfasalazine.[3,4] BSZ and OSZ are both prodrugs of mesalazine, meaning that active moiety is released upon the metabolism of the drug. BSZ comprises of carrier 4-aminobenzoyl-β-alanine and mesalazine linked together by an azo bond, while OSZ comprises of two mesalazine molecules connected by azo bond as well. These prodrugs do not tend to be absorbed in the upper part of the gastrointestinal tract and the majority of administered drug reaches the colon where the enzyme azoreductase breaks the azo bond and releases the anti-inflammatory moiety, mesalazine, directly to the site of inflammation.[2,5]

It is well known that IBD patients suffer from malabsorption of necessary nutrients, such as proteins, fats, carbohydrates, vitamins and minerals.[6–8] Among all mentioned, in this work we are going to focus on the malabsorption of vitamins, more specifically vitamin B_9, also known as folic acid (FA). FA plays an important role in

maintaining of normal functioning of the human body and it is mostly ingested through regular nutrition, followed by its absorption in the small intestine. However, as IBD damages parts of the gastrointestinal tract, the small intestine can be affected too, resulting in a reduced area of healthy sidewalls to absorb the FA. Reduced concentration of FA in IBD patients can increase the chance of developing colorectal cancer or megaloblastic anaemia, as well as defects in newborns.[9–11] FA deficiency is easily controlled and supplemented by taking a FA oral dosage form during regular therapy of IBD, however, elevated doses of 1–5 mg per day are mandatory compared to the regular intake of 0.4 mg per day.[12]

As simple as it sounds that taking only one additional pill during therapy can solve the problem of malabsorbed FA, therapy nonadherence is one of the biggest threats to effective treatment of not only IBD, but also a variety of diseases that are based on polytherapy.[13–15] One of the ways this problem was approached was the development of so-called fixed-dose combinations (FDCs).[16] FDC is a drug product consisting of two, or more, active pharmaceutical ingredients (API) combined in a single formulation, whether in a form of tablet, capsule, or other dosage forms. During the development of such formulation, it has to be ensured that developed FDC has the same therapeutical outcome as it would be if two drugs were taken separately. To secure the safety and efficacy of FDC, during the development the emphasis is on physicochemical stability evaluation of active ingredients when present in the physical blend.[17]

Various methods have been used so far for the investigation of drug-drug and drug-excipient compatibility when present in binary blends. Differential scanning calorimetry (DSC) is a thermal technique that offers information about possible physical changes in prepared blends.[18–20] Isothermal stress testing (IST) followed by chromatographic analysis enables monitoring of degradation rate of components present in blends while spectroscopic technique such as Fourier-transform infrared spectroscopy (FTIR) is a non-destructive method for fast screening of samples and offers information regarding physicochemical stability.[18,20,21] X-ray powder diffraction (XRPD) is another non-destructive method used to get an insight into possible changes of polymorphic forms of drugs.[22,23] Dissolution studies followed by chromatographic analysis can offer an insight into the release rate of APIs from prepared formulation as well as their stability in biologically relevant media.[20,24]

In this work, physicochemical stability of binary blends containing FA and one of the 5-aminosalicylates, BSZ or OSZ, will be examined using the above-mentioned analytical techniques. The aim of such study would open the path to the development of BSZ/FA and OSZ/FA FDCs.

EXPERIMENTAL

Reagents and Chemicals

BSZ analytical standard (> 98 %) (TCI Co., Ltd., Tokyo, Japan), OSZ European Pharmacopoeia Reference Standard (neat) (EDQM, Strasbourg, France) and FA standard (100.6 %) (meets United States Pharmacopoeia (USP) specifications) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Premid® 750 mg capsules (Almirall, S.A., Barcelona, Spain) and Dipentum® 250 mg capsules (UCB Pharma Ltd., Dunstable, United Kingdom) containing BSZ and OSZ, respectively, were used for the preparation of FDCs. For preparation of samples, buffers, mobile phase and dissolution media following chemicals were used: ultrapure water (< 0.1 μS cm^{-1}) purified with Series Ultra Clear and Integra Ultra-pure water system (SG Water, Barsbuttel, Germany), methanol HPLC grade solvent (≥ 99.9 %) (Avantor, Deventer, Netherlands), formic acid for HPLC (98 – 100 %) (Merck, Kenilworth, NJ, USA), sodium hydroxide pellets (≥ 98.0 %) (Sigma-Aldrich), di-sodium hydrogen phosphate dihydrate (≥ 99.0 %) and sodium dihydrogen phosphate dihydrate (≥ 99.0 %) (Kemika, Zagreb, Croatia).

Preparation of Stock and Working Solutions, Blends and Formulations

For adjustment and validation of chromatographic method, stock solution containing 100 μg mL^{-1} of BSZ, OSZ and FA was prepared by weighing and dissolving each standard in 1 mM NaOH followed by 1-minute sonication in an ultrasonic bath (Elmasonic XtraTT, Biosan, Riga, Latvia). Working solutions were prepared by diluting the stock solution down to 0.5 μg mL^{-1} to achieve acceptable concentration range of calibration curve for upcoming chromatographic analyses.

Blends containing BSZ and FA standards as well as those containing OSZ and FA standards were prepared in 10:1, 5:1, 2:1, 1:1, 1:2, 1:5 and 1:10 ratio (the first number represents the share of 5-ASA, second the share of FA) by accurately weighing (using MX5 microbalance scale by Mettler Toledo, Columbus, OH, USA) and mixing both standards using mortar and pestle to secure complete homogenisation.

Proposed BSZ/FA and OSZ/FA FDCs were prepared by emptying and weighing the content of 20 capsules of 5-ASA followed by the addition of weighed amount of FA and homogenisation. Capsules were refilled with prepared blends and closed. Prepared FDCs contained 750 mg of BSZ and 1.25 mg of FA or 250 mg of OSZ and 1.25 mg of FA. Homogeneity of prepared blends was examined by chromatographically analysing six samples of each blend, where relative standard deviation (RSD) values of obtained recoveries for each blend did not exceed 0.9 %.

Chromatographic Conditions

HPLC system Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) coupled with diode-array detector (DAD) was used for analysis of samples. For all chromatographic analyses and data processing, ChemStation software was used. Samples were analysed using the slightly modified version of our previously published method.[25] The linearity range was expanded for BSZ, OSZ and FA for the method to be suitable for the intended use. To confirm the suitability of the modified method, accuracy and precision tests were performed.

Differential Scanning Calorimetry (DSC)

DSC measurements were performed on Perkin-Elmer Diamond differential scanning calorimeter (Perkin Elmer, Inc., Waltham, MA, USA). Analyses were conducted on BSZ, OSZ and FA standards as well as their blends in the ratio of 10:1, 5:1, 2:1 and 1:1, in favour of 5-ASA. Sample amount of approximately 3 mg was weighed directly into a 50 µL aluminium pan and sealed with a pierced aluminium lid. The heating temperature for measurement of BSZ and its blends ranged from 25 to 250 °C whilst for OSZ and its blends temperature ranged from 25 to 360 °C. Pure nitrogen at a flow rate of 25 mL min^{-1} was used as a purging gas with a heating rate of 10 °C min^{-1}.

X-ray Powder Diffraction (XRPD)

Diffractograms of BSZ, OSZ and FA, as well as their 1:1 blend, were obtained on XRD 6000 diffractometer by Shimadzu (Kyoto, Japan) equipped with a wide-focus X-ray tube with CuKα radiation and monochromator, operating at a voltage of 40 kV and current of 30 mA in a step scan mode between 2 and 42 °2θ with steps of 0.02 °2θ and counting time of 0.6 s.

Isothermal Stress Testing (IST)

IST was performed on BSZ, OSZ and FA standards as well as on all prepared blends. Samples were spread in a thin layer on a glass dish, visually inspected and placed in an ES-20/60 Orbital Shaker-Incubator by Biosan at an elevated temperature of 50 °C for 4 weeks. After the defined period samples were removed from the heating chamber and inspected for any visual changes. Samples were prepared for chromatographic analysis by accurately weighing and dissolving 1 mg of heated sample in 10 mL of 1 mM NaOH followed by 1-minute sonication.

Fourier Transform Infrared (FTIR) Analysis

FTIR spectrums of IST stressed samples were measured using Fourier Transform Infrared Spectrophotometer FTIR-8400S by Shimadzu, equipped with PIKE MIRacle™ universal ATR by PIKE Technologies (Madison, WI, USA). Each FTIR spectra was obtained as the average of 45 scans, with the resolution of 2 cm^{-1} in a range from 500 – 4000 cm^{-1}.

For interpretation of obtained FTIR spectra XLSTAT statistical software by Addinsoft (New York, USA) was used. Principal component analysis (PCA) approach followed by cluster analysis (CA) was used on spectral region from 550 – 1800 cm^{-1} (using Pearson correlation, Euclidean distance measure and Ward's agglomerative clustering). A 9 × 625 matrix was created. The number of rows represents the used 5-ASA and FA as well as their blends, whilst spectral data was placed in columns.

In vitro Dissolution Studies

Testing of drug release from prepared BSZ and OSZ based FDCs was performed on USP 2 dissolution apparatus LDLT-A10 (Labtron Equipment Ltd., Fleet, UK) as per USP method for dissolution of BSZ Capsules.[26] Studies were performed in 900 mL of buffered dissolution media with the pH set at 6.8 (using FiveEasy pH meter by Mettler Toledo, Columbus, OH, USA) and thermostated at 37.0 °C with the constant rotation speed of 50 rpm. During the dissolution of prepared FDCs, 5 mL aliquot was drawn from the dissolution vessel every 10 minutes over 30 minutes of dissolution time, filtered through 0.22 µm PTFE syringe filter (Obrnuta Faza d.o.o., Pazin, Croatia) directly into HPLC vial and analysed.

RESULTS AND DISCUSSION

Chromatographic Method Modification

For all chromatographic measurements used in this study, our previously published in-house method was modified regarding the linearity range.[25] Linearity was broadened to cover the wider concentration range for OSZ, BSZ and FA since the initial method was developed for quality control of the prepared samples, however, this modified version will be applied for monitoring of assay in blends of different ratio. Final linearity of the method was from 0.5 – 100.0 µg mL^{-1}. As none of the chromatographic conditions such as gradient, flow rate, temperature and detection wavelengths were changed, full validation of the method was not necessary, however, verification of the method was done regarding the precision and accuracy, to confirm the applicability of new calibration curves for further calculations. Method parameters such as linearity, accuracy, precision, limit of quantification (LOQ) and limit of detection (LOD) were retested according to the ICH guideline (Table 1).[27]

High linearity was achieved across the desired range with the values of correlation coefficient (*r*) being higher than 0.9999. The lowest concentration points of calibration

Table 1. Method verification data

Analyte	Linearity range (μg mL^{-1})	Correlation equation	*r*	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
BSZ	0.5 – 100.0	$y = 31.726\,x - 1.753$	0.9999	0.14	0.5
OSZ	0.5 – 100.0	$y = 37.857\,x - 0.934$	0.9999	0.16	0.5
FA	0.5 – 100.0	$y = 30.306\,x + 0.313$	0.9999	0.17	0.5

Analyte	Precision as RSD (%)		Accuracy as recovery ± RSD (%)		
	Intra-day precision (n = 6)	Inter-day precision (n = 9)	low (n = 3)	medium (n = 3)	high (n = 3)
BSZ	1.0	1.0	100.8 ± 1.8	101.9 ± 0.9	101.0 ± 2.0
OSZ	1.3	1.2	99.7 ± 1.9	101.7 ± 1.3	98.0 ± 2.3
FA	1.1	1.0	99.9 ± 0.9	101.5 ± 1.0	98.7 ± 2.7

curves (0.5 μg mL^{-1}) were set as limits of quantification, whilst limits of detection are somewhat lower (< 0.17 μg mL^{-1}). Precision of the method was retested as intra- and inter-day precision at the concentration of 50 μg mL^{-1}. Intra-day precision was calculated as RSD of six individually prepared and analysed samples, whilst inter-day precision was expressed as RSD of three samples prepared and analysed each day, for three consecutive days. Low RSD values for both intra- (< 1.3 %) and inter-day precision (< 1.2 %) imply that the method is precise. The accuracy of the method was tested in triplicate on three concentration levels, lowest (0.5 μg mL^{-1}), medium (50.0 μg mL^{-1}) and highest (100.0 μg mL^{-1}) to cover the whole range of calibration curve. Recoveries ranged from 98.0 up to 101.9 % with the RSD (%) values lower than 2.7 %, which implies that the method and calibration curves can be used for accurate recovery of unknown concentrations.

Thermal Analysis Measurements

Application of thermoanalytical techniques such as DSC in the screening of drug-drug and drug-excipient compatibility has been commonly used in the last decade. The principle of screening is based on comparing exothermic/endothermic peaks present in the thermogram of pure compounds to those obtained by measuring the blends. Results of DSC measurements are present in Figure 1.

BSZ showed characteristic peak with the maximum at 189.5 °C which occurs due to the dehydration of BSZ dihydrate (Figure 1a). With the further heating of the sample no specific peaks were observed. To verify that BSZ has no specific melting temperature Stuart™ melting point apparatus SMP3 (Bibby Sterilin Ltd., Staffordshire, UK) was used. It was observed that BSZ started decomposing at the temperatures above 276.0 °C where the colour of BSZ gradually turned black from starting orange-yellow. Melting of the BSZ was not observed with further heating of the sample up to 360.0 °C. On the other hand, FA showed a characteristic DSC peak at 200.0 °C which corresponds to its melting point. Precise conclusions cannot be made regarding the BSZ/FA compatibility because BSZ decomposes before it melts, therefore omitting the information regarding the physical changes of BSZ itself. The fact that FA characteristic peak (198.9 – 201.9 °C) and observed BSZ peak (185.7 – 190.0 °C) did not shift notably in measured blends might indicate that there is no physical interaction between the two compounds. Any physical change in BSZ or FA would result in a shift of the observed characteristic peaks, indicating that there was a change in crystal structure of the compounds, resulting in different temperature of water release from the structure in case of BSZ or different melting temperature of FA.

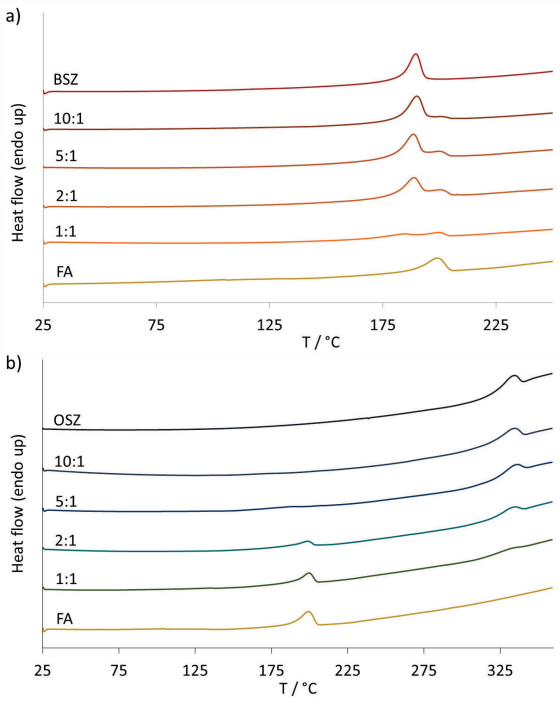

Figure 1. DSC thermograms of a) BSZ, FA and BSZ/FA blends and b) OSZ, FA and OSZ/FA blends.

OSZ showed an endothermic peak at 333.2 °C which refers to its melting point (Figure 1b). OSZ/FA blends showed peaks with little to no change in peak temperatures. OSZ peak temperatures ranged from 332.1 to 334.1 °C whilst FA peak temperatures ranged from 198.9 up to 199.2 °C, showing minor changes in peak temperatures. The broad FA peak is not observed in 5:1 and 10:1 blend, which is mainly due to the low sensitivity of the DSC instrument.

Results presented above could imply that FA is compatible with both BSZ and OSZ, although reaching conclusions solely on results obtained from DSC measurements could mislead the further development process. DSC in combination with other techniques such as XRPD and IST followed by FTIR and chromatographic analysis creates a wider picture where more concise and backed up conclusions can be made.

XRPD results

XRPD, a non-destructive, non-thermal analytical technique, plays an important role in the drug development process in the preformulation stage for characterisation of crystalline nature of materials used for blend preparation as well as for determining polymorphic nature of APIs. Polymorphic form of API highly impacts its physical properties, therefore it is mandatory to secure their polymorphic stability. APIs that come in crystalline form exhibit unique X-ray diffraction patterns, described with diffraction angles (2θ) and intensities of observed peaks. XRPD is a common technique for determining the possible drug-drug and drug-excipients interactions.[28,29] Any physical interactions between the two APIs or API and excipient would result in the change of their characteristic X-ray diffractograms, whether in the form of peak shifting, occurrence of new peaks, or disappearance of existing peaks. Obtained diffractograms show high-intensity sharp peaks characteristic for crystalline APIs (Figure 2).

BSZ shows high-intensity peaks at 2θ angles of 4.60, 12.96, 15.14, 18.14, 26.32 and 26.98° whilst FA showed characteristic peaks at 10.74, 12,98, 26.66 and 27.72°. OSZ has shown characteristic peaks at the 2θ angle of 6.48, 13.00, 16.56 and 26.56°. X-ray diffractograms of both BSZ/FA and OSZ/FA blends showed all characteristic peaks with no shift in 2θ values. Moreover, no new peaks were observed with no occurrence of so-called 'halo', which would indicate the presence of amorphous phase. Pearson's correlation was calculated between obtained diffractogram of blend and theoretical diffractogram (calculated as the average of two spectra of pure compounds). Obtained correlation coefficients (*r*) for BSZ/FA and OSZ/FA blends are 0.99 and 0.98, respectively. High correlation value between observed and theoretical spectra indicates lack of component interaction.

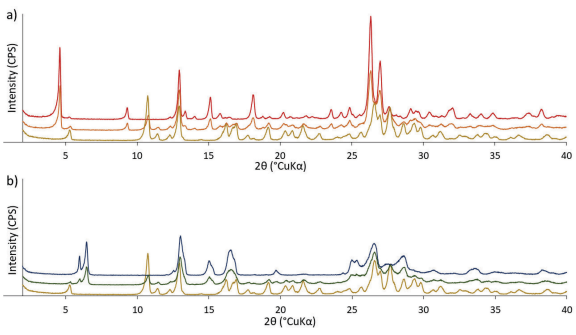

Figure 2. XRPD spectrograms of a) BSZ (red), BSZ/FA (orange) and FA (yellow) and b) OSZ (blue), OSZ/FA (green) and FA (yellow).

Obtained results imply that there were no chemical interactions or even changes in the polymorphic forms of both BSZ and OSZ, as well as in the polymorphic form of FA, although they all exhibit polymorphism [30–32], which confirms our assumptions based on DSC screening.

IST followed by chromatographic analysis

Long term exposure of samples to elevated temperature greatly increases the kinetics of potential reactions and therefore emphasizes the possible drug-drug incompatibility. The best approach to get an insight into the chemical changes of the samples after IST is their quantitative analysis where notable drops of assays imply that components of the binary mixtures decomposed or chemically reacted. Recoveries of OSZ, BSZ, FA, and all their blends are present in Table 2.

Recoveries of all analysed samples do not show a trend in assay changes depending on the share of FA or one of the 5-ASA in the blend. Recoveries of BSZ from BSZ/FA blends are in the range from 98.2 up to 101.3 % with the RSD (%) values lower than 2.4 %, whilst recoveries of FA from those same blends were from 98.3 to 102.5 % with the RSD (%) values not exceeding 2.2 %. Also, by visually inspecting the obtained chromatograms, no new unknown peaks emerged. Recoveries of OSZ from its blends are in the range from 98.3 to 103.7 % with the highest RSD (%) value of 1.0 %. Likewise, recovery of FA from OSZ/FA blends ranged from 97.9 to 103.0 % with the RSD (%) values lower than 1.7 % showing that no chemical interaction between OSZ and FA occurred. Chromatograms obtained from all analyses showed no new unknown peaks, implying that there was no formation of new products when BSZ or OSZ and FA are present in the same blend, which was confirmed with the peak purity analysis (> 998). Considering the absence of new peaks and recoveries within the blends it can be said that there was no chemical interaction between investigated 5-ASAs and FA.

Table 2. Chromatographic analysis results of samples conducted to IST

Sample	5-ASA:FA	Recoveries of 5-ASA (mean ± RSD %)	Recoveries of FA (mean ± RSD %)
API	BSZ	99.4 ± 1.9	/
	OSZ	99.9 ± 1.6	/
	FA	/	98.8 ± 0.6
BSZ/FA blends	10:1	99.6 ± 0.6	99.2 ± 0.4
	5:1	98.4 ± 2.7	102.5 ± 2.2
	2:1	98.8 ± 1.0	98.3 ± 1.6
	1:1	98.2 ± 2.4	98.4 ± 1.4
	1:2	100.6 ± 1.6	98.6 ± 2.0
	1:5	99.2 ± 1.0	100.0 ± 1.7
	1:10	101.3 ± 0.7	98.9 ± 0.8
OSZ/FA blends	10:1	99.8 ± 1.2	97.9 ± 1.7
	5:1	99.2 ± 0.2	103.7 ± 2.4
	2:1	99.1 ± 2.0	103.0 ± 0.1
	1:1	99.9 ± 2.4	99.7 ± 3.6
	1:2	98.3 ± 1.0	101.6 ± 1.9
	1:5	103.7 ± 0.6	99.6 ± 1.9
	1:10	102.6 ± 0.4	98.7 ± 1.7

IST Followed by FTIR and Statistical Analysis

FTIR is a commonly used method for the investigation of possible physicochemical interactions between two compounds, based on measuring the FTIR spectra of pure compounds as well as their blends, followed by the comparison of obtained spectra. Any changes in the FTIR spectra of measured blends, compared to spectra of pure substances, such as disappearance of characteristic peaks or occurrence of new peaks would indicate that there was a change in the molecules due to their chemical reaction and creation of new products. FTIR spectra obtained from measurements of pure substances and their blends are shown in Figure 3.

BSZ shows characteristic absorption bands at 1639, 1574 and 1484 cm^{-1} characteristic for C=O, C=C and N=N azo bond, respectively, whilst OSZ shows a wide absorption band at 1583 cm^{-1} characteristic for aromatic C=C bond as well as sharp peak at 1482 cm^{-1} which is related to the present azo bond. FA absorption bands characteristic for C=O and amide N-H bonds are observed at 1690 and 1603 cm^{-1} whilst band at 1482 cm^{-1} is present due to the pterin ring.[33] Characteristic peaks of BSZ, OSZ and FA can be observed in the prepared blends, with no shifting, disappearance, or occurrence of new peaks.

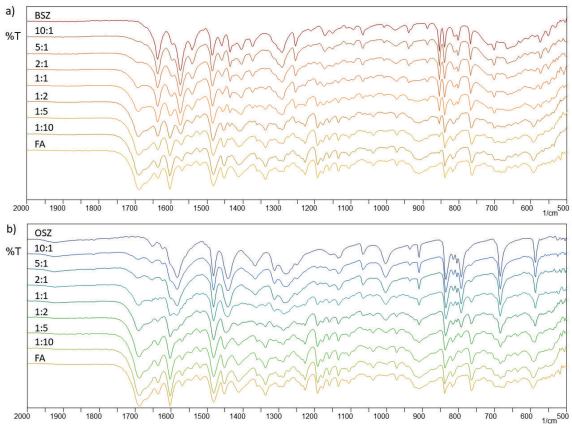

Figure 3. FTIR spectra of a) BSZ, FA and their blends, and b) OSZ, FA and their blends.

Although BSZ, OSZ and FA are not considered large molecules, their complex FTIR spectra are hard to interpret by visual analysis, especially in the case of prepared blends. The interpretation of spectra of physical mixtures may lead to false conclusions due to the overlapping of bands present at similar wavenumbers. Therefore, without going into deeper visual analysis of obtained spectra whereas said above we cannot arrive at an unambiguous conclusion, we turned to a statistical approach with the aim of better interpretation of obtained spectra. Multivariate statistical analysis tools, such as PCA and CA, were already used and are well described in the literature for the interpretation of obtained data.[21,34] The results of PCA and CA calculations are shown in Figure 4.

In the case of BSZ, FA and their blends, the first principal component (PC1) explained 89.63 % of data

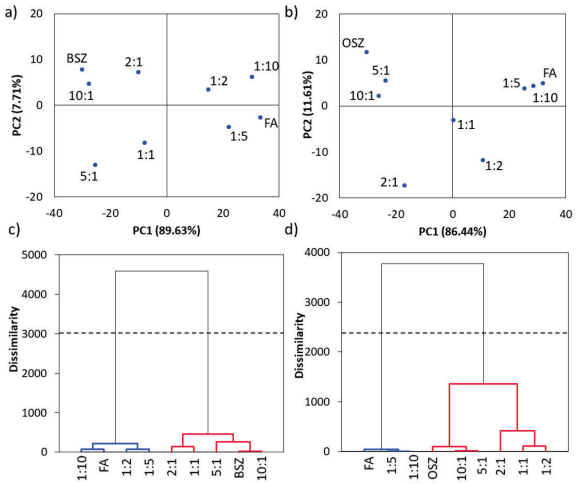

Figure 4. PCA results of a) BSZ, FA and their blends and b) OSZ, FA and their blends as well as accompanying CA results of c) BSZ and d) OSZ based samples.

variability, whilst the second (PC2) explained only 7.71 % variability. As it can be seen in Figure 4a, BSZ is placed on the left end with the most negative PC1 value (-30.12), whilst FA with the most positive value (33.47) is placed on the right end of the plot, both representing the values of pure compounds. BSZ/FA blends are positioned in between, where blends with the greater BSZ share are positioned closer to the pure BSZ, whilst blends with a greater share of FA are placed closer to FA. All blends are positioned in the plot in the increasing PC1 values as the BSZ share reduces and FA share in blends increases, with the 1:1 being placed between measured blends. Dendrogram present in Figure 4c reflects the results of the PCA in the form of clusters. Two major clusters were formed with low dissimilarity values within each cluster. First cluster (blue) shows the similarity between FA and blends where FA is in a greater share, whilst the second cluster (red) shows the similarity between BSZ and blends with a greater BSZ share. BSZ/FA 1:1 blend is placed in the cluster with BSZ because of the dominance of its high-intensity peaks in obtained spectra.

In the case of OSZ, similar results were obtained, where PC1 explains 86.44 % data variability whilst PC2 explains 11.61 %. As in the case of BSZ, OSZ is placed far to the left with the most negative PC1 value (–30.27) and FA is placed on the opposite side with the most positive PC1 value (31.96) (Figure 4b). All blends are placed between OSZ and FA in the increasing PC1 value as the OSZ share in blends reduces and FA share in blends increases. Dendrogram obtained with CA (Figure 4d) shows 3 clusters with strong linkages. First (blue) consisting of FA and blends with the high FA content, second (red) consists of OSZ and its high content blends whilst the third (red) cluster consists of 2:1, 1:1 and 1:2 blends. The first two clusters reflect the similarity between pure compounds and their dominant blends, where the third cluster shows the similarity between 1:1 blend and blends with slightly higher content of either OSZ or FA. The formation of this cluster is a result of similar absorption bands present in both OSZ and FA spectra at around 1600 cm^{-1} and in the ranges of 1500 – 1400 cm^{-1} and 850 – 550 cm^{-1}.

Placement of pure compounds and their blends in the PCA plot, as well as the formation of clusters in CA dendrogram, are in favour of BSZ/FA and OSZ/FA compatibility. Any deviation from the obtained order in which blends are positioned would indicate incompatibility between compounds, as well as the formation of clusters with random blends. The absence of new peaks in the obtained FTIR spectra indicate that there was no chemical interaction between compounds as well as there was no disappearance of peaks present in the spectra of pure compounds.

Drug Dissolution Testing

In vitro dissolution study is often used to display the successful release of the API from its final formulation. In dissolution testing of FDCs, the release of both APIs is monitored during the defined period to ensure that the desired amount of both APIs is dissolved. In addition to the monitoring of drug release, dissolution studies are also used for testing the stability of two APIs when present in the same solution of the pH similar to those in the gastrointestinal tract.[20,24] Although there is no official method published for dissolution testing of OSZ capsules, BSZ official USP method was applied for both tests. Based on the fact that both BSZ and OSZ have similar logk_w values (3.01 and 3.25, respectively),[25] similar solubility was expected considering the pH of the used dissolution media (6.8), also, both drugs come in the same dosage forms without modified release and the same excipients present in the formulation. Also, sink conditions are achieved with the defined volume of the dissolution media, considering the high olsalazine solubility in neutral media.[35]

Results obtained from the dissolution of twelve prepared BSZ/FA and OSZ/FA FDCs are presented in Figure 5. As per criteria for BSZ Capsules stated in the USP, more than 80 % of the API has to be released from the formulation after the 30-minute test time. As it can be seen

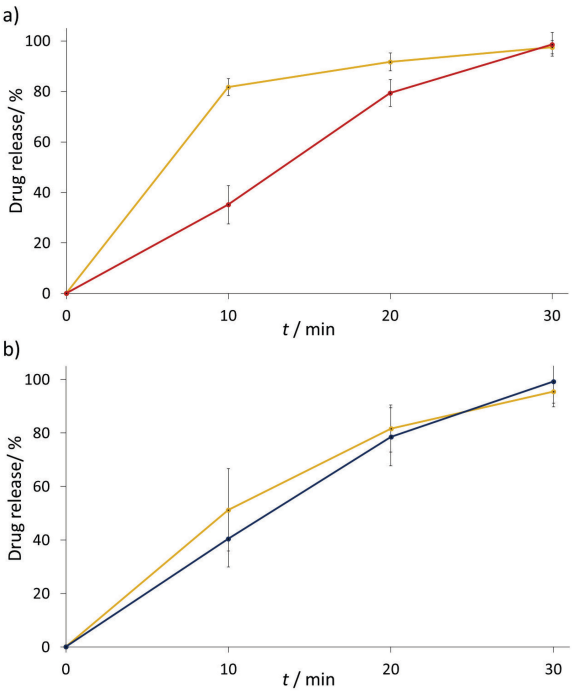

Figure 5. Drug release profiles of a) BSZ (red line) and FA (yellow line) and b) OSZ (blue line) and FA from prepared FDCs (n = 12) with the accompanied standard deviation ranges.

from the results all drug releases are in the range from 91.9 up to 102.0 %. FA was as well released almost in total after 30 minutes with the recoveries ranging from 94.9 up to 101.6 %. A in the case of OSZ/FA FDCs, OSZ was released in the range from 89.6 up to 102.8 % whilst FA was released from 90.9 up to 103.4 %. Faster release of FA from BSZ/FA FDC is due to the greater solubility of BSZ itself compared to the solubility of OSZ as well as the fact that BSZ finished product used for preparation of FDCs contains colloidal anhydrous silica as excipient as well, which serves as an anti-caking agent, enabling better solubility of both compounds.

These results indicate that FA can be in the same formulation containing BSZ or OSZ. Considering that BSZ and OSZ capsules contain anhydrous silica and magnesium stearate as excipients, which are commonly used for the preparation of FA finished products, FA stability in their presence is not compromised. The results obtained from dissolution studies imply that FDCs containing FA and BSZ or OSZ are stable, and the liberation of both drugs is satisfactory, although to confirm such conclusions further *in vivo* studies should be performed.

CONCLUSIONS

This research was focused on investigating the physicochemical compatibility between FA and two 5-ASAs, OSZ and BSZ. Various analytical techniques were used for mentioned purpose, from thermal such as DSC and IST, to spectroscopic such as FTIR and XRPD as well as chromatographic analysis. Although DSC measurements provided ambiguous results, those obtained with other techniques showed that 5-ASA and FA are physiochemically compatible with no interferences between them. This work opens the path to development of BSZ/FA and OSZ/FA FDCs with the aim of reducing the number of drugs IBD patients need to take during therapy, however further studies are mandatory in regard of developing final formulation as well as bioavailability, safety and efficacy of such product.

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6. GENERALNA RASPRAVA

Uzimajući u obzir nezadovoljavajuću adherenciju pacijenata koji boluju od upalnih bolesti crijeva te povećani rizik od razvoja drugih bolesti, kao posljedica nedostatka folne kiseline, u ovom istraživanju razvijene su analitičke metode za ispitivanje kompatibilnosti 5-aminosalicilata i folne kiseline kao sastavni dio razvoja njihovih fiksnih kombinacija. Predložene fiksne kombinacije mesalazin/folna kiselina, sulfasalazin/folna kiselina, olsalazin/folna kiselina te balsalazid/folna kiselina, sadržavale bi 5-aminosalicilat i folnu kiselinu u omjeru koji bi kroz redovnu terapiju osigurao nadomjestak malapsorbiranih folata te spriječio relaps bolesti. Predložene fiksne kombinacije ne bi se koristile u fazi liječenja akutnih epizoda jer režim doziranja u toj fazi ovisi o težini slučaja. S obzirom na činjenicu da je razvoj takvih proizvoda složen, dugotrajan i financijski zahtjevan proces, ovo istraživanje usmjereno je na ispitivanje fizikalno-kemijske kompatibilnosti folne kiseline i 5-aminosalicilata, korištenjem termalnih, spektroskopskih i kromatografskih tehnika. Kao preliminarno istraživanje, provedeno je ispitivanje terapijske kompatibilnosti korištenjem biomimetičke kromatografije kako bi se ispitalo potencijalno kompetitivno vezanje ispitivanih lijekova za ista vezna mjesta na proteinima plazme, što nam daje informaciju o međusobnom utjecaju na njihovu farmakokinetiku.

6.1. Ispitivanje kompetitivnog vezanja 5-aminosalicilata i folne kiseline primjenom biomimetičke kromatografije

Ispitivanje potencijalnog kompetitivnog vezanja između folne kiseline i 5-aminosalicilata provelo se korištenjem biomimetičke kromatografije (engl. *Biomimetic Chromatography*), odnosno studijama frontalne analize (engl. *Frontal Analysis*) i zonske eluacije (engl. *Zonal Elution*). Ovaj pristup omogućuje *in-vitro* uvid u obim vezanja lijekova za proteine plazme (engl. *Plasma Protein Binding*, PPB), odnosno za humani serumski albumin (HSA) i α-kiseli glikoprotein (AGP), kao najzastupljenije proteine u krvnoj plazmi (HSA 35–50 g/L i AGP 0,6–1,2 g/L) te ispitivanje kompetitivnog vezanja različitih lijekova za ista vezna mjesta na tim proteinima kao i moguće alosteričke modulacije proteina (132, 133). Izračunati obimi vezanja 5-aminosalicilata i folne kiseline za ispitivane proteine pokazali su kako se prolijekovi mesalazina, pogotovo sulfasalazin i olsalazin u velikom obimu vežu za oba proteina plazme ($PPB_{HSA,\ SASP}$ = 93,6 %, $PPB_{HSA,\ OSZ}$ = 96,0 % i $PPB_{AGP,\ SASP}$ = 96,4 %, $PPB_{AGP,\ OSZ}$ = 90,3 %) dok su mesalazin i balsalazid te folna kiselina pokazali veći afinitet za HSA protein ($PPB_{HSA,\ MSZ}$ = 61,4 %, $PPB_{HSA,\ BSZ}$ = 91,6 % i $PPB_{HSA,\ FA}$ = 69,4 %) u odnosu na AGP ($PPB_{AGP,\ MSZ}$ = 6,22 %, $PPB_{AGP,\ BSZ}$ = 77,6 % i $PPB_{AGP,\ FA}$ = 3,45 %) (**Rad 1**, Tablica 1). Vrijednosti PPB-a dobivene ovom tehnikom pokazuju podudaranje s vrijednostima dostupnim u literaturi i

sažetcima opisa svojstava lijekova (engl. *Summary of Product Characteristics*, SPC) objavljenim od strane regulatornih agencija; mesalazin 43–74 % (134–137), sulfasalazin > 99,3 % (138), olsalazin >99 % (137), balsalazid >99 % (139) i folna kiselina 50–70 % (140, 141). Afinitet folne kiseline, ali i 5-aminosalicilata za vezanje na isti protein plazme (HSA) otvara mogućnost kompetitivnog vezanja za ista vezna mjesta na HSA proteinu, što u konačnici može utjecati na farmakokinetiku ispitivanih spjeva (142, 143). Primjenom frontalne analize utvrđeno je kako se folna kiselina veže na jedno vezno mjesto na HSA proteinu uz konstantu vezanja od 1,64 (±0,03) $\times 10^4$ M^{-1}. Konstanta vezanja folne kiseline za HSA protein prvi je puta izmjerena ovom tehnikom te je u skladu s vrijednostima u literaturi dobivenim korištenjem drugih tehnika (0,53 – 9,70 $\times 10^4$ M^{-1}) (144–148). Moguća kompeticija za isto vezno mjesto ispitano je korištenjem zonske eluacije te je utvrđeno da nema razlike u vezanju 5-aminosalicilata za proteine plazme u prisutnosti folne kiseline što potvrđuje činjenica da se folna kiselina veže na mjesto koje se nalazi u Domeni I na HSA proteinu, dok se sulfasalazin veže na domenu IIb, a balsalazid i mesalazin na domenu IIIa (149–151). Dobiveni rezultati upućuju na izostanak farmakokinetičke interakcije između ispitivanih spojeva, međutim potrebno je provesti *in-vivo* studije kako bi se potvrdila navedena pretpostavka.

6.2. Ispitivanje fizikalno-kemijske kompatibilnosti 5-aminosalicilata i folne kiseline

6.2.1. HPLC metode za određivanje sadržaja i onečišćenja 5-aminosalicilata i folne kiseline

Studije fizikalno-kemijske kompatibilnosti provedene su na pripremljenim smjesama standarada i smjesama usitnjenih gotovih dozirnih oblika 5-aminosalicilata i folne kiseline. U svrhu usporedbe i uočavanja potencijalnih interakcija u pripremljenim smjesama sve studije su provedene i na čistim sastavnicama. Za potrebe kromatografskih analiza razvijene su tri nove HPLC metode koje su validirane prema smjernicama definiranim u ICH Q2 (R1) (152). Iako postoji niz razvijenih metoda za individualno određivanje sadržaja 5-aminosalicilata i folne kiseline, korištenjem raznih analitičkih tehnika (153, 154, 163–166, 155–162), razvijena je nova HPLC metoda za simultano određivanje sva četiri 5-aminosalicilata i folne kiseline (**Rad 1**). Također, razvijena je metoda u svrhu simultanog praćenja sadržaja folne kiseline te mesalazina i njegovih devet onečišćenja (**Rad 2**) kao i metoda za simultano praćenje sadržaja folne kiseline te sulfasalazina i njegova dva onečišćenja (**Rad 3**). Iako postoje razvijene metode za određivanje 5-aminsalicilata i njihovih onečišćenja, ovo su prve razvijene za simultano praćenje i sadržaja folne kiseline (167–169).

6.2.2. Primjena termalnih tehnika – DSC i TG/DTA

Primjenom termalnih tehnika, DSC te simultane TG/DTA ispitane su potencijalne interakcije između sastavnica smjesa. Interakcije između folne kiseline i mesalazina, olsalazina te balsalazida ispitane su DSC tehnikom. Iako nema službeno definiranih kriterija koji definiraju koliki pomak upućuje na nekompatibilnost, pomak karakterističnog pika od više od 5,0 °C se smatra kao značajna promjena koja ukazuje na potencijalnu interakciju sastavnica (94). U termogramima smjesa mesalazina i folne kiseline došlo je do značajnog pomaka u temperaturi karakterističnih pikova tališta iako su svi pikovi zadržali svoj oblik. Pik mesalazina (287,6 °C) u smjesama 5:1 i 1:1 pokazao je pomak od 13,8 °C te 21,0 °C prema nižim temperaturama dok se pik folne kiseline (199,7 °C) također pomaknuo prema nižim temperaturama u pripremljenim smjesama za 22,6 °C i 5,2 °C, pri čemu je uočeno kako je pomak pika pojedine sastavnice značajniji što je udio druge sastavnice u smjesi veći (**Rad 2**, Slika 2).

U pripremljenim smjesama olsalazina i folne kiseline (10:1, 5:1, 2:1 te 1:1) nije uočen značajan pomak pikova njihovih tališta niti promjena oblika. Pomak pika olsalazina (333,2 °C) od 1,1 °C zabilježen je u smjesi u kojoj je udio folne kiseline najveći (1:1) dok u je slučaju folne kiseline (199,3 °C) najveći zabilježeni pomak iznosio 0,3 °C u smjesi 2:1. U smjesama 5:1 i 10:1 pik folne kiseline nije bilo moguće opisati zbog njezinog niskog udjela u smjesama te niske osjetljivosti samog instrumenta (**Rad 4**, Slika 1b).

U odnosu na druge ispitivane spojeve balsalazid nije pokazivao karakterističan pik tališta, što je i provjereno korištenjem aparature za određivanje tališta, pri čemu nije uočeno taljenje spoja u rasponu od 25,0 do 360,0 °C nego samo promjena boje iz narančasto-žute u crnu pri temperaturama višim od 276,0 °C kao posljedica termalnog raspada. Iako nema karakterističnog pika tališta na termogramu je uočen pik dehidracije spoja (189,5 °C) s obzirom da je ispitivani standard dihidrat. U slučaju izostanka pika tališta, nekompatibilnost spojeva može se ispitivati praćenjem drugih karakterističnih pikova uz istu pretpostavku da njihov pomak indicira interakcije između sastavnica smjesa (170). U pripremljenim smjesama (10:1, 5:1, 2:1 te 1:1) najveći pomak pika dehidracije balsalazida iznosio je 4.3 °C, dok se pik tališta folne kiseline (199,1 °C) nije značajno pomaknuo (<3.0 °C) (**Rad 4**, Slika 1a).

Zbog izrazito endotermnog taljenja sulfasalazina, praćenog egzotermnim raspadom (171, 172), bilo je potrebno smanjiti količinu uzorka koji se mjerio kako bi se izbjeglo potencijalno oštećenje DSC instrumenta, čime se i utjecalo na intenzitet pika folne kiseline koji je bio slabo vidljiv. S ciljem mjerenja veće količine uzorka, smjesa sulfasalazina i folne kiseline u omjeru 1:1 mjerena je na robusnijem TG/DTA uređaju. Pik tališta sulfasalazina (259,0 °C) pokazao je

pomak od 9,6 °C dok pik folne kiseline (199,0 °C) nije pokazao značajan pomak (1,6 °C) (**Rad 3**, Slika 3). TG mjerenja nisu pokazala znatnu razliku između krivulja čistih sastavnica i smjese. Uočeni su svi karakteristični padovi masa, pri čemu niti jedan pad mase nije bio izraženiji u smjesi u odnosu na padove masa mjerenih čistih sastavnica (**Rad 3**, Slika 3).

Korištenjem termalnih tehnika zabilježene su promjene u termogramima smjesa mesalazina i folne kiseline te sulfasalazina i folne kiseline, što ukazuje na potencijalne interakcije, dok u slučaju olsalazina i balsalazida nisu uočene značajne promjene. Termalne tehnike jednostavne su i brze, međutim, zbog zagrijavanja uzoraka na visoke temperature, često mogu ukazati na interakcije koje se u stvarnim uvjetima ne događaju, stoga zaključke donesene na temelju termoanalitičkih mjerenja treba potvrditi korištenjem drugih komplementarnih tehnika (93, 173, 174).

6.2.3. Studije prisilne razgradnje

Potencijalne interakcije, utvrđene termalnim tehnikama, između folne kiselina i sulfasalazina te mesalazina dodatno su ispitane primjenom prisilne razgradnje. U sklopu studije prisilne razgradnje uzorci (standardi, smjese standarada te smjese usitnjenih gotovih dozirnih oblika) su razgrađivani u kiselim (0,1 M HCl), lužnatim (0,1 M NaOH) i oksidativnim uvjetima (3 % H_2O_2) te se ispitivala termalna stabilnost i fotostabilnost na otopinama i krutinama. Smjese su pripremljene u omjeru 500:1 u korist 5-aminosalicilata. Taj omjer predstavlja omjer u kojem bi bile pripremljene predložene fiksne kombinacije.

Mesalazin je pokazao nestabilnost, odnosno pad sadržaja u lužnatim (9,4 % nakon jednog sata) i oksidativnim (9,1 % nakon jednog sata) uvjetima te termalnu nestabilnost (15,4 % nakon 18 sati) prilikom prisilne razgradnje otopina, dok se folna kiselina pokazala nestabilnom u kiselim uvjetima (10,6 % nakon pola sata) te fotonestabilna (18,3 % nakon 4 sata), što je u skladu s literaturnim zapisima (168, 175–177). Smjese standarada i gotovih dozirnih oblika izložene prisilnoj razgradnji nisu pokazale veću nestabilnost u odnosu na zasebno izložene sastavnice, osim folne kiseline koja se pokazala stabilnijom u otopini s mesalazinom (do 4,6 % razgradnje u 12 satnom razdoblju u odnosu na 18,3 % u četverosatnom razdoblju) što je posljedica veće apsorpcije zračenja od strane mesalazina zbog njegove znatno veće koncentracije u otopini (**Rad 2**, Tablica 3). Analizom razgradnih profila nije uočeno nastajanje novih razgradnih produkata prilikom prisilne razgradnje smjesa niti povećanje pikova razgradnih produkata mesalazina, dok je vrijednost čistoće svih pikova bila iznad 998.

Sulfasalazin se pokazao stabilan u svim razgradnim uvjetima kroz sedam dana pri čemu je maksimalna razgradnja od 1,9 % uočena prilikom termalne razgradnje dok je folna kiselina

pokazala stabilnost u lužnatim i termalnim uvjetima (2,7 i 1,1 % nakon 7 dana) te se pokazala fotostabilnom u krutom obliku (2,9 % nakon 7 dana) (**Rad 3**, Tablica 4) (167). Izuzetno visoka stabilnost sulfasalazina posljedica je para-položaja diazo skupine u odnosu na sulfonilnu skupinu. Ovakvo para usmjerenje dviju skupina omogućuje delokalizaciju elektrona i štiti sumpor iz sulfonilne skupine od nukleofilnog napada (178). U razoblju od jednog dana uočena je razgradnja folne kiseline u kiselim (21,6 %) i oksidativnim (11,7 %) uvjetima te prilikom izlaganja svjetlu (10,0 %). Prisilne razgranje smjese standarada i gotovih dozirnih oblika nisu pokazale drugačije obime razgradnje osim prilikom termalnog izlaganja, gdje je došlo do 100 % razgradnje folne kiseline nakon jednog dana, u usporedbi s 1,1 % razgradnje folne kiseline u sedmodnevnom razdoblju kada je termalno stresirana sama. Ovaj se fenomen može opravdati činjenicom da je izmjerena pH vrijednost pripremljene otopine sulfasalazina i folne kiseline 2,85 (u usporedbi s čistim otapalom čiji je pH 6,35), pri čemu je sulfasalazin odgovoran za stvaranje kiselih uvjeta u kojima se folna kiselina raspada, a samo zagrijavanje uzorka značajno povećava kinetiku reakcije, što u konačnici dovodi do potpune razgradnje folne kiseline u kratkom vremenskom razdoblju. Na temelju ovih spoznaja, može se pretpostaviti da je razgradnja folne kiseline strogo povezana s pH otopine i temperaturom, a ne rezultat kemijske interakcije sa sulfasalazinom. Utjecaj pH na nestabilnost folne kiseline kao i utjecaj pH u kombinaciji s povišenim temperaturama dobro je opisan u literaturi (179–182). Međutim, s obzirom na to da sulfasalazin snižava pH otopine, to se mora uzeti u obzir tijekom postupka razvoja i pripreme formulacije i dozirnog oblika. U izmjerenim kromatogramima nije uočeno nastajanje novih razgradnih produkata uz zadovoljavajuću čistoću svih pikova (>998) što u kombinaciji s dobivenim sličnim obimima razgradnje skreće pozornost na izostanak interakcija između sulfasalazina i folne kiseline.

6.2.4. Studije izotermalne razgradnje

Izotermalne studije razgradnje provedene su na čistim sastavnicama sulfasalazina, mesalazina i folne kiseline te na smjesama standarada i gotovih dozirnih oblika pripremljenim u omjeru 500:1. Svi uzorci su vizualno pregledani prije i nakon skladištenja u izotermalnim uvjetima te nisu uočene promjena u izgledu uzoraka. Analitički povrati dobiveni kromatografskom analizom uzoraka mesalazina (engl. *Recovery*) kreću se u rasponu od 98,8 % do 103,6 % s maksimalnih 2,2 % relativne standardne devijacije (engl. *Relative Standard Deviation*), dok se povrati folne kiseline kreću od 99,5 % do 102,0 % s maksimalnih 1.9 % relativne standardne devijacije (**Rad 2**, Tablica 4). U slučaju sulfasalazina i njegovih smjesa povrat je bio u rasponu od 98,4 % do 102,5 % s maksimalnom relativnom standardnom

devijacijom od 2,1 % dok su povrati folne kiseline bili u rasponu od 98,4 % do 101,6 % s maksimalnom relativnom standardnom devijacijom od 2,7 % (**Rad 3**, Tablica 3). Ovi rezultati pokazuju da nije bilo kemijske reakcije između mesalazina i folne kiseline kao i sulfasalazina i folne kiseline te uzimajući u obzir omjer u kojem su smjese pripremljene (500:1) svaka interakcija bi dovela do značajnog pada sadržaja folne kiseline.

Za potrebe izotermalne razgradnje balsalazida i olsalazina pripremljene su smjese u omjeru 1:1 te omjerima 10:1, 5:1, 2:1 u korist i 5-aminosaliclata i folne kiseline. Kromatografskom analizom smjesa balsalazida i folne kiseline nakon izotermalne razgradnje analitički povrati bili su u rasponu od 98,2 % do 102,5 % s maksimalnom relativnom standardnom devijacijom od 2,7 %, dok u slučaju olsalazina i folne kiseline povrati su bili u rasponu od 97,9 % do 103,7 % uz relativnu standardnu devijaciju ne veću od 3,6 % (**Rad 4**, Tablica 2). Ovakvi rezultati također upućuju na izostanak kemijske interakcije između 5-aminosalicilata i folne kiseline, uzimajući u obzir dužinu trajanja primijenjenog izotermalnog stresa te primijenjenu temperaturu (4 tjedna na 50,0 °C).

6.2.5. Primjena infracrvene spektroskopije - FTIR

Nakon kromatografske analize uzoraka izloženih izotermalnoj razgradnji slijedila je analiza infracrvenom spektroskopijom standarada sulfasalazina i folne kiseline te njihovih smjesa (**Rad 3**, Slika 4) kao i olsalazina i balsalazida i njihovih smjesa s folnom kiselinom (**Rad 4**, Slika 3a i 3b). Zbog preklapanja karakterističnih vrpci 5-aminosalicilata i folne kiseline za interpretaciju FTIR spektara primijenjena je statistička analiza. Rezultat analize glavnih komponenata su eigenvektori koji opisuju varijabilnost dobivenih podataka, pri čemu je eigenvektor koji ima najveću varijabilnost podataka glavna komponenta analize (183). U slučaju analiziranih uzoraka, čiste sastavnice su zauzele krajnje vrijednosti, odnosno najpozitivniju (folna kiselina) i najnegativniju (5-aminosalicilati), te one predstavljaju FTIR spektre čistih sastavnica. Dobivene vrijednosti FTIR spektara analiziranih smjesa smještene su između vrijednosti čistih sastavnica s time da vrijednost najbližu vrijednosti čiste sastavnice imaju smjese 10:1 ili 1:10, odnosno one koje imaju najveći udio te iste čiste sastavnice, dok se smjesa 1:1 nalazi u sredini a ostale smjese su poredane prema udjelu sastavnica (5-aminosalicilat, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10 te folna kiselina) (**Rad 3**, Slika 5a i **Rad 4**, Slika 4a i 4b). Takav raspored smjesa potvrđuje sličnost između FTIR spektara susjednih smjesa, dok bi nasumičan razmještaj ukazao na promjene u spektrima, odnosno na prisutnost novih kemijskih veza kao posljedica kemijske interakcije sastavnica. Analizu glavnih komponenata prati klaster analiza koja prikazuje jačinu sličnosti, odnosno različitosti, između

dobivenih spektara. Klaster analiza pokazala je formiranje dva glavna klastera, jedan koji obuhvaća 5-aminosalicilat i smjese s njegovim većim udjelom te drugi koji sadrži folnu kiselinu i smjese s njezinim većim udjelom dok su podklasteri formirani povezivanjem najsličnijih uzoraka (čista sastavnica i 10:1, 5:1 i 2:1, itd.). U svim slučajevima smjesa 1:1 je pridružena klasteru 5-aminsalicalata, što je posljedica prisutnosti intenzivnih apsorpcijskih vrpci koje preklapaju vrpce karakteristične za folnu kiselinu (**Rad 3**, Slika 5b), dok je iz istog razloga, u slučaju olsalazina, smjesa 1:2 (smjesa s većim udjelom folne kiseline), pridružena klasteru koji sadrži smjese u omjerima 2:1, 1:1 te 1:2, ukazujući na sličnost između smjese 1:1 te smjesa s malo većim udjelom pojedine sastavnice (**Rad 4**, Slika 4d). Odsutnost kemijske interakcije između sastavnica pripremljenih smjesa vidljiva je iz kromatografske analize uzoraka nakon izotermalnog stresiranja, što je i potvrđeno rezultatima dobivenih korištenjem infracrvene spektrometrije.

6.2.6. Primjena rendgenske difrakcije - XRPD

Za dodatnu provjeru kompatibilnosti između folne kiseline te olsalazina i balsalazida primijenjena je tehnika rendgenske difrakcije na prahu. Difraktogrami čistih sastavnica pokazali su intenzivne pikove karakteristične za kristalinične tvari. Difraktogrami smjesa pripremljenih u 1:1 omjeru pokazali su sve karakteristične pikove bez pomaka u vrijednosti difrakcijskog kuta (2θ) (**Rad 4**, Slika 2) te nisu primijećeni novonastali pikovi niti pojava takozvanog "halo" efekta, što upućuje na pojavu amorfne faze (184). Kako bi potvrdili sličnost difraktograma čistih sastavnica i smjesa izračunata je Pearsonova korelacija između eksperimentalno dobivenog difraktograma smjese i teoretskog difraktograma, koji je izračunat kao prosjek dva spektra čistih sastavnica (npr. balsalazida i folne kiseline). Dobiveni koeficijent korelacije (r) u slučaju balsalazida i folne kiseline iznosi 0,99 a u slučaju olsalazina i folne kiseline 0,98. Dobivene vrijednosti koeficijenta korelacije između eksperimentalnog i teoretskog difraktograma upućuju na njihovu sličnost što potvrđuje odsutnost kemijskih interakcija između sastavnica smjesa i promjenu njihovih polimorfnih oblika, iako su za sve njih u literaturi nađeni/opisani različiti kristalni oblici posjeduju (185–187).

6.2.7. Studije stabilnosti u biološki relevantnom mediju

Studije stabilnosti u biološki relevantnom mediju provedene su na dozirnim oblicima 5-aminosalicilata, folne kiseline te fiksnim kombinacijama pripremljenim u ciljanom omjeru. U svrhu kvantifikacije oslobođene frakcije iz dozirnog oblika korištene su prethodno razvijene kromatografske metode za simultano određivanje sadržaja 5-aminosalicilata i folne kiseline. Studije otapanja mesalazina i sulfasalazina te studije njihovog simultanog otapanja s folnom

kiselinom provedene su prema metodama opisanim u američkoj farmakopeji (engl. *United States Pharmacopoeia*, USP) (188). Profili oslobađanja pojedinačnih dozirnih oblika uspoređeni su s profilima dobivenim istovremenim otapanjem dva dozirna oblika, kako bi se uočile promjene te utvrdila njihova stabilnost kada su istovremeno prisutni u biološki relevantnom mediju. Usporedba se temeljila na računanju faktora različitosti (f_1) te faktora sličnosti (f_2) između krivulja dobivenih otapanjem pojedinačnih dozirnih oblika i krivulja dobivenih njihovim istovremenim otapanjem. Faktor različitosti između krivulja mesalazina (**Rad 2**, Slika 5a) i krivulja folne kiseline (**Rad 2**, Slika 5b) iznosio je 4,51 % odnosno 3,16 %, dok u slučaju sulfasalazina i folne kiseline (**Rad 3**, Slika 6a i 6b) faktor različitosti je iznosio 1,60 % odnosno 3,01 %, što je unutar prihvatljivog kriterija od 0–15 % (189). Faktori sličnosti od 82,09 % i 77,01 % dobiveni su usporedbom krivulja u studijama mesalazina i folne kiseline dok su faktori sličnosti od 92,40 % te 77,91 % dobiveni u studijama sulfasalazina i folne kiseline. Faktor sličnosti od 100 % predstavlja dvije identične krivulje, a vrijednost faktora sličnosti ne smije biti manja od 50 % (190). Visoka sličnost, odnosno mala različitost, između krivulja oslobađanja dobivenih samostalnim otapanjem dozirnih oblika i njihovih kombinacija, kao i odsutnost novih pikova razgradnih produkata ukazuje na izostanak interakcija između sastavnica.

U slučaju olsalazina i balsalazida studije stabilnosti u biološki relevantnim medijima provedene su na pripremljenim dozirnim oblicima predloženih fiksnih kombinacija. Olsalazin i balsalazid dolaze u obliku kapsula što je omogućilo lakšu pripremu fiksnih kombinacija. Također, pomoćne tvari koje se uz olsalazin i balsalazid nalaze u kapsulama nabavljenih dozirnih oblika su magnezijev stearat i koloidna bezvodna silika, koji se koriste i u proizvodnji tableta koje sadrže folnu kiselinu, stoga njezina stabilnost u njihovoj prisutnosti nije upitna (137, 141, 191). Analitički povrati nakon provedbe studija otapanja pripremljenih dvanaest fiksnih kombinacija balsalazida i folne kiseline, u biološki relevantnom mediju, bili su u rasponu od 91,9 % do 102,0 % za balsalazid te od 94,9 % do 101,6 % za folnu kiselinu (**Rad 4**, Slika 5a). Visoki analitički povrati obiju sastavnica navode na njihovo zadovoljavajuće oslobađanje iz dozirnog oblika, dok visoki povrati folne kiseline stavljaju naglasak na izostanak kemijske interakcije između ispitivanih spojeva, uzevši u obzir da je kombinacija pripremljena u omjeru 750:1 u korist balsalazida. Analitički povrati u studijama provedenim na pripremljenim fiksnim kombinacijama olsalazina i folne kiseline također su pokazali zadovoljavajuće oslobađanje lijeka iz dozirnog oblika. Olsalazin se oslobodio u rasponu od 89,6 % do 102,8 % dok se folna kiselina oslobodila u rasponu od 93,4 % do 103,4 % (**Rad 4**, Slika 5b). Kao i u slučaju balsalazida, visoki analitički pronosi folne kiseline ukazuju na izostanak

kemijske interakcije između olsalazina i folne kiseline. Uzevši u obzir omjer u kojem se sastavnice fiksne kombinacije nalaze u dozirnom obliku (750:1 i 250:1), u slučaju kemijske interakcije došlo bi do značajnog pada analitičkog povrata folne kiseline.

7. ZAKLJUČCI

U ovom doktorskom radu provedeno je niz eksperimenata s ciljem utvrđivanja fizikalno-kemijske kompatibilnosti folne kiseline i četiri 5-aminosalicilata, mesalazina, sulfasalazina, olsalazina i balsalazida, kao uvod u razvoj fiksnih kombinacija lijekova.

Upotrebom biomimetičke kromatografije utvrđeno je da se ispitivani spojevi više vežu za HSA protein u odnosu na AGP, pri čemu je ispitivanje kompetitivnog vezanja bilo usmjereno na HSA protein. Primjenom frontalne analize i zonske eluacije nije utvrđeno da dolazi do vezanja ispitivanih spojeva na isto mjesto na HSA proteinu, što ide u prilog razvoju predloženih fiksnih kombinacija jer se ne očekuju promjene u njihovoj farmakokinetici.

Uspješno su validirane nove HPLC metode razvijene u svrhu praćenja sadržaja ispitivanih spojeva te njihovih onečišćenja koje su se koristile u analizi ispitivanih uzoraka.

Korištenjem termalnih tehnika uočene su promjene u termogramima smjesa mesalazina i folne kiseline te sulfasalazina i folne kiseline, što ukazuje na potencijalne interakcije, dok u slučaju olsalazina i balsalazida nisu uočene značajne promjene.

Provedene studije prisilne razgradnje čistih sastavnica mesalazina, sulfasalazina i folne kiselina kao i pripremljenih smjesa nisu ukazale na interakciju između ispitivanih sastavnica smjesa. Sličan obim razgradnje te visoke vrijednosti čistoće pikova ukazuju na odsutnost kemijske reakcije između sastavnica smjesa.

Kromatografskom i spektroskopskom analizom izotermalno stresiranih uzoraka pokušala se dodatno utvrditi potencijalna kemijska interakcija između 5-aminosalicilata i folne kiseline. Kromatografskom analizom stresiranih smjesa nije uočena značajna razlika u analitičkim povratima u odnosu na povrate dobivene stresiranjem samih sastavnica. Također stresirani uzorci na bazi sulfasalazina, olsalazina i balsalazida su analizirani pomoću FTIR-a te analizom dobivenih spektara uz pomoć statističke obrade podataka nije utvrđena interakcija između sastavnica. Moguće interakcije između folne kiseline te olsalazina i balsalazida ispitane su XRPD tehnikom. Snimljeni difraktogrami nisu pokazali interakcije između ispitivanih spojeva, što je i potvrđeno statističkom obradnom podataka.

Primjenom studije stabilnosti u biološki relevantnom mediju nisu uočene interakcije između ispitivanih sastavnica niti međusoban utjecaj na njihovo oslobađanje iz dozirnog oblika.

Na temelju dobivenih rezultata može se pretpostaviti da su ispitivani 5-aminosalicilati i folna kiselina fizikalno-kemijski kompatibilni. Također takav zaključak može se još dodatno potvrditi provedbom stabilitetnih studija na pripremljenim smjesama aktivnih komponenti, pripremljenim formulacijama te gotovim dozirnom oblicima.

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9. Biografija

Mario-Livio Jeličić rođen je 18. ožujka 1994. u Zagrebu. Nakon maturiranja u Prirodoslovnoj školi Vladimira Preloga upisuje Fakultet kemijskog inženjerstva i tehnologije, smjer Primijenjena kemija. 2015. godine završava preddiplomski studij te iste godine upisuje diplomski studij, smjer Specifični materijali i napredne tehnologije. Diplomski rad izrađuje na matičnom fakultetu na Zavodu za analitičku kemiju pod mentorstvom izv. prof. dr. sc. Šime Ukića. Diplomski rad brani 2017. godine čime stječe akademski naziv magistar primijenjene kemije. Tijekom studija radi kao demonstrator na Zavodu za analitičku kemiju te aktivno sudjeluje u znanstveno-istraživačkim projektima. Pred kraj studija se zapošljava u Plivi kao tehnička podrška u Odjelu za registraciju lijekova gdje i nastavlja raditi nakon završetka fakulteta kao stručnjak za regulatorne poslove. 2018. godine odlazi iz Plive te se zapošljava na Farmaceutsko-biokemijskom fakultetu kao doktorand na projektu Hrvatske zaklade za znanost - Razvoj naprednih analitičkih metoda za lijekove i biološki aktivne tvari u liječenju upalnih bolesti crijeva. U sklopu doktorskog studija aktivno je sudjelovao na domaćim i međunarodnim znanstvenim skupovima. Autor i koautor je na ukupno 13 znanstvenih radova.

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Doktorski rad

ODREĐIVANJE FIZIKALNO-KEMIJSKE KOMPATIBILNOSTI 5-AMINOSALICILATA I FOLNE KISELINE KAO PREDUVJET RAZVOJA FIKSNE KOMBINACIJE LIJEKOVA

Mario-Livio Jeličić

SAŽETAK

Crohnova bolest i ulcerozni kolitis, poznatiji kao upalne bolesti crijeva, predstavljaju kronične upalne bolesti gastrointestinalnog trakta. Zacjeljivanje sluznice i postizanje duboke remisije omogućuje se ciljanom terapijom, međutim postoje skupine pacijenata kojima koristi isključivo liječenje protuupalnim lijekovima iz skupine 5-aminosalicilata; mesalazin i njegovi prolijekovi sulfasalazin, balsalazid i olsalazin. Nedostatak folne kiseline primijećen je u više od polovine oboljelih od Crohnove bolesti i više od trećine pacijenata s ulceroznim kolitisom. Njezin nedostatak može dovesti do razvoja megaloblastične anemije, a povezan je i s povećanom prevalencijom karcinoma debelog crijeva, stoga se uz redovitu terapiju često propisuju povišene doze folne kiseline od 1 do 5 mg na dan. Razvojem fiksnih kombinacija lijekova potencijalni rizik od nepridržavanja propisane terapije nastoji se svesti na najmanju moguću mjeru, što je posebno važno kod pacijenata koji boluju od kroničnih bolesti gdje se terapija sastoji od više lijekova iz različitih farmakoloških skupina. Razvoj fiksnih kombinacija lijekova složen je postupak u kojem važnu ulogu ima ispitivanje fizikalno-kemijskih svojstava svakog pojedinog lijeka kao i njihovih smjesa. U ovom radu predložene su analitičke metode kao podrška razvoju fiksnih kombinacija 5-aminosalicilata i folne kiseline pri čemu su korištene različite analitičke tehnike, poput termalnih, spektroskopskih te kromatografskih, s ciljem ispitivanja fizikalno-kemijske kompatibilnosti 5-aminosalicilata i folne kiseline. Provedena istraživanja pokazala su da su ispitivani 5-aminosalicilati; mesalazin, sulfasalazin, balsalazid i olsalazin, fizikalno-kemijski kompatibilni s folnom kiselinom, što otvara mogućnost za daljnja ispitivanja potrebna za razvoj predloženih fiksnih kombinacija.

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Rad sadrži:	101 stranicu, 19 grafičkih prikaza, 13 tablica i 191 literaturni navod. Izvornik je na hrvatskom jeziku.
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Basic documentation card

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Doctoral dissertation

PHYSICOCHEMICAL COMPATIBILITY DETERMINATION OF 5-AMINOSALICYLATES AND FOLIC ACID AS A PREREQUISITE FOR THE DEVELOPMENT OF A FIXED-DOSE COMBINATION

Mario-Livio Jeličić

SUMMARY

Crohn's disease and ulcerative colitis, better known as inflammatory bowel diseases, are chronic inflammatory diseases of the gastrointestinal tract. Healing of the mucosa and achieving deep remission is achieved by targeted therapy, however there are groups of patients who benefit exclusively from treatment with anti-inflammatory drugs from the group of 5-aminosalicylates; mesalazine and its prodrugs sulfasalazine, balsalazide and olsalazine. Folic acid deficiency has been observed in more than half of Crohn's disease patients and more than a third of patients with ulcerative colitis. Its deficiency can lead to the development of megaloblastic anemia, and is associated with an increased prevalence of colorectal cancer, so in regular therapy, increased doses of folic acid of 1 to 5 mg per day are often prescribed. With the development of fixed-dose combinations, the potential risk of non-compliance with prescribed therapy is minimized, which is especially important in patients with chronic diseases where therapy consists of several drugs from different pharmacological groups. The development of fixed drug combinations is a complex process in which an important role is played by the examination of the physicochemical properties of each individual drug as well as their mixtures. In this paper, analytical methods are proposed to support the development of fixed combinations of 5-aminosalicylate and folic acid using various analytical techniques, such as thermal, spectroscopic and chromatographic, to test the physicochemical compatibility of 5-aminosalicylate and folic acid. Studies have shown that 5-aminosalicylates; mesalazine, sulfasalazine, balsalazide and olsalazine, are physicochemically compatible with folic acid, which opens the possibility for further testing required for the development of the proposed fixed combinations.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

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