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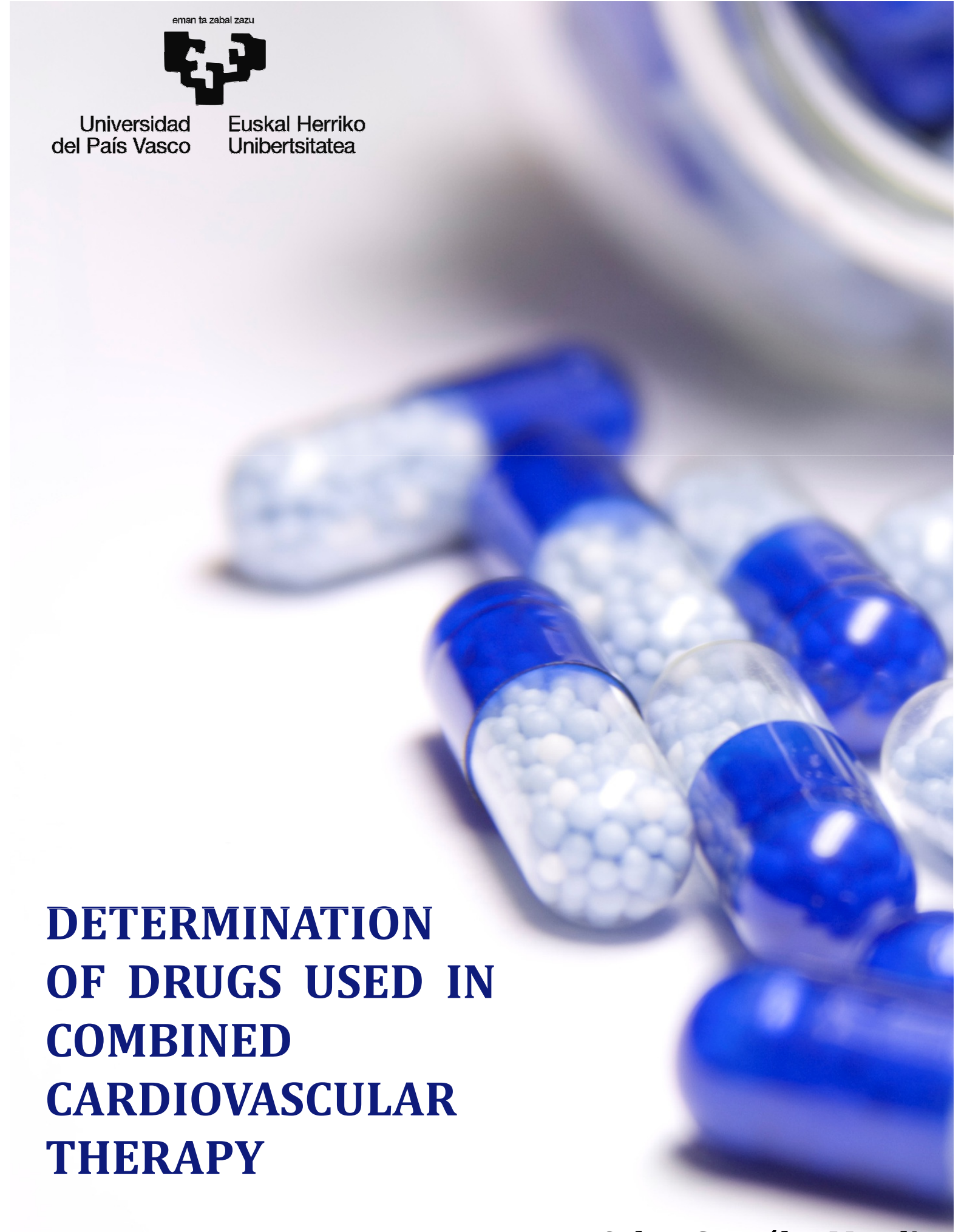
Universidad del País Vasco

Euskal Herriko Unibertsitatea



DETERMINATION OF DRUGS USED IN COMBINED CARDIOVASCULAR THERAPY

Oskar González Mendia

June 2011

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ZTF-FCT
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Facultad de Ciencia y Tecnología

DETERMINATION OF DRUGS USED IN COMBINED CARDIOVASCULAR THERAPY

Report to compete for the Ph. D. degree

Oscar González Mendia

June 2011

Tingues sempre al cor la idea d'Ítaca.
Has d'arribar-hi, és el teu destí,
però no forcis gens la travessia.
És preferible que duri molts anys,
que siguis vell quan fondegis l'illa,
ric de tot el que hauràs guanyat fent el camí,
sense esperar que et doni més riqueses.
Ítaca t'ha donat el bell viatge,
sense ella no hauries sortit.
I si la trobes pobra, no és que Ítaca
t'hagi enganyat. Savi, com bé t'has fet,
sabràs el que volen dir les Ítaques.

Ítaca

ESKERONAK

Behin lan hau bukatuta bidean ezagutu ditudan pertsona guztiei eskerrak emateko aukera daukat hurrengo lerroetan. Protokoloari jarraituz tesi zuzendariekin hasiko naiz, Rosa Alonso, orain dela zazpi! urte pasatu nintzen zure bulegotik lehenengo aldiz eta oraindik ere zure ikasleekin borrokatu behar bertan sartzeko… Eskerrik asko lan hau burutzeko aukera emategatik. Itxaso Maguregui, arte ederretako oasi horretan sartuta, oraindik estetika kontuetan asko hobetu behar dut baina behintzat zeozer ikasi dugu. Y la tercera en discordia, Rosa Jimenez, quién te iba a decir hace unos años que te iba a echar del despacho!

Laborategikideen txanda da orain. Gorka, HPLC modular horrekin hasi eta orain doctor honoris causa en UPLC izango zara. Eskerrik asko laguntzagatik eta badakizu gaur egungo haurrek tesiak dakartela besapean. Nerea Ferreirós, masa espektrometrian maisu, Leioatik Freiburg-era zure urratsak jarraitu nituen eta bertan primeran hartu ninduzun, Vielen Dank! Estitxu, auzokide, ez zara nitaz aspertuko? Hurrengo geltokia: metabolomika. Mis otras pequeñas padawan, Sion y Maite, os debo una gran parte de este trabajo. Azken egun hauetan laborategian nire umore txarra jasan duzuenoi: Nerea, Mariluz, Itxaso Jr, Itxaso Sanro, Txesko, Maialen, hemendik aurrera “15” minututako kafeetara bueltatuko naiz. Nahiz eta jada laborategian ez egon hemendik pasatu diren guztiei eskerrak emateko parada aprobetxatuko dut: Oiana, Juan, Bea, Gorka, Christian, Irene…

Pasilloa gurutzatu eta mintegiko jendea agurtu nahi nuke orain. Pertsona gehiegi pasatu zarete hortik guztiak aipatzeko baina “la vieja guardia” (Asier,

Maitane, Joxean…) oraindik ere erne ta tinko. Lunch eta tesi asko ditugu aurretik elkarrekin ospatzeko. Olatz eta Aresatz, eskerrik asko irakaskuntzarekin eskainitako laguntzagatik. Eta Arantzari, azken hilabete hauetako presioa nigaz jasateagatik, ezin dozu esan aurretik daukazuna ezagutzen ez duzunik.

Departamentuko beste taldeaz ez naiz ahazten. Cristina, herrikide, zure taxia utzi nuen zure alboan duzun neskarena hartzeko. Vilory, animo tesiarekin, siempre nos quedaran las tostadas. Bea (te sigo metiendo en el mismo grupo) gracias por la ayuda con el masas, el ion-mobility lo dejamos para otra ocasión.

Marta, el promès és deute, he acabat abans però de segur que el pèptids no se't resisteixen molt mes.

Herzlichen Dank an die Luete vom Rechtsmedizin Labor aus Freiburg: Herr Weinmann, Sebastian (wir enden zusammen!), Heike, Maria, Arianne und an alle die Leute ich dort kennengelernt habe: Xaolai, Jan, Basil...

Eta bukatzeko beti gertu izan dudan familiari eskerrak eman behar diot. Ama eta aita, gezurra dirudien arren azkenean bukatu dut, eh? Amaia, gutxi barru zure neba doktorea dela esan dezakezu Bartzelonatik, que fort!

Eskerrik asko danoi, bihotz-bihotzez

SUMMARY

Throughout the history the humanity has undergone deep changes in its living conditions: the lifestyle is more and more sedentary, the physical activity decreases and the diet is richer in saturated fat and sugars. Therefore, the fact that cardiovascular diseases have become the first cause of mortality worldwide can be easily understood.

In The Basque Country, cardiovascular illnesses caused 31% of the deaths in 2008, being noteworthy the fact that 87% of these deaths were people over 69 years. In the USA, a country with a sedentary lifestyle and bad eating habits in general, 80 million inhabitants suffer from this kind of diseases and around 800000 people died as consequence of them in 2007.

The risk of suffering from cardiovascular illnesses is strongly related to some factors such as hypertension, high cholesterol levels, diabetes, obesity… Unfortunately, these factors often appear together increasing the risk of a cardiovascular accident. The combination of these different risk factors is known as **metabolic syndrome** and it is considered a pandemic by many experts due to the high prevalence worldwide.

Metabolic syndrome was first mentioned by Dr. Reaven in 1988, but it was not until 1998 that the World Health Organization published the first harmonized definition. Nevertheless, diagnosing this ilness following this criterion is hazardous and expensive and, therefore, the definition released in the NCEP-ATPIII (*National Cholesterol Education Program's Adult Treatment Panel III*) is more widely used.

The five risk factors taken into account by this definition are gathered in the following table. When 3 of these 5 parameters are out of range metabolic syndrome is diagnosed. Furthermore, there are other factors that can increase the chances of a cardiovascular disorder such as tobacco, caffeine and alcohol consume, lack of physical activity and the aforementioned age.

Risk factor	Limit values
Hypertension	>135/85 mmHg (Systolic/diastolic)
Abdominal obesity*	>102cm (Male) >88cm (Female)
Triglycerides	>150 mg/dL
HDL cholesterol	<40 mg/dL (Male) <50 mg/dL (Female)
Fasting glucose	>100 mg/dL

*Depending on the ethnia

Hence, metabolic syndrome is a cluster of different medical illnesses: hypertension, dyslipidemia (abnormal levels of lipids), diabetes and obesity. The first step to fight against them is a change in the lifestyle and in the diet, but when it is not enough a medical treatment is necessary. Obviously, since different symptoms must be treated, different drugs must be prescribed, which is known as **combined cardiovascular therapy**. Generally, in this therapy, antihypertensives are used to reduce blood pressure, lipid lowering drugs to control fat levels and

antidiabetics to decrease glucose levels. Furthermore, antiplatelets and anticoagulants are commonly used to avoid thrombus formation that can cause a circulatory collapse.

The monitoring of the plasmatic concentrations of these drugs is crucial for understanding their pharmacokinetics and pharmacodynamics. Moreover, in the case of several compounds used in combined cardiovascular therapy, it is also important to detect misuse of these drugs (i.e. for doping), to reveal accidental or intentional intoxications and to obtain valuable information about possible interactions and secondary effects derived from the co-administration of several drugs which share metabolic and/or excretion pathways.

Therefore, the simultaneous determination of these analytes turns interesting, but this is a complex task since the analysis of multiple substances with different physicochemical properties and physiological behaviour is always a challenge for the analytical chemist. The complexity of the biological matrices and the difference in the expected concentrations (from several milligrams to a few micrograms per litre) of some analytes require the development of extremely sensitive and selective determination methods. These could be the limiting factors behind the scarce number of publications focused on the simultaneous screening of a high number of drugs belonging to different compound classes. However, several analytical methods have been reported for the simultaneous determination of the most frequently prescribed associations.

The aim of this work is to fill the gap existing in this field of the drug analysis, developing analytical methods capable of quantifying the different drugs prescribed in combined cardiovascular therapy simultaneously. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been the technique of choice throughout the main part of this work, due to the high sensitivity and selectivity requirements.

Starting from this point the work developed can be summarized in the following points:

- Study of the mass fragmentation of the compounds of interest.
- Development of LC-MS/MS methods for the simultaneous determination of cardiovascular drugs in human plasma.
- Study of the photometric and fluorimetric properties of the compounds of interest.
- Systematic optimization of an Ultra High Performance Liquid Chromatography method.
- Application of the Solid Phase Extraction to the sample treatment.
- Study of the degradation of the lipid lowering drug fluvastatin in acidic media.

ABBREVIATIONS

ACEI: Angiotensin-Converting Enzyme Inhibitor

ACN: Acetonitrile

AEB: Ameriketako Estatu Batuak

AEBI: Angiotensinaren Entzima Bihurtzailearen Inhibitzaileak

AHA: American Heart Association

AHA-II: Angiotensina-II Hartzailearen Antagonistak

ANOVA: Analysis Of Variance

ARA-II: Angiotensin Receptor Antagonists

C_{max}: Maximum plasmatic concentration

CE: Capillary Electrophoresis

CE: Collision Energy

CEP: Collision Cell Entrance Potential

CXP: Collision Cell Exit Potential

DEE: Desbiazio Estandar Erlatiboa

DP: Declustering Potential

EAE: Euskal Autonomi Erkidegoa

ECD: Electron Capture Detector

EP: Entrance Potential

EPI: Enhanced Product Ion Scans

ER: Errore Erlatiboa

ESI: Electrospray Ionization

FDA: Food and Drug Administration

FLUO: Fluorescence Detector

FWHM: Full Width at Half Maximum

GC: Gas Chromatography

HDL: High Density Lipoproteins

HMGCoA: 3-hydroxy-3-methyl-glutaryl coenzyme A

HPLC: High Performance Liquid Chromatography

HRMS: High Resolution Mass Spectrometry

ICH: International Conference on Harmonisation

IS Voltage: Ion Spray Voltage

IS: Internal Standard

JNC: Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure

LC: Liquid Chromatography

LDL: Low Density Lipoproteins

LLE: Liquid Liquid Extraction

LLME: Liquid Liquid Microextraction

LOD: Limit of Detection

LOQ: Limit of Quantitation

m/z: Mass Charge Ratio

MALDI: Matrix Assisted Laser Desorption Ionization

MeOH: Methanol

MRM: Multiple Reaction Monitoring

MS: Mass Spectrometry

NCEP-ATP III: National Cholesterol Education Program's Adult Treatment Panel III

OME: Osasun Munduko Erakundea

OVAT: One Variable At a Time

PA: Presioa Arteriala

PAD: Persio Arterial Diastolikoa

PAS: Presio Arterial Sistolikoa

PDA: Photodiode Array Detector

PFP: Pentafluorophenyl

ppm: Parts Per Million

PPT: Protein Precipitation

Q: Quadrupole

QC: Quality Control

RE: Relative Error

R_s: Resolution

RSD: Relative Standard Deviation

S/N: Signal to Noise Ratio

SPE: Solid Phase Extraction

SPME: Solid Phase Microextraction

t_{max}: Maximum plasmatic concentration time

TG: Triglyceride

TIC: Total Ion Chromatogram

TOF: Time of Flight

t_r: retention time

UHPLC: Ultra High Performance Liquid Chromatography

UPLC: Ultra Performance Liquid Chromatography

UV: Ultraviolet

VLDL: Very Low Density Lipoproteins

WHO: World Health Organization

DETERMINATION OF DRUGS USED IN COMBINED CARDIOVASCULAR THERAPY

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CHAPTER I

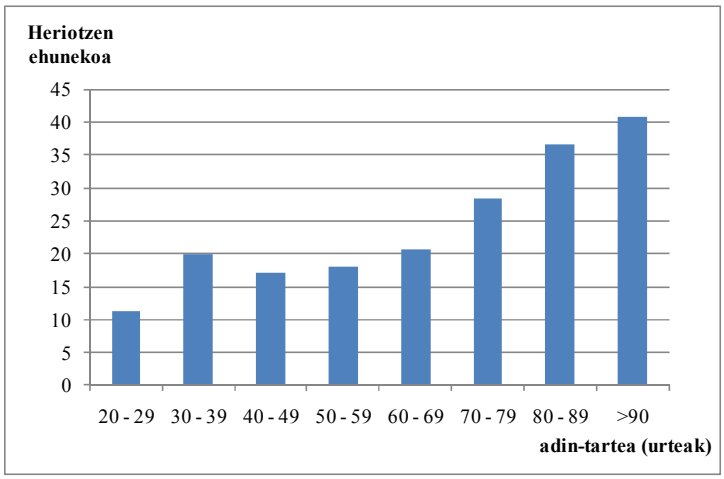
SARRERA ETA HELBURUAK

1.1. Sindrome metabolikoa eta eritasun kardiobaskularrak

Urteetan zehar gizadiak etengabeko eboluzioa garatu du bere bizi-baldintzetan: bere ehiztari eta nomada ohiturak bizitza sedentario eta erosoagatik ordezkatu ditu; esku-lanak lan mekanikoari utzi dio bidea eta aise aktibitateek aktibitate fisikoak baztertu dituzte. Gainera, kontuan izanda gure dieta gantz saturatuetan eta azukreetan aberastu dela, ulertzekoa da azken urteetan eritasun kardiobaskularrak lurralde garatuetan lehenengo heriotza arrazoi bihurtu izana.

Hala ere, pentsa daitekeenaren kontra, gaitz kardiobaskularrek eragindako heriotzen % 80 garapen bidean dauden lurraldeetan gertatzen dira. Arrazoiak herrialde hauetan prebentzio eta tratamendu osasun-zerbitzu egokiak eskaintzeko zailtasunak eta egoera ekonomikoak eragindako dieta desorekatuak dira [1].

Euskal Autonomi Erkidegoan (EAE) 2008ko datuen arabera 6000 biztanle inguru hil ziren arazo zirkulatorioak direla eta, hildako guztien % 31. Eritasun hauek pairatzeko arriskua adinarekin estuki lotuta dago, izan ere, hildako horien % 87ek 69 urte baino gehiago zuten (1.1.Irudia). Era berean, sexuaren arabera ezberdintasun nabarmenak daude, gizonezkoen artean heriotza-tasa % 28koa den bitartean emakumeen artean % 36koa da [2].



1.1. Irudia. 2008. Urtean eritasun kardiobaskularrek EAEn kausatutako heriotzak adinaren arabera

Gaixotasun kardiobaskularren arazoaren isla dira Ameriketako Estatu Batuak (AEB), lurralde sedentarioa eta dieta txarrekoa orokorrean. Bertan 80 miloi lagun baino gehiagok pairatzen dituzte eritasun kardiobaskularrak eta 2007an zortziehun mila biztanle hil ziren hauen ondorioz. Gutxi balitz, eritasun hauen aurkako tratamenduak 207.000 miloi dolarreko kostua ekarri zuen. Lurralde horretan gizartea arazoaz jabearazteko sortutako *American Heart Association* (AHA) eritasun kardiobaskularri buruzko informazio iturri ezinbestekoa bilakatu da, urtero egoerari buruzko estatistika oparo argitaratzen dituelarik [3]. Zorionez, datu berrienen arabera, hildakoen kopuruak beherakada nabarmena jasan du azken bosturtekoan, baina oraindik ere gaitz kardiobaskularrek lehen heriotza-arrazoia izaten jarraitzen dute AEBn.

Eritasun kardiobaskularrak

Kardiopatia iskemikoa: Bihotzeko gaixotasun ohikoena. Arteria koronarioen buxadurak miokardioak behar duen odola iristen uzten ez duenean gertatzen da. Odol fluxua murrizten denean *bularreko angina* agertzen da eta guztiz mozten denean *bihotzekoa*. Kardiopatia iskemikoa pairatzen duten erien heren batek bat-bateko heriotza izaten dute.

Garuneko istripu baskularrak: Burmuinera odola eramaten duten odol-hodien gaixotasunen ondorioz gertatzen direnak. Odol fluxua eteten denean *garun-infartua* gertatzen da eta odol-hodi entzefaliko bat apurtzen denean *apoplexia*.

Arteriopatia periferikoa: Bihotzetik gorputz-adarretara odola eramaten duten arterien buxaduragatik gertatzen da.

Tronbosi benoso sakona: Orokorrean hanken zain batean odol koagulu bat sortzen denean gertatzen da, koagulu hau biriketara iritsi eta *biriketako tronboenbolismoa* sor dezake.

Sukar erreumatiko akutua: Bihotzeko muskuluak eta balbulak kaltetzen direnean estreptokoko infekzio baten ondorioz.

Bihotzeko sortzetiko malformazioa: Jaiotzako unetik bihotzean malformazioak daudenean.

Ikerkuntza-lan honen ikuspuntutik azken biek ez dute garrantzi handirik ohiko terapia kardiobaskularraren bidez tratatzen ez direlako.

Eritasun kardiobaskularren jatorria

Eritasun kadiobaskularrak pairatzeko aukera hainbat arrisku-faktoreren menpekoa da: hipertentsioa, kolesterola, diabetesa, loditasuna… Arrisku-faktore hauek ez dira normalean banaka agertzen, konbinatuta baizik eta gaixotasun kardiobaskularren azaltzea errazten dute [4]. Arrisku-faktoreen konbinaketa hau da, hain zuzen ere, ***sindrome metabolikoa*** bezala ezagutzen dena.

Aipatu berri diren arrisku-faktoreak gure bizi-aztura negatiboen ondorioz agertzen dira batez ere: dieta desorekatua, aktibitate fisiko eza, nikotina eta kafeina bezalako substantzia kaltegarrien kontsumoa… Gainera adinak ere rol garrantzitsua jokatzen du, urteen poderioz gure odol-hodiak hondatzen baitira eritasun hauen agertzea ahalbidetuz.

Sindrome metabolikoa

Azaldu den bezala, sindrome metabolikoa eritasun kardiobaskularrak pairatzeko aukera areagotzen duten arrisku-faktoreen konbinaketa da. Hala ere, komunitate zientifikoan ez dago adostasun handirik gaixotasun hau diagnostikatzeko ikuspuntu ezberdinak daudelako arrisku-faktoreak zeintzuk diren definitzerakoan. Eritasun berri samar honen lehenengo aipamena Reaven doktoreak [5] egin zuen 1988. urtean eta hortik aurrera definizio ezberdinak agertu dira baita izen ezberdinak ere: X-sindromea, intsulina-erresistzentzia sindromea,

sindrome plurimetabolikoa, laukote hiltzailea… Lehenengo definizio bateratua ez zen 1998. urtera arte plazaratuko Osasunaren Munduko Erakundearen (OME) eskutik [6]. Dena den, sindrome metabolikoa irizpide honen arabera detektatzea neketsua eta garestia izateagatik gehien erabiltzen den definizioa NCEP-ATPIII (*National Cholesterol Education Program's Adult Treatment Panel III*) txostenean agertzen dena da. Txosten hau AEB-ko *National Heart, Lung, and Blood Institute* eta AHA erakundeek sindrome metabolikoaren definizioa berrikusteko 2001. urtean antolatutako kongresuan sortu zen eta hainbat aldaketa jasan ditu azken urteetan. 1.1.Taulan ikus daitekeen definizioa 5 aldagai kontuan hartzen ditu: loditasuna [7], hipertentsioa eta triglizerido, HDL kolesterol eta glukosa mailak. Parametro hauetatik hiru mugaz kanpo daudenean sindrome metabolikoa diagnostikatzen da [8].

1.1. Taula NCEP-ATPIII-ko irizpideak sindrome metabolikoa identifikatzeko

Parametroak	Mugak
Hipertentsioa	>135/85 mmHg (Sistolikoa/diastolikoa)
Loditasun abdominala*	>102cm (Gizonezkoak) >88cm (Emakumezkoak)
Triglizeridoak	>150 mg/dL
HDL kolesterola	<40 mg/dL (Gizonezkoak) <50 mg/dL (Emakumezkoak)
Glukosa baraurik	>100 mg/dL

*Etniaren arabera balioak alda daitezke

Ikerketa ezberdinen arabera, munduan populazio helduaren % 20-30-ak gaitz hau jasaten duela uste da eta hauek eritasun kardiobaskularrak pairatzeko aukera bikoitza daukate [9]. Mundu mailan aurrera eraman diren ikerketa ezberdinen artean, ospetsuena eta sakonena Massachusetts-eko Framingham herrian 1948. urtean hasi zena da [10]. Lortutako emaitzei esker gaixotasun kardiobaskularrak pairatzeko aukerak aurresateko programa garatu dute, gure adina, sexua, kolesterol maila, eta abar kontuan hartuta [11].

Sindrome metabolikoa zer den azalduta bere osagaiak sakonki aztertuko dira, banan-banan.

1.1.1. Hipertentsioa

Gure gorputzeko odol-hodietan odolak zirkulatzen duen indarrari presio arteriala deritzo. Indar hori gehiegizkoa denean, eta zeharkatzen dituen organoak erasaten dituenean, hipertentsioa gertatzen da.

Presio arterialaren (PA) kuantifikazioa bi parametroen neurketaren bidez egiten da. Bihotza uzkurtzen denean odola garraio sisteman bultzatzen da, momentu honetan presioa maximoa da eta presio sistolikoa edo altua (PAS) bezala ezagutzen da. Bihotza atsedenaldian dagoenean presio diastolikoa edo presio minimoa neurtzen (PAD) da.

OMEk 1999an Hipertentsiorako Tratamenduaren Gidan plazararaturikoaren arabera, presio arterial normala era honetan definitzen da: pertsona heldu batentzat 140 mmHg baino baxuagoko PAS eta 90 mmHg baino baxuagoko PAD.

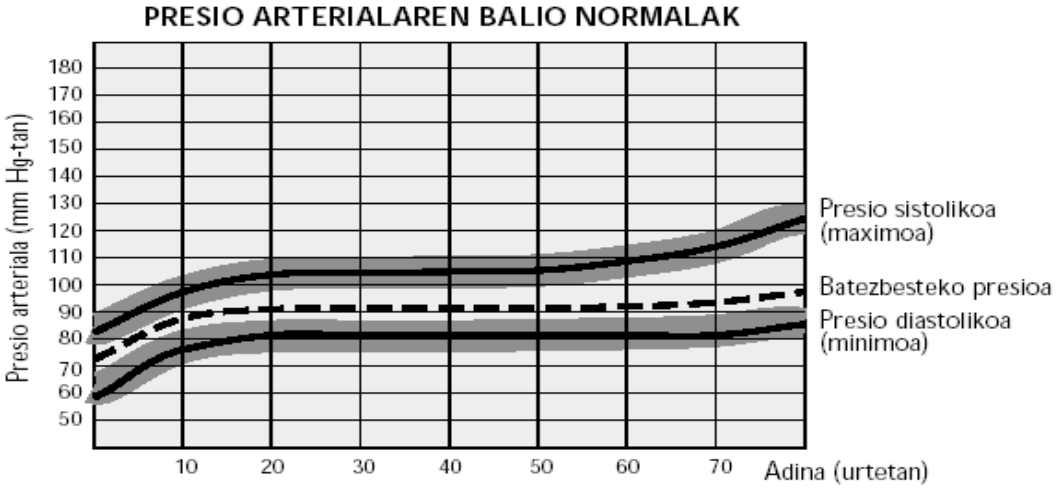
140/90 mmHg balioetatik gorako kasuetan (muga hauek barne), hipertentsioaz hitz egin behar da. Balio antzekoak agertzen dira “Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure” (JNC) komisioaren azken txostenean [12], ezberdintasuna pre-hipertentsio tarte bat ezartzen dela da. Hau berez ez da gaixotasun bezala kontuan hartzen, baina hipertentsioa garatzeko arrisku handia duten pertsonak identifikatzeko erabiltzen da. 1.2 Taulan jasotzen dira JNC-k presio arteriala sailkatzeko ezarritako irizpideak.

1.2. Taula JNC-ren presio arterialaren sailkapena

Sailkapena	PAS (mmHg)	PAD (mmHg)
Normala	< 120	<80
Pre-hipertentsioa	120-139	80-89
Hipertentsioa I	140-159	90-100
Hipertentsioa II	>160	>100

Hipertentsio arteriala fisiopatologia ezaguneko aldakuntza hemodinamikoa da. Egoera patologiko ezberdinen ondorioa izan daitekeenez, gaixotasun baten ezaugarri klinikotzat har daiteke. Hipertentsioak ondoeza baskularraren (nerbio-sistema zentrala eta bihotzeko gaixotasun iskemikoak nagusiki) ondorioz sortzen diren eritasunak pairatzeko arriskua potentziatzen du, hots, arrisku faktore nabarmena da. Badaude hipertentsioa eragiten duten hainbat faktore, hala nola pisua, sexua, sedentarismoa, estresa, gatz-kontsumoa, alkohola... orokorrean bizi ohiturak aldatzearekin zuzenduko liratekeenak.

Hipertentsioa kontrolatzea erraza izan daitekeen arren, gaitz honek bere baitan arrisku inplizitua dakar: apenas ematen du sintomarik. Hori dela bide, kezka nagusia ez da hipertentsio arterial zorrotza duten pertsonen kopuru erlatiboki baxua, baizik eta tentsio arteriala arinki altua duen gizarteko espektro zabala. Populazioaren % 15-19 hipertentsoa da, joera hori adinarekin handitzen delarik odol hodiek jasaten duten endekapena eta aldaketa hormonalen ondorioz (1.2 Irudia). Hala ere bost pertsonetatik batek tentsioa altua duen arren horietatik % 40k baino ez daki hipertentsoa dela. Gainera, hipertentsoak direla dakiten % 60 horietatik erdia ez da medikuarenera joaten. Beraz, ez du medikazio edo tratamendurik hartzen. Eta azkenik, medikuarenera doazenetatik erdiak baino ez du behar bezala ondo jasotzen tratamendua [13].



1.2.Irudia Bizitzan presio arterialaren bilakaera

1.1.2. Dislipemia

Dislipemia edozein lipido plasmatikoren kontzentrazioa bere balio normaletik urruntzen denean gertatzen da. Eritasun kardiobaskularren ikuspuntutik, kolesterolean edo triglizeridoetan gertatzen diren aldaketak dira interesgarriak, hauek baitira gaitz askoren errudun.

Kolesterola, organismoaren mintzak osatzeko ezinbestekoa den lipidoa, sistema zirkulatorioaren zehar garraiatzen da, lipidoz eta proteinez osaturiko lipoproteinei esker [14]. Hiru lipoproteina mota bereiz daitezke bere dentsitateren arabera, bakoitzak betebehar ezberdina izanik:

- *LDL edo dentsitate baxuko lipoproteinak*: Kolesterolaren % 70 daramate, kolesterola gibelatik garraiatzen dute eta arterietan jalki dezakete, honela ateroma deritzoten lipido metaketak eratuz, hauek arteriak buxatzen dituztenean sortzen dira miokardio infartuak eta tronbosiak. Bere ondorio kaltegarriengatik “kolesterol txarra” bezala ezagutzen dira, zenbat eta LDL gutxiago egon orduan eta eritasun kardiobaskular arrisku gutxiago dago.

- *HDL edo dentsitate altuko lipoproteinak*: Kolesterolaren % 20 daramate, bere betebeharra gantzak gibelara eramatea da eta badirudi horri esker ateromak sahiesten dituztela, horregatik “kolesterol ona” deitzen zaie.

- *VLDL edo dentsitate oso baxuko lipoproteinak*: Kolesterolaren % 10 daramate, gehien bat triglizeridoz osatuta daude eta hauen kontzentrazioekin estuki erlazionatzen dira, LDL-ak bezala gantzak arteriak

depositatu ditzakete eta horregatik “kolesterol txarraren” multzoan sartzen dira.

Dislipemia, kolesteremia edo hiperlipidemia; triglizeridoek edota LDL-ek 1.3 Taulan ikus daitezkeen balio limiteak gainditzen dituztenean hasten da [15], honekin batera, orokorrean, HDL-en kontzentrazioen murrizketa ematen da. Beraz, alde batetik “kolesterol txarraren” eragin kaltegarria handiagotzen da eta bestetik “kolesterol onaren” lan onuragarria txikitzen da. Baldintza hauetan eritasun kardiobakularra jasateko aukerak areagotzen dira.

1.3. Taula Dislipemiarekin erlazionatutako lipidoen kontzentrazioen sailkapena

Sailkapena	Kolesterol totala (mg/dL)	HDL kolesterola (mg/dL)
Egokia	< 200	>60
Muga	200-239	40-60
Ezegokia	>240	<40 (gizonak) <50 (emakumeak)

Laburbilduz, dislipemia barruan sindrome metabolikoak barneratzen dituen bi arrisku faktore daude: triglizerido maila altuak eta HDL maila baxuak.

1.1.3. Diabetesa

Sindrome metabolikoaren arrisku-faktoreren artean eztabaida gehien sortu dituena da diabetesa. OME-k emandako definizioaren arabera diabetes mellitusa edo intsulinari erresistentzia izatea beharrezko baldintza zen sindrome

metabolikoa diagnostikatzeko. Hurrengo definizioen arabera ordea, beste arrisku-faktoreak bezala kontuan hartu da.

Diabetes Mellitusa edo 2. motako diabetesa, metabolismoaren nahasmendu bat da glukosa-maila odolean altua izateagatik (hipergluzemia) bereizten dena. Maila anomalo hauek zelulek, gorputzeko azukre kontzentrazioak kontrolatzen duen entzimaren, intsulinaren, aurkako erresistentzia garatu dutelako agertzen dira. Gorputzak ekoizten duen intsulina nahikoa ez izateagatik ere agertu daiteke. Diabetesa izateko aurrejoera genetiko handia egon arren bizimoduak ere eragin zuzena dauka. Dieta egokia jarraitzeak eta kirola egiteak gaixotasuna saihesten laguntzen du: diabetikoen % 55 inguruk loditasuna pairatzen dutela kalkulatzen da.

OME-k odoleko glukosa mailaren arabera egiten duen diabetesaren sailkapena 1.4. Taulan agertzen da. Bertan bi egoera prediabetiko ezberdintzen dira. Glukosa maila alteratuta bakarrik baraurik neurtzen den bitartean, glukosarekiko tolerantzia anormala diagnostikatzeko 75 g-ko azukre dosia hartu eta bi ordutara odoleko glukosa maila neurtu behar da. Kontzentrazioa 140-199 mg/dL tartean baldin badago gaixotasun hau diagnostikatzen da [6]. Bi egoera prediabetiko hauek denbora laburrean diabetes mellitusa garatzeko joera adierazten dute. Sindrome metabolikoaren diagnostikoan glukosa maila 100-110 mg/dL-tik gora egotearekin nahikoa da arrisku-faktore gisa hartzeko.

1.4. Taula OME-ren odoleko glukosa mailen araberako sailkapena

Sailkapena	Glukosa maila baraurik (mg/dL)
Normala	< 110
Glukosa maila alteratuta	110-126
Glukosarekiko tolerantzia anormala	>126
Diabete Mellitus	>126

Diabetesa eta sindrome metabolikoen arteko loturari dagokionez desadostasunak egon diren arren argi dago zerikusirik daukatela [16]. Izan ere, sindrome metabolikoa duten eta diabetikoak ez diren gaixoen artean, diabetesa garatzeko aukerak bost aldiz igotzen dira. Gainera, bi gaixotasun hauek aldi berean jasaten duten eriek gaitz kardiobaskularrak pairatzeko arrisku gehiago daukate [9].

1.2. SINDROME METABOLIKOAREN TRATAMENDUA

Sindrome metabolikoaren aurkako lehenengo neurria bizimoduaren aldaketa izan beharko litzateke eta bakarrik tratamendu farmakologikoa erabiltzea lortutako emaitzak behar bezalakoak ez direnean.

1.2.1. Terapia ez-farmakologikoa

Sindrome metabolikoa kontrolatzeko bizimoduan aldaketa txikiak baina garrantzitsuak ezarri behar dira. Ikerketa batzuen arabera pisu galtze txikiak

(% 5-10) sindromearen hainbat arrisku faktoreren hobekuntza dakar [17]. Beraz, dieta eta ariketa fisikoa ezinbestekoak dira gaixotasunari aurre egiteko.

NCEP-ak proposatutako dieta 1.5. Taulan ikus daiteke [18], funtsezkoa da azukre eta gantz saturatuen kontsumoa jaistea fruituena eta barazkiena igotzen den bitartean. Orokorrean egunero kaloria kopurua 500-1000 inguruan txikitzea komeni da.

1.5. Taula Sindrome metabolikoari aurre egiteko NCEP erakundeak proposatutako dieta

Elikagaia	Gomendatutako dosia
Gantz saturatuak	< % 7 Kcal totalak
Gantz poliinsaturatuak	≈ % 10 Kcal totalak
Gantz monoinsaturatuak	≈ % 20 Kcal totalak
Gantz kopuru osoa	% 25-35 Kcal totalak
Karbohidratoak	% 50-60 Kcal totalak
Proteinak	≈ % 15 Kcal totalak
Fibra	20-30 g/egun
Kolesterola	200 mg/egun
Kaloria kopuru totala	“prebentzio” pisua eusteko

Ariketa fisikoak, pisua galtzera laguntzeaz gain, sindrome metabolikoaren faktore guztiak kontrolatzera laguntzen du, horregatik gomendatzen da egunero gutxienez 30 minutu ariketa fisiko aerobikoa egitea eta posible bada ordu bat baino gehiago. Kontrolatu behar diren beste ohitura txarrak tabakoa, alkohola, kafeina eta gatzaren kontsumoa dira, hauek presio arteriala igotzen baitute. Izan

ere, tabakoa kontrolatu daitekeen heriotza goiztiarraren arrazoi garrantzitsuena da lurralde garatuetan [19]. Sinetsi daitekeenaren aurka heriotza gehiago eragiten ditu gaitz kardiobaskularrekin erlazionatuta, biriketako minbiziarekin baino. Droga honen kaltea argi ikusten da, Europa mailan 2004. urtetik hona ezarri diren tabakoaren aurkako legeen ondorioz bihotzekoen kopurua % 15 murriztu dela jakinda [20].

Dieta edo ariketa fisikoaren bidez pisu murrizketa lortzen ez bada apetitu depresoretara jo daiteke. Hauek gose sentsazioa murrizten dute eta sindrome metaboliko tratatzeko erabili daitezke farmako espezifikoagoak erabili gabe. Dena den, terapia farmakologikoa erabiltzeak ez du esan nahi dietarik eta ariketarik egin behar ez denik, izan ere hauek tratamendu farmakologikoa arrakasta izateko nahitaezkoak dira.

1.2.2. Terapia farmakologikoa

Sindrome metabolikoaren ezaugarri garrantzitsuenetarikoa arrisku faktore ezberdinez osatuta dagoela da. Hauek tratamendu ezberdinak behar dituzte eta ondorioz ezinezkoa da medikamendu bakarrarekin tratatzea, hori dela eta farmako ezberdinez osaturiko **terapia kardiobaskular konbinatua** beharrezkoa da. Orokorrean, terapia honetan, anithipertentsiboak erabiltzen dira presio arteriala gutxitzeko, hipolipemianteak gorputzeko gantz kontzentrazioak kontrolatzeko, eta intsulina edo antidiabetikoak glukosa mailak murrizteko. Gainera, nahiz eta inongo arrisku-faktoreri aurre ez egin, antikoagulatzaileak eta antiagregatzaile plaketarioak erabiltzen dira sarritan arterien buxadura ekiditzeko [21,22].

Sindrome metabolikoari aurre egiteko erabiltzen diren medikamentuek izugarrizko garrantzia dute gaur egungo industria farmazeutikoan. Mundu mailan 2010. urtean diru gehien mugiarazten duten 20 farmakoen artean, 7 terapia kardiobaskular konbinatuan erabiltzen dira. Guztien gainetik, Lipitor© nabarmentzen da, azken urteotan salmenta zerrenden lehen postua abantaila handiz okupatu duen hipolipemiantea (atorvastatina) [23].

1.2.2.1. Antihipertentsiboak

Farmako antihipertentsiboak aspaldi hasi ziren terapia kardiobaskularrean erabiltzen eta ondorioz ugariak eta ezagunak dira. Beren helburua presio arteriala egunean zehar egonkor mantentzea da arrisku tartetik kanpo, presio arteriala ziklikoki aldatzen baita lotan gaudenean minimoa izanik eta esnatzean igoz [24]. Presio arteriala kontrolatzeko terapia bizitza osorako da, hori dela eta ezin da bertan behera utzi eta hori gerta ez dadin eriaren betetzea errazten duten tratamenduak erabili behar dira.

Hipertentsioaren aurkako farmako anitzak beren eragite-mekanismoaren arabera sailka daitezke [25]:

- Diuretikoak: Eragite-mekanismoa odol-bolumenaren murrizketan oinarritzen da. Lehenengo aukera izan ohi dira naiz eta erabiltzen ziren dosi handiek eragindako efektu kaltegarriek kolokan ipini zituzten. Gaur egun terapia konbinatuan oso erabiliak dira beren efektu hipotentsorea gehigarria baita [26]. Familia honen barnean azpitalde ezberdinak daude: tiazidak (*hidroklorotiazida*), potasio aurreztaileak (*amilorida*)...

- Angiotensina-II Hartzailearen Antagonistak (AHA-II) edo “sartanak”: AT_1-hartzailearen blokatze espezifiko-selektiboaren bidez angiotensina-II-ren ekintzak inhibitzen dute, presio arterialaren igoeraren arduradun nagusiena dena. AHA-II gehienek itzulezina edo ez lehiakorra den blokatzea eragiten dute, Angiotensina-II maila igo arren blokatze hau itzulgarri bihurtzen ez delarik [27]. Farmako hauen artean *valsartan, telmisartan, candesartan*… aurkitzen dira.

- Angiotensinaren Entzima Bihurtzailearen Inhibitzaileek (AEBI) edo “priloek”: Angiotensina-I Angiotensina-II-n bihurtarazten duen entzima lehiakorki blokeatzen dute, honen sintesia oztopatuz [28]. Farmako hauen artean *enalapril, ramipril*… daude.

- Kaltzioaren antagonistak: Mintz zelularraren zehar Ca^{+2} iragatea galarazten dute. Efektu antihipertentsibo printzipala potentziatzen duen efektu natriuretikoa eragiteko ahalmena dute. Salgai dauden farmako askoren artean *amlodipino, lercandipino, lacidipino…* daude.

- Beta blokeatzaileak: Presio arteriala murrizten dute bihotzaren uzkurdura motelduz. Efektu antihipertentsiboa ere duen renina-angiotensina-aldosterona ardatzaren inhibizio partzialean eragiteaz gain, kolesterolaren metabolismoan efektu positibo bat eragiten dute. Erabilienak *bisoprolol* eta *atenolol* dira.

Estatu mailan paziente hipertentsoen artean egindako ikerketa batek gehien erabiltzen diren antihipertentsiboak AEBI-ak direla erakutsi du, ondoren, eta orden honetan: AHA-II, kaltzioaren antagonistak, tiazidak eta beta blokeatzaileak [29].

Askotan terapia eraginkorra izateko farmakoen konbinazioa beharrezkoa da, eta horretan diuretikoek berebiziko garrantzia dute, beste antihipertentsiboekin erabilita efektu sinergikoa lortzen baita (efektu totala banakako efektuen batuera baino handiagoa da). Beraz, terapia antihipertentsibo konbinatuan diuretiko bat erabili ohi da beste antihipertentsibo batekin, konbinazio arruntena tiazidek eta AHA-IIek (edo AEBI) osatutakoa izanda [30].

1.2.2.2 Hipolipemianteak

Nahiz eta eritasun kardiobaskularren eta kolesterolaren arteko harremana aspalditik ezagutu, farmako hipolipemianteen erabilera ez zen zabaldu lehen mailako prebentzio neurri bezala hartu ez ziren arte. Espainian 90eko hamarkadan kontsumoa izugarri igo zen, oraindik joera horrek jarraitzen duelarik [31]. Agertu ziren lehenengo medikamentu hipolipemianteak fibratoak izan ziren 60ko hamarkadan eta beste batzuk agertu arren (azido nikotinikoaren deribatuak, behazun-azidoen bahitzaileak...) kontsumituenak izan ziren estatinak, iritsi arte. Hauek tratamendu hipolipemiatearen merkatua goitik behera aldatu zuten eta egun, erabiltzen diren farmako hipolipemianteen % 90 dira.

2003. urtean kolesterolaren absortzioa galerazten zuen ezetimiba izeneko farmakoa agertu zen. Gantzen aurkako terapia aldatu behar zuen droga honek

harrera ona izan zuen baina estatinak ordezkatu gabe. Izan ere, ezetimiba gehien bat beste estatina batekin hartzen da konbinazio eraginkorra osatuz. Hipolipemianteen ezaugarri ezberdinak jarraian azaltzen dira:

- Fibratoak: PPARα errezeptore nuklearraren transkripzioa aktibatzen dute, honek gatz azidoak oxidatzen eta garraiatzen dituen proteinetan eta lipasa lipoproteikoan eragina du, honi esker VLDL eta LDLen katabolismoa handitzen da HDLen ekoizpenarekin batera. Triglizeridoen maila murrizteko oso aproposak dira baina miopatiak sor ditzakete. Farmako hauen artean *gemfibroziloa* da kontsumituena [32].

- Behazun azidoen bahitzaileak: behazun azidoen iraizketa igoaraziz kolesterolaren degradazioa eragiten dute eta horrela LDLena. Triglizeridoen maila ez da aldatzen eta HDLena apur bat igo daiteke. Hala ere gorputzak duen tolerantzia txarragatik oso gutxi erabiltzen dira, *colestiramina* eta *colestipol* dira ohikoenak [33].

- Azido nikotinikoaren deribatuak: VLDLen sintesia inhibitzen dute eta triglizeridoen garraioa gibelara oztopatzen dute, horrela triglizerido eta LDL mailak jaisten dira eta HDLenak igo. Emaitza oso onak lortu arren efektu sekundarioak direla eta estatu mailan bere erabilera kasu zehatzetara mugatuta dago. Familia honetako farmako erabiliena *niceritrol* da [34].

- Estatinak: Hidroxi-metil-glutaril koentzima A (HMGCoA) reduktasa inhibitzen dute, kolesterolaren sintesian funtsezkoa dena, honen ondorioz

LDLak eta VLDLak murrizten dira eta HDLak igotzen dira. Gaur egun dislipemiaren aurkako farmako onenak dira batez ere LDLa txikitu behar denean, gainera efektu antihipertentsiboa dutenez terapia konbinaturako oso interesgarriak dira. Honez gain medikuntzaren beste arlo garrantzitsu askotan ikertzen hasiak dira efektu terapeutiko asko eransten zaielako, batez ere HIESA, Alzehimer eta minbiziaren aurkako tratamenduetan. Gaur egun merkatuan dauden estatinak *Atorvastatina, Fluvastatina, Lovastatina, Pravastatina*, *Rosuvastatina* eta *Simvastatina* dira. 2001. urtean *Cerivastatina* merkatutik kendu zuten albo-ondorio arriskutsuak eragiten zituelako. Hala ere, ez dirudi beste estatinek inongo arazo larririk eragiten dutenik kasu batzuetan miopatiak (min muskularrak) kausatu eta transaminasak igo ditzaketen arren.

- Ezetimiba: Enterozitoetan dagoen Niemann Pick C1L1 proteina inhibitzen du hesteen kolesterol xurgaketa oztopatuz, honen ondorioz LDL gutxiago sortzen dira. VLDL mailak ere arinki txikitzen dira eta HDL-enak igo. Gehien bat estatinak txarto toleratzen direnean edo hauekin batera hartzen dira, medikamentu nahiko berria denez bere albo-ondorioei buruzko informazio gutxi dago [35].

- Beste hipolipemiante batzuk: Badaude oso gutxi erabiltzen edo frogetan dauden beste hipolipeminate batzuk, adibidez arritmiak kausatzen zituen eta merkatutik at dagoen probukol antioxidatzailea, arrainen gantz azido poliinsaturatuak, heparinak...

Gogoratu behar da gaixotasun kardiobaskularrak saihesteko “kolesterol txarra” (LDL) txikitzea komeni dela eta “kolesterol ona” (HDL) igotzea, triglizeridoekin (TG) erlazionatuta dagoen VLDL ere murriztu beharra dago. Hainbat ikerketek frogatu dute estatinak hilkortasuna gehien murrizten duten hipolipemianteak direla, seguru asko LDLa gehiago murrizteagatik eta presio arterialean eragin handiagoa izateagatik [36].

1.2.2.3 Antidiabetikoak

Orokorrean diabetesaren aurka gehien erabiltzen den tratamendua intsulinan oinarrituta dago. Hala ere, Diabetes Mellitusaren kasuan bakarrik erabiltzen da hau intsulinaren menpekoa denean edo ahozko antidiabetikoak eraginkorrak ez direnean. Azken farmako hauen artean bi multzo sailka daitezke, alde batetik intsulinaren jariatzea areagotzen dituztenak (sulfonilureak eta metiglinidak) eta bestetik intsulinaren aurreko erantzuna hobetzen dituztenak (biguanidak eta tiazolidindionak) [37].

- Sulfonilureak: Langerhasen irletako β-zelulen intsulina jariatzea eragiten dute. Eriak loditasun arazorik ez baditu lehen-aukerako tratamendu bezala erabiltzen dira. Hauen artean erabilienak *glibenclamida*, *gliquidona* eta *glimepirida* dira.

- Metiglinidak: Sulfonilureen portaera berdina dute, ezberdintasun bakarra zelula-hartzaileen beste gune batera lotzen direla da. Beren efektua azkarragoa da baina laburragoa ere. Epe luzerako ikasketa gutxi daudenez eta garestiagoak direnez ez dira gehiegi erabiltzen. Gainera

ezin dira sulfonilureekin batera hartu onura gehigarririk lortzen ez delako. *Repaglidina* da familia honen farmakorik ezagunena

- Biguanidak: Intsulinaren jariatzea piztu ordez glukosaren ekoizpen hepatikoa murrizten dute, hesteetan honen absortzioa murriztuz. Gainera lipido mailetan efektu lagungarria dute. Biguaniden artean *metformina* dago, farmako antidiabetikoen artean erabilienetariko bat. Egokia da loditasun arazoak dituzten pazienteentzat pisua handitzen ez duen antidiabetiko bakarra izateagatik.

- Tiazolidindionak: Gantz arrean, muskulu eskeletikoan eta gibelean intsulinaren aurkako erresistentzia txikitzen lan egiten dute PPARg hartzaile nuklearrari lotuz. Gibelaren glukosa ekoizpena murrizten dute eta honen erabilera periferian areagotzen dute. Familia honetako farmakoen artean *Rosiglitazona* zen ezagunena baina estatuan 2010-eko Abenduaren 29an bere merkaturatzea eten egin zen infartu arriskua igotzen zuen susmoagatik [38,39].

1.2.2.4. Antikoagulanteak eta antiagregatzaile plaketarioak

Farmako hauek ez dute, berez, inolako efektu onuragarririk sindrome metabolikoa osatzen duten arrisku-faktoreen aurka. Hala ere, oso erabilgarriak dira gaitz onen tronboak bezalako ondorio hilgarriak ekidin ditzaketelako. Nahiz eta antikoagulanteak eta antiagregatzaileak efektu berdina izan beraien mekanismoak guztiz ezberdinak dira eta kasu ezberdinetan erabiltzen dira.

Antikoagulanteak bihotzekoa, garun-infartu, tronbosi benoso sakona eta biriketako tronboenbolismoa saihesteko erabiltzen dira. Alde batetik ahoz hartzen diren K bitaminaren antagonistak daude. Izenak dion bezala K bitaminaren menpekoak diren koagulazio faktoreen aktibazioa galarazten dutenak. *Acenocoumarol* da erabiliena Europa mailan eta *Warfarina* AEBetan. Beste aldetik, injektagarriak diren *heparina* bezalako antikoagulanteak daude, ospitale mailan gehiago erabiltzen direnak.

Antiagregatzaile plaketarioak tronbosi arteriala eta arteroeskleriosia saihesteko erabiltzen dira plaketen elkarketa galarazten baitute tronboen sorkuntza ekidinez. Antikoagulatzaileek ezin dute lan hau egin zirkulazio arterialean efektu gutxi baitute. Dudarik gabe kostu-eraginkortasun onena eskaintzen duen farmakoa *aspirina* da. Hau, efektu analgesikoa izateaz gain dosi txikiagoetan terapia kardiobaskularrean erabili daiteke. Izan ere, lehenengo aukerako farmakoa da eta beste medikamentuak (*clopidogrel, ticlopidine…*) bakarrik erabiltzen dira aspirinarekin arazoak daudenean.

1.3. ANALISI FARMAZEUTIKOA

Farmako batek, sintetizatzen hasten denetik gero merkatuan agertu arte, kontrol prozesu zaila jasaten du. Farmako berri bat merkaturatzea 670 miloi euro inguru kostatzen da eta lehenengo pausuetatik salgai egotera bataz beste 15 urte pasatzen dira. Gainera, sintetizatzen diren 5000 farmakoetatik bakarrik 10 iristen dira fase klinikora eta 10 horietatik soilik hiruk berreskuratzen dute “I+G”-n (Ikerkuntza eta Garapena) inbertitutako dirua [40]. Prozesu luze honetan kimika

analitikoak garrantzi bizia dauka hainbat etapetan: farmakoaren egonkortasunaren segimenduan, ezpurutasunen analisian, farmakozinetika eta farmakodinamikaren ikerketan… Etapa bakoitzerako analisi metodo ezberdinak garatuko dira, beti ere beharrezkoa den sentikortasuna eta selektibitatea eskainiz. Hau bereziki garrantzitsua da fluido biologikoak analizatzean (plasma, gernua) farmakoak (eta metabolitoak) oso kontzentrazio baxuetan egon daitezkeelako eta interferentzia ugari daudelako [41].

Analisi metodo farmazeutikoak mota ezberdinetakoak izan daitezke. Metodo espektrofotometrikoak oso erabiliak izan dira formulazio farmazeutikoen printzipio aktiboaren kuantifikazioan, egonkortasun frogetan eta purutasun analisietan [42]. Hala ere, teknika hauen selektibitate ezak bere erabilera asko mugatzen du inolako banaketa teknikarik erabiltzen ez denean matrizean egon daitezkeen beste substantziek interferitzen baitute. Metodo elektroanalitikoak ere baliagarriak izan daitezke hainbat kasutan, teknika voltamperometrikoak adibidez, konposatu elektroaktiboen determinazioan erabili daitezke [43]. Beste aldetik, metodo inmunologikoak oso sinpleak eta erabiltzeko errazak dira baina garestiak dira eta aplikazio mugatua daukate.

Dena den, analisi metodo guztien artean erabilienak teknika hibridoak dira (hyphenated-techniques). Hauek banaketa teknika baten eta detekzio teknika baten (edo gehiago) konbinazioz sortzen dira [44,45]. 1960.ko hamarkadan gas kromatografia-masa espektrometria (GC-MS) agertu zenetik asko dira aurkitu daitezkeen mota honetako instrumentuak: bereizmen altuko likido kromatografia-

masa espektrometria (HPLC-MS), elektroforesi kapilarra-masa espektrometria (CE-MS), gas kromatografia-elektroi harrapaketa detektagailua (GC-ECD)…

Teknika hauen guztien artean LC-MS da arrakastatsuena analisi farmazeutikoan eskaintzen duen aldakortasunagatik. Analisi azkarrak eta oso sentikorrak eskaintzeaz gain, metabolitoen *in-vitro* eta *in-vivo* ikerkuntzetarako, analisi kiralerako eta ezpurutasunen eta degradazio produktuen identifikaziorako oso erabilgarria da [46-48]. Gainera, ESI eta MALDI ionizazio metodoen garapenarekin lehen analizatu ezin ziren biomolekulak (petpidoak, azido nukleikoak…) aztertu daitezke, metabolomika, proteomika eta genomika bezalako diziplina hasiberrientzako ezinbestekoa dena [49].

Detektagailu espektrofotometrikoak akoplatuta duten likido kromatografia sistemak ere oso erabilgarriak dira. Nahiz eta aurrekoen aukera guztiak ez eskaini, askoz ere merkeagoak direnez analisi farmazeutikoko laborategi gehienetan aurki daitezke diodo edo fluoreszentzia detektagailuak akoplatuta duten HPLC sistemak [50].

Banaketa teknikei dagokionez kromatografia likidoan 2004. urtean aurrerapen handia egon zen: presio ultra handia eta 2 mikra azpiko partikulak konbinatzen dituen ultra-bereizmen altuko kromatografia (UPLC edo UHPLC). Honi esker, analisi azkarragoak eta bereizmen hobekoak lortzen dira, denbora eta erreaktiboak (“kimika berdea”) aurreztuz. Eboluzio honen eskutik heldu ziren detekzio tekniken hobekuntzak, banaketa teknikek eskaintzen zuten abiadurari egokitzeko [51].

Beste aldetik, analisi prozesu batean, analisi teknikaz gain laginaren aurretratamendua kontuan hartu beharreko etapa da. Analisi farmazeutikoaren bezalako arlo zabalean makina bat aurretratamendu teknika aurkitu daitezke bibliografian matrize biologikoen analisirako. Analisia LC-MS-en bidez egiten bada laginaren injekzio-zuzena egin daiteke edo plasmaren kasuan proteinen prezipitazio soilaren ostean [52]. Likido-likido eta fase solidozko erauzketak (LL/SPE) tradizionalak ere oso ohikoak dira eta azken urteotan hauen miniaturizaziotik sortutako likido-likido edo fase solidozko mikroerauzketak (LLME edo SDME/SPME) zabaltzen ari dira [53-56]. Zentzu honetan garatzen ari diren beste teknika eraberritzaileak badaude: *Hollow-fiber*, *Pipette tip extraction, Stir Bar Sorptive Extraction* [57-63]…

1.4. METODO BIOANALITIKOEN BALIDAZIOA

Farmako baten edo bere metabolitoen determinaziorako garatu den metodo analitikoak honen bioerabilgarritasunari, farmakozinetikari eta metabolismoari buruzko informazioa lortzen laguntzen du. Lortutako informazioa hain garrantzitsua da, non farmakoaren merkaturatzea baimendu edo eragotzi baitezake. Ondorioz, metodo analitikoaren fidagarritasuna ziurtatu behar da, horretan datza, izan ere metodoen balidazioa.

Nahiz eta metodo bioanalitikoen betebeharreko baldintza den, ez dago momentuz metodo bioanalitikoen balidaziorako gida bateratu bat. Hala ere, gehien erabiltzen diren irizpideak ICH [64] (International Conference on Harmonisation) eta FDA [65] (Food and Drug Administration) erakundeenak dira. Irizpide hauek

jarraituz metodoa farmakoaren determinaziorako, aztergai den matrizean, emaitza fidagarriak eskaintzeko gai denetz ikusten da.

Orokorrean, matrize biologikoetan analitoen determinazio kuantitatiborako garatutako metodoetan hurrengo parametroak kontuan izaten dira.

Selektibitatea: Determinatzen diren analitoak eta interferitu dezaketen substantziak banatzeko ahalmena da. Metodo analitiko kromatografiko baten selektibitate maila, banaketa kromatografikoaren kalitatearen eta erabilitako detekzio motaren berezko selektibitatearen araberakoa da. Modu honetan, arreta handiagoa jarri beharko da selektibitate eskaseko detektagailu bat (UM adibidez, eta are gehiago uhin luzera baxuetan) erabiltzerakoan selektibitate handiagoko batekin aritzerakoan baino (MS). Selektibitate gutxiko detektagailuekin, beraz, erauzketa prozesu eta banaketa kromatografiko hobeak beharrezkoak dira alde batetik interferentzia gehiago eliminatzeko eta bestetik, hauek agertzen direnean, analitoetatik bereizteko.

Gainera, farmakoen analisian garrantzitsua da analitoak koadministratu daitekeen bestelako konposatuekin aztertzea, hauekin interferitzen dutenetz ikusteko. Puntu honek terapia konbinatuan bere biziko garrantzia du.

Linealtasuna: Kalibrazio kurba, senaile analitikoaren eta analito kontzentrazioaren arteko erlazioa modu sinpleenean ezartzen duen funtzioa da. Linealtasuna, ezarritako eremu batean, metodoak, kalibrazio ereduaren bitartez, analitoaren kontzentrazioarekiko zuzenki proportzionalak diren seinaleak emateko gaitasuna da.

Lan-tartea: Goiko eta beheko kontzentrazio mugen arteko tartea da. Bertan metodoak linealtasun, zehaztasun eta prezisio egokia eskaintzen duela frogatuta egon behar da.

Zehaztasuna: Kontzentrazio teorikoa eta analisian lortutako emaitzaren arteko ezberdintasun maila da. Errore erlatiboa (%ER) bezala adierazten da: kontzentrazio teorikoaren eta neurtutako kontzentrazioen arteko portzentajezko ezberdintasuna. Orokorrean, metodo bioanalitikoetan egunean eta egun ezberdinen arteko zehaztasuna ebaluatu behar da, onartzen den %ER maximoa % 15koa delarik (kuantifikazio mugan % 20koa izan daiteke).

Prezisioa: Prozedura berdinari jarraituz lortu diren datu analitikoen arteko bat etortze maila (edo errepikakortasuna) da eta desbiazio estandar erlatiboaren bidez (%DEE) adierazten da. Orokorrean, metodo bioanalitikoetan egunean eta egun ezberdinen arteko prezisioa ebaluatu behar da, onartzen den %DEE maximoa % 15koa delarik (kuantifikazio mugan % 20koa izan daiteke),

Detekzio eta kuantifikazio mugak: Zehaztasun eta doitasun onargarriarekin detektatu eta kuantifikatu daitezkeen kontzentrazio baxuenak, hurrenez hurren. Metodo bioanalitikoetan kuantifikazio mugak garrantzi gehiago du, detekzio limitearen kontzeptua gutxitan eskatzen delarik. Parametro hauek metodoaren sentikortasunarekin lotuta daude.

Egonkortasuna: Fluido biologiko baten baitan aurkitzen den analito baten egonkortasuna, hainbat faktoreren menpekoa da. Hala nola, kontserbazio egoera, analitoaren propietate fisiko-kimikoak, matrize biologiko mota, fluidoa biltzen duen

ontzia eta lagin-prestaketa egiterakoan gertatzen diren manipulazioen araberakoa. Analitoaren egonkortasuna prozesu analitiko osoan zehar txekeatzea beharrezkoa denez egonkortasun azterketa ezberdinak egin behar dira balidazio prozesuan:

- Izozte-desizoketa prozesuaren egonkortasuna
- Epe luzerako egonkortasuna
- Tratamendu-etaparen zehar laginaren egonkortasuna
- Lagin prozesatuaren egonkortasuna
- Estandar disoluzioen egonkortasuna

Parametro hauetaz gain, ***berreskurapena*** eta ***matrize efektua*** ere kontuan izan behar dira nahiz eta balidazioari begira hauei buruzko irizpideak ondo definituta ez egon. Berreskurapena analitoen erauzketa etaparen efikazia da eta berez ez da parametro kritikoa balidazioari begira, baina metodoaren sentikortasunean eragin zuzena duenez aztertu beharreko parametroa da. Matrize efektua edo supresio ionikoa bakarrik kuantifikazioa masa espektrometriaren bidez egiten denean kontuan hartzen da. Honen bidez matrizean dauden konposatu endogenoek analitoaren seinale analitikoan duten eragina ebaluatzen da [66].

Sendotasuna metodo analitikoak analisian zehar gerta litezkeen aldaketa txikien aurrean duen erantzuna da [67-69]. Hots, fase mugikorren konposizioaren, pH balioen, tenperaturaren, eta abarren aldaketa arinek nolako eragina duten

lortutako emaitzetan. Kasu honetan ere, balidazio gidek ez dute onartze irizpide zehatzik ezartzen.

1.5 HELBURUAK

Sindrome metabolikoa gaixotasun pandemikoa da gaur egun eta honi aurre egiteko gehienetan terapia kardiobaskular konbinatua erabiltzea beharrezkoa da. Ikuspuntu farmazeutiko eta medikutik, hartzen diren farmakoen printzipio aktiboen eta metabolitoen kontzentrazio plasmatikoak determinatzea oso baliagarria izan daiteke. Adibidez, farmakoen farmakozinetika eta farmakodinamika ulertzeko, tratamenduaren eraginkortasuna aztertzeko beharrezkoak direnak. Gainera, bide metabolikoa partekatzen duten drogen kasuan koadministrazioaren ondorioz sortutako elkarrekintzak edo albo-efektuei buruzko informazio baliagarria lortu daiteke. Horretaz gain, ustekabezko edo nahita eragindako intoxikazioak edo dopina bezalako okerreko erabilerak detektatzeko baliogarriak dira.

Hau guztia kontuan izanda, farmako kardiobaskularren aldibereko analisia ahalbidetzen duten metodo analitikoen garapena garrantzitsua da. Hala ere, hau ez da lan erraza propietate fisiko-kimiko eta portaera fisiologiko ezberdinak dituzten konposatuen analisia orokorrean erronka latza baita. Gainera, matrize biologikoen konplexutasunak eta analitoek izan dezaketen kontzentrazio baxuek metodo selektibo eta sentikorrak erabilarazten dituzte. Zailtasun hauek farmako kardiobaskularren aldibereko analisiari buruzko publikazio gutxi egotea [70-73] eragin dute, nahiz eta ohiko konbinazioen analisia ahalbidetzen duten hainbat

metodo analitiko garatu diren [74-77]. Gure ikerkuntza laborategia farmakoen analisiaren alor honetan dagoen hutsunea betetzen saiatu da azken urteetan metodo analitiko ezberdinak garatuz (Ikusi laugarren eranskina "*Appendix IV")* [78,79].

Ikerkuntza-lan honen helburua, beraz, giza plasman terapia kardiobaskular konbinatuan erabiltzen diren farmakoen aldibereko analisirako metodo analitikoen garapena izan da. Kontuan izanda xede hori lortzeko behar den selektibitatea eta sentikortasuna, lan honetan zehar gehien erabili izan den teknika analitikoa LC-MS/MS izan da.

Lan honetan zehar hurrengo pausuak garatu dira:

- Aztergai diren analitoen MS zatiketen ikasketa.
- Terapia kardiobaskular konbinatuan erabiltzen diren farmako ezberdinen analisirako LC-MS/MS metodoak garatzea.
- Analitoen ikasketa fotometrikoa eta fluorimetrikoa.
- Metodo kromatografikoen optimizazio sistematikoa.
- Ingurune azidoan Fluvastatina farmakoaren degradazioa aztertzea eta bere degradazio produktuak karakterizatzea.

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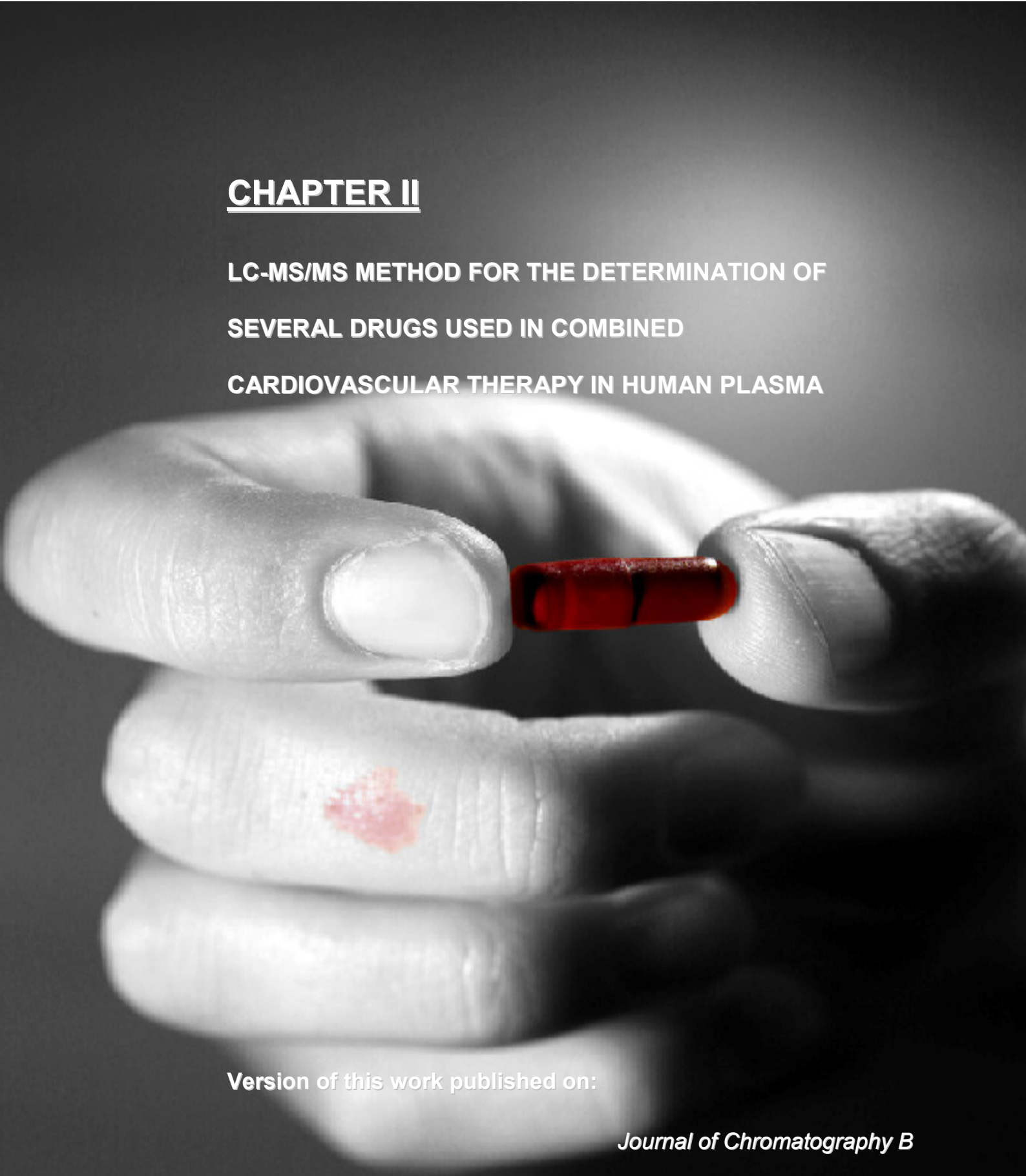
CHAPTER II

LC-MS/MS METHOD FOR THE DETERMINATION OF SEVERAL DRUGS USED IN COMBINED CARDIOVASCULAR THERAPY IN HUMAN PLASMA



Version of this work published on:

Journal of Chromatography B

Volume 878, Issue 28, 15 October 2010, Pages 2685-2692

Abstract

A simple, fast and validated method is reported for the simultaneous analysis, in human plasma, of several drugs usually combined in cardiovascular therapy (atenolol, bisoprolol, hydrochlorothiazide, chlorthalidone, salicylic acid, enalapril and its active metabolite enalaprilat, valsartan and fluvastatin) using high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI) working in multiple reaction monitoring mode (MRM). Separation of analytes and internal standard (pravastatin) was performed on a Luna C18(2) (150 x 4.6 mm, 3 µm) column using a gradient elution mode with a run time of 15 min. The mobile phase consisted of a mixture of acetonitrile and water containing 0.01% formic acid and 10 mM ammonium formate at pH 4.1. Sample treatment consisted of a simple protein precipitation with acetonitrile, enabling a fast analysis. The method showed good linearity, precision (between 0.7% and 12.7% RSD%) and accuracy (between 0.9% and 14.0% RE%). Recoveries were within 68-106% range and the ion-suppression was not higher than 22% for any analyte. The method was successfully applied to plasma samples obtained from patients under combined cardiovascular treatment.

Keywords: Metabolic syndrome, bioanalysis, LC-MS/MS, validation, cardiovascular therapy.

2.1. Introduction

Cardiovascular diseases are the first cause of mortality worldwide, causing around the 30% of the global deaths, a number which will significantly increase in the following years according to the World Health Organization (WHO). These illnesses have been traditionally associated to the western society, but in fact 80% of the deaths take place in low- and middle-income countries due to the troubles to access medicines and their unhealthier diet [1,2].

Actually, inappropriate diet and other bad habits like alcohol and tobacco consumption are some of the factors closely related to the risk of suffering from a cardiovascular illness. Other important risk factors are hypertension, dyslipidemia and diabetes. The suffering of some of these pathologies simultaneously is known as metabolic syndrome [3,4].

Due to the factors involved, different drugs must be used to fight the metabolic syndrome, thus a combined cardiovascular therapy is necessary. This therapy usually involves different antihypertensive (diuretics, angiotensin II receptor antagonists (ARA-II) and β-blockers), lipid lowering drugs (statins, ezetimibe), antiplatelet (salicylic acid, clopidogrel) and antidiabetic (metfomin, glibenclamide) drugs [5,6].

The monitoring of the plasmatic concentrations of cardiovascular drugs is crucial for understanding their pharmacokinetics and pharmacodynamics. Moreover, it provides valuable information about possible interactions. Therefore the simultaneous determination of these analytes turns interesting. This is a

complex task since analytes from different families have different physicochemical properties. The complexity of the biological matrices and the low concentrations of some analytes require the development of sensitive and selective determination methods. In this context, one of the most suitable techniques to achieve this objective is the liquid chromatography-tandem mass spectrometry (LC-MS/MS).

On the other hand, although quantitative analysis for different drug families have been widely developed, very few analytical methods have been focused on the simultaneous analysis of drugs from different families [7-9]. In this work, a simple and fast method for the determination of drugs used in combined cardiovascular therapy has been developed taking into account some of the most usually prescribed drugs in our geographical area: atenolol, bisoprolol (β-blockers), hydrochlorothiazide, chlorthalidone (diuretics), salicylic acid (active metabolite of aspirin, antiplatelet), enalapril (ACEI) and its active metabolite enalaprilat, valsartan (ARA-II), and fluvastatin (statin).

Although most of these drugs have been individually analyzed in plasma by LC coupled to mass spectrometry techniques [10-14], in this work, a simple and fast LC-MS/MS method has been developed for their simultaneous analysis in human plasma. The suitability of the method has been demonstrated by validation, carried out following the guidelines proposed by Food and Drugs Administration (FDA) [15] and International Conference on Harmonisation (ICH) [16]. The method has also been successfully applied to samples obtained from patients under cardiovascular treatment.

2.2. Experimental

2.2.1 Instrumentation

The chromatographic system consisted on a Waters Alliance 2695 separation module connected to a Waters 996 photodiode array detector (PDA) and to a Micromass Quatro Micro tandem quadrupole mass spectrometer operated in electrospray ionization mode (Milford, MA, USA). Chromatograms were recorded by means of a computer and treated with the aid of the software MassLynx 4.0 from Waters.

A Phenomenex Luna C18(2), 150 mm x 4.6 mm I.D., 3 µm, 100 Å column was used to perform the separation. A Phenomenex C18 , 4x3 mm, Security guard cartridge was placed prior to the analytical column in order to prevent its degradation.

Plasma samples were centrifuged in an Eppendorf model 5804R centrifuge (Hamburg, Germany) after protein precipitation procedure (PPT). The supernatant was evaporated under a nitrogen stream using a Zymark Turbovap evaporator LV (Barcelona, Spain).

The pH was measured with a Crison GPL 22 pH-meter (Barcelona, Spain) using a Crison glass-combined electrode model 5209 with a reference system Ag/AgCl and KCl 3 M saturated in AgCl as electrolyte.

2.2.2 Chemical and reagents

Sodium fluvastatin and valsartan were kindly supplied by Novartis Pharma AG (Basel, Switzerland) and bisoprolol fumarate by Merck (Darmstadt, Germany). Chlorthalidone was kindly supplied by Ciba-Geigy (Barcelona, Spain). Enalapril maleate, atenolol, sodium pravastatin, salicylic acid and hydrochlorothiazide were supplied by Sigma-Aldirch (St. Louis, MO, USA). Enalaprilat was synthesized in our laboratories and characterized by ^{1}H-NMR, ^{13}C-NMR and elemental analysis. Chemical structures of these compounds are shown in Figure 2.1.

Ammonium formate, 99% purity, was purchased from Alfa Aesar (Karlsruhe, Germany) and formic acid, LC-MS quality, from Fluka (Buchs, Switzerland).

HPLC quality methanol and acetonitrile were obtained from VWR (Barcelona, Spain). Purified water from a Milli-Q Element A10 water system (Millipore, Milford, MA, USA) was used in the preparation of buffer and reagent solutions.

Drug-free human plasma samples were purchased from the Blood Bank of Galdakao Hospital (Biscay, Basque Country) and collected in polypropylene tubes to be frozen at -20 ºC.

2.2.3. Standard solutions and spiked plasma samples

Standard solutions between 500 and 10000 mg/L were prepared in 100% acetonitrile for valsartan and salicylic acid, and in acetonitrile:water (90:10) for the

other drugs. These solutions were diluted with acetonitrile:water to obtain the necessary multicomponent working solutions for spiking plasma. A 6 mg/L pravastatin solution in acetonitrile was also prepared from a 1000 mg/L stock solution to use as internal standard (IS).

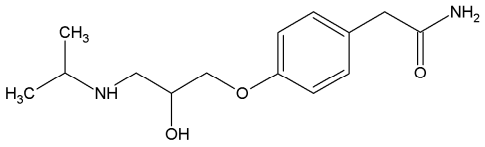
In order to obtain representative plasma for method development, a plasma pool was prepared by mixing in a proportional way eight plasmas obtained from different healthy volunteers.

During the optimization step of the extraction procedure plasma samples were spiked with 950 µg/L of each analyte.

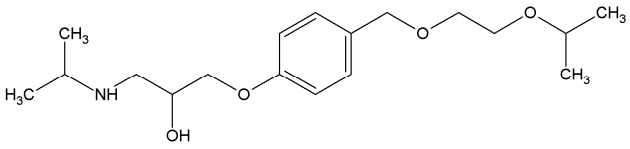
Calibration standards were prepared by spiking a pool plasma with the working solutions. Calibration curve for atenolol was built from 2 to 1000 µg/L (n=8), enalaprilat from 2.5 to 250 µg/L (n=7), salicylic acid from 187.5 to 7500 µg/L (n=7), hydrochlorothiazide from 20 to 2000 µg/L, chlorthalidone from 5 to 500 µg/L (n=7), enalapril from 3.5 to 350 µg/L (n=7), bisoprolol from 1.5 to 150 µg/L (n=7), valsartan from 10 to 5000 µg/L (n=9) and fluvastatin from 1 to 500 µg/L (n=8). In all the cases the IS was added (1000 µg/L) prior to the extraction procedure.

Quality Control (QC) samples used for stability assays were prepared by spiking a drug-free plasma with all the analytes, in low and high concentrations, using the appropriate working standard solution volumes, taking into account their working ranges.

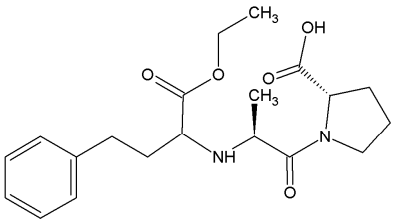
Atenolol



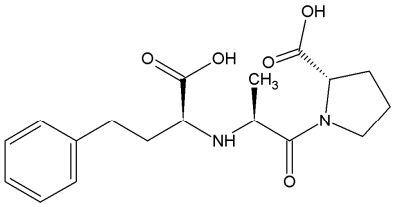
Bisoprolol



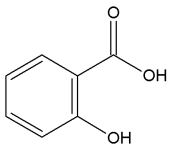
Enalapril



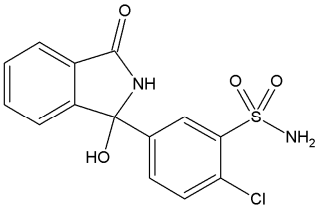
Enalaprilat



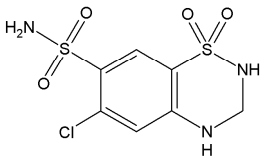
Salicylic acid



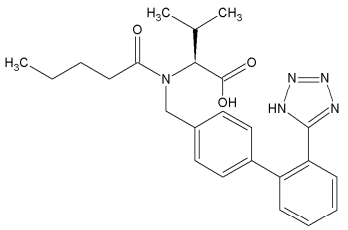
Chlorthalidone



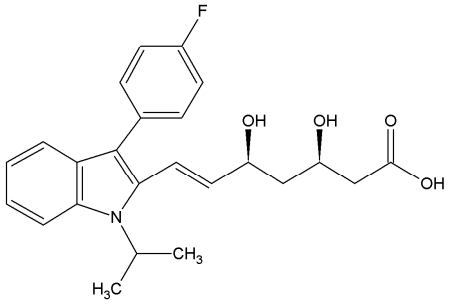
Hydrochlorothiazide



Valsartan



Fluvastatin



Pravastatin (I.S.)

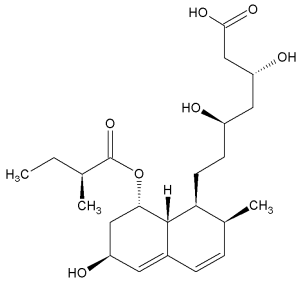


Figure 2.1. Chemical structures of the analyzed compounds

2.2.4. Chromatographic conditions

The mobile phase consisted of solvent A (acetonitrile with 0.01% formic acid and 10 mM ammonium formate) and solvent B (0.01% formic acid with 10 mM ammonium formate (pH 4.1)) delivered in gradient mode (Table 2.1) at a flow rate of 0.8 mL/min. This chromatographic separation was optimized based on a previously developed method for chlorthalidone, fluvastatin and valsartan [7]. After the PDA the flow was split so that only 0.28 mL/min reached the mass spectrometer. The mobile phases were prepared by a 1:20 dilution of a 0.2% formic acid and 200 mM ammonium formate water solution. Before the chromatographic use, the mobile phases were filtered through a 0.45 µm type HVLP Durapore membrane filter from Millipore.

Table 2.1. Optimized gradient for LC-MS/MS analysis

Time (min)	Organic phase (%)	Aqueous phase (%)
0	5	95
2	28	72
6	40	60
10	90	10
15	90	10
16	5	95
20	5	95

During the chromatographic analysis, samples were kept at 10 ± 1 ºC in the autosampler and the injected volume was 30 µL. The chromatographic separation

was performed at 40 ± 1 ºC. The column was re-equilibrated for 6 min after the gradient separation.

2.2.5. MS conditions optimization

In order to optimize the transitions used for quantitation purposes, analytes' fragmentation was studied by direct infusion of 10 µg/mL standard solutions in methanol. First of all, mass scans were carried out using different cone voltages (5, 15, 30 and 45 V), both in ESI+ and ESI-. After choosing the adequate cone voltage and ionization mode, product ions scans were carried out with different collision energies (5, 15, 30 and 45 eV) in order to find the most intense fragment for each precursor ion. Then, these fragmentations were used in the multiple reaction monitoring (MRM) method.

During the optimization processes the following parameters were fixed according to the manufacturer's recommendations: capillary voltage: 3.2 kV (for ESI+) or 2.6 (for ESI-); desolvation gas (N_2) flow: 450 L/h, source temperature: 120 ºC and desolvation temperature: 300 ºC. The scan time for each analyte was set at 1.0 s for mass and daughters scans.

2.2.6. Extraction procedure

2.2.6.1. Stability of the compounds

Since extraction procedure involves sample evaporation under a N_2 stream, stability of the analytes at different temperatures was studied prior to the optimization process.

For this purpose 800 µg/L concentration samples in acetonitrile: water (60:40) were prepared, simulating the conditions required for protein precipitation (PPT). These samples were kept in the turbovap at 60, 70 or 80 ºC (n=3) for a time slightly longer than they needed for a total evaporation (40, 30 and 20 minutes respectively). Then, they were reconstituted with 100 µL methanol:aqueous phase (60:40) and injected in the LC system. The stability of the compounds was tested by comparing the obtained chromatographic responses with those obtained for the standard solutions.

2.2.6.2. Optimized extraction procedure

Different parameters involved in the extraction procedure were studied by experimental design, using *The Unscrambler* program [17]: precipitant agent (methanol and acetonitrile), precipitant agent's temperature (room and fridge) and centrifugation temperature (20 ºC and 4 ºC).

The extraction procedure was carried out as follows: A 0.5 mL plasma aliquot was spiked with 100 µL IS (pravastatin) to achieve concentration of 1000 µg/L. Next, 0.65 mL acetonitrile, at room temperature, was added followed by

vortex-mixing and centrifugation for 5 minutes at 10000 rpm and 20 ºC. Supernatant was transferred to 6 mL glass tube and it was evaporated to dryness under N_2 stream at 80 ºC for 15 minutes. The eluent was then reconstituted with 100 µL methanol: aqueous phase (60:40) and vortex mixed in order to obtain the correct dissolution of all the compounds. Then, it was filtered and transferred to autosampler vials. 30 µL aliquots were injected into the LC system for analysis.

2.2.7. Validation

In order to demonstrate the suitability of the developed analytical method, validation was carried out following FDA [15] and ICH [16] recommendations: linearity, working range, intra and inter assay accuracy and precision, limit of quantitation (LOQ), selectivity and stability were tested for each analyte. Recovery and matrix effect were also studied.

Recovery and matrix effect were determined at three different concentrations following the strategies reported by Matuszewski et al. [18]. Three sets of samples were used for this purpose: standards prepared in reconstitution solution (A, n=3), plasma samples spiked after the PPT and before the evaporation step (B, n=5) and plasma samples spiked before PPT (C, n=5). Recovery was calculated by comparing the areas of B and C samples (Rec(%) = C_{area}/B_{area} x100) and matrix effect by comparing the areas of A and B samples (ME (%)= B_{area}/A_{area} x100). A matrix effect value higher than 100% indicates enhancement, whereas a lower one indicates suppression effects.

The method's selectivity was tested by analyzing, under optimized chromatographic conditions, blank human plasma samples from six different sources, and by comparing them with spiked plasma samples at a concentration close to the LOQ.

Taking into account the wide concentration ranges, $1/x^2$ statistical weight was applied in order to obtain the most reliable calibration curves [19]. Calibration curves consisting of triplicate calibration standards for each concentration were analyzed on three different days for linearity studies. They were built by plotting the corrected areas for each concentration level versus the nominal concentration of each calibration standard, taking into account the selected weighting factor.

LOQ were calculated by interpolating the value obtained from multiplying 10 times the average signal-obtained from 6 different drug free plasma samples in the calibration curve. Then, LOQ were tested in order to prove that they fulfil the validation criteria: relative standard deviation (RSD) and RE < 20% and signals at least 10 times higher than the blank's response. The working ranges were defined considering the LOQ, the normal therapeutic dosage and the time needed to achieve the maximum plasmatic levels [7,10-14,20-24].

In each batch, three samples, corresponding to low, medium and high concentration levels, were assayed in sets of five replicates in order to evaluate the intra and inter day accuracy and precision. The deviation of the mean from the true value, expressed as RE, served to measure the accuracy. In the same way RSD was used to express the precision.

Short-term stability (bench top, room temperature), long-term stability (frozen at the intended storage temperature for 1 month), stability after three freeze-thaw cycles and stability in the autosampler were tested at low and high concentrations. The procedure also included an evaluation of analytes stability in the stock solutions.

2.2.8. Plasma sample collection

14 blood samples were collected from different patients under treatment with a combination of the studied drugs between 1 and 20 h after the oral intake of the drugs. Blood samples were immediately transferred into tubes containing ethylenediaminetetraacetic acid (EDTA) and gently mixed. Then, they were centrifuged at 3500 rpm for 10 minutes at 4 ºC. The plasma supernatant was carefully separated from blood cells and collected in polypropylene tubes to be frozen at -20 ºC until analysis. This procedure underwent the revision of the ethical committee of the Basque Country University.

2.3. Results

2.3.1 MS fragmentation and MRM conditions

The mass scans obtained for each analyte using different cone voltages and the product scans for each molecular ion (daughter scans) at different collision energies are attached at the end of this chapter.

According to these mass spectra, the optimal precursor and fragment ions for quantitation and confirmation were chosen. The results are shown in Table 2.2,

together with their optimal cone voltage, collision energy and ionization mode. The dwell time for the analysis was set at 0.2 s and the inter-scan delay at 0.1 s. In Figure 2.2 a chromatogram obtained for a calibration standard under optimized conditions can be observed.

Table 2.2. Optimal MS/MS conditions for analyzed compounds and their quantitation limits. First row corresponds to quantitation transition and the second one to the confirmation transition.

Analyte	ESI mode	Precursor ion (m/z)	Fragment ion (m/z)	Cone Voltage (V)	Collision Energy (eV)	LOQ (µg/L)
Atenolol	+	267.0	144.9	30	30	2.0
		267.0	132.9	30	30	
Enalaprilat	+	349.0	206.0	15	15	2.5
		349.0	117.0	15	30	
Salicylic acid	-	136.8	92.7	15	15	75.0
		136.8	64.5	15	30	
Hydrochlorothiazide	-	295.8	268.8	30	15	20.0
		295.8	204.8	30	15	
Chlorthalidone	+	338.8	321.8	15	15	5.0
		338.8	242.9	15	30	
Enalapril	+	376.9	234.0	15	15	3.5
		376.9	129.9	15	30	
Bisoprolol	+	326.0	115.8	30	15	1.5
		326.0	73.6	30	30	
Valsartan	+	436.0	234.9	15	15	2.0
		436.0	207.0	15	30	
Fluvastatin	+	411.9	224.0	15	30	1.0
		411.9	266.0	15	15	
Pravastatin (I.S.)	-	422.9	321.0	30	15	-

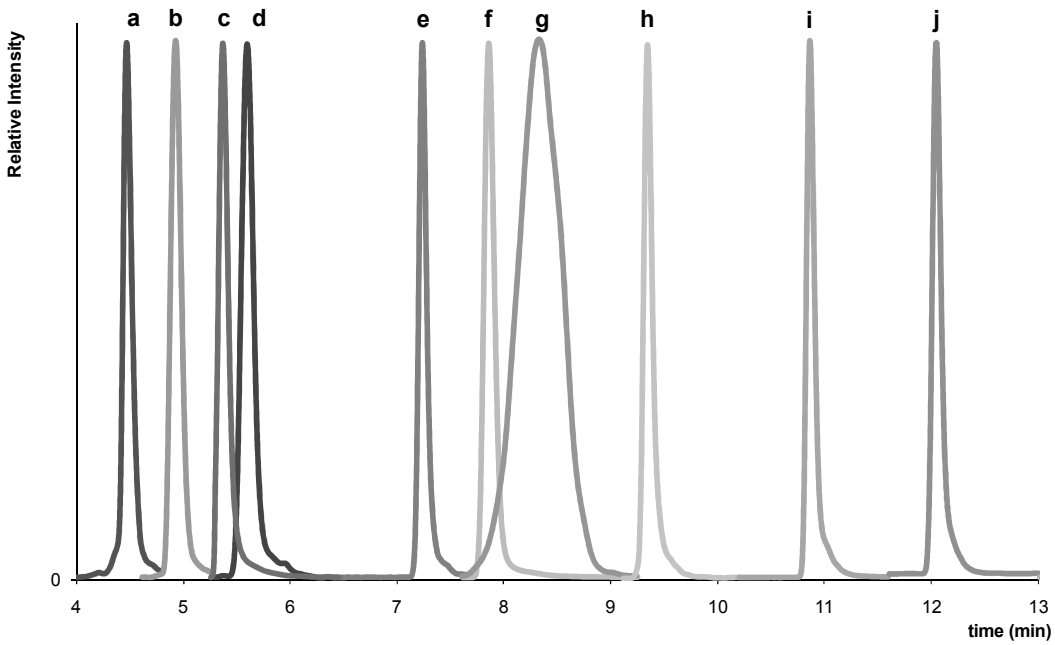


Figure 2.2. LC–MS/MS chromatogram corresponding to a calibration standard solution (normalized heights): atenolol (a), enalaprilat (b), salicylic acid(c), hydrohlorothiazide (d), chlorthalidone (e), bisoprolol (f), enalapril (g), pravastatin (I.S) (h), valsartan (i) and fluvastatin (j). LC and MS conditions described in section 2.3.1.

2.3.2 Extraction procedure

The relative errors obtained by comparing the chromatographic signals from samples evaporated at 80 ºC for 20 minutes with those ones obtained from standard solutions were lower than 3.5%. Therefore, no degradation of the analytes was observed and 80 ºC was chosen as the temperature for the evaporation step since it allowed the fastest extraction procedure.

In the sample treatment, optimization step neither of the parameters studied by experimental design showed a significant effect in the analytes recoveries within the studied ranges at 95 % confidence level. Thus, the parameters were chosen in order to achieve the simplest sample treatment

2.3.3 Validation

2.3.3.1. Recovery and matrix effect

Obtained recoveries and matrix effect for each analyte at three different concentration levels are shown in Table 2.3 Recoveries ranged between 68% and 106% and matrix effect between 78% and 119%. Non significant differences in those values were observed among different concentration levels for most of the analytes. Matrix effect values suggest ion suppression for all the analytes except for the fluvastatin. Higher RSD values for this analyte could be attributed to its photodegradation [25,26].

Table 2.3. Recovery percentages (R%) and matrix effect (ME%) obtained for all the cardiovascular drugs studied at three different concentration levels. Expressed as mean value ± standard deviation (s).

	Low Concentration		Middle Concentration		High Concentration	
Compound	**R% (±s)**	**ME% (±s)**	**R% (±s)**	**ME% (±s)**	**R% (±s)**	**ME% (±s)**
Atenolol	79 ± 3	78 ± 3	90 ± 3	80 ± 2	89 ± 3	93 ± 5
Enalaprilat	76 ± 5	88 ± 5	68 ± 9	82 ± 3	76 ± 2	82 ± 4
Salicylic acid	85 ± 6	85 ± 7	85 ± 2	84 ± 4	85 ± 1	91 ± 3
Hydrochlorothiazide	100 ± 7	98 ± 7	97 ± 11	97 ± 5	97 ± 7	97 ± 6
Chlorthalidone	91 ± 6	84 ± 8	87 ± 8	82 ± 3	93 ± 4	84 ± 3
Enalapril	91 ± 3	92 ± 5	93 ± 9	85 ± 2	90 ± 2	87 ± 3
Bisoprolol	98 ± 4	93 ± 4	96 ± 4	89 ± 1	93 ± 3	92 ± 2
Valsartan	88 ± 7	97 ± 3	92 ± 11	90 ± 6	88 ± 12	88 ± 2
Fluvastatin	96 ± 8	107 ± 6	106 ± 8	102 ± 13	97 ± 10	119 ± 16

2.3.3.2 Selectivity

In the present study, selectivity has been studied by analyzing 6 plasma samples from different healthy volunteers. As the ICH guideline requires [16], the studied blanks showed neither area values higher than 20% of the LOQ's areas at the analyte's retention times nor higher than 5% of the IS area at its corresponding retention time. Representative chromatograms obtained from drug-free plasma and plasma sample spiked with a concentration equivalent to the LOQ are shown for each analyte in Figure 2.3.

2.3.3.3 Linearity, LOQ and working range

LOQ calculated from a relationship S/N equal to 10 are shown in Table 2.2 and the chromatographic signals obtained for a plasma sample spiked with these concentrations in Figure 2.3 Salicylic acid showed the highest quantitation limit, it might be due to its poor ionization and to the fact that the fragment ion is quite small facilitating the appearance of interferences. Anyway, concentration of salicylic acid is usually quite higher than this LOQ and it would not be a problem when analyzing samples of patients under treatment with aspirin.

Calibration standards did not exceed the limit values established by FDA and ICH neither for the accuracy or the precision. Therefore the models were accepted for the linear ranges established: from 2 to 1000 µg/L for atenolol, from 2.5 to 250 µg/L for enalaprilat, from 187.5 to 7500 µg/L for salicylic acid, from 20 to 2000 µg/L for hydrochlorothiazide, from 5 to 500 µg/L for chlorthalidone, from 3.5

to 350 µg/L for enalapril, from 1.5 to 150 µg/L for bisoprolol, from 10 to 5000 µg/L for valsartan and from 1 to 500 µg/L for fluvastatin.

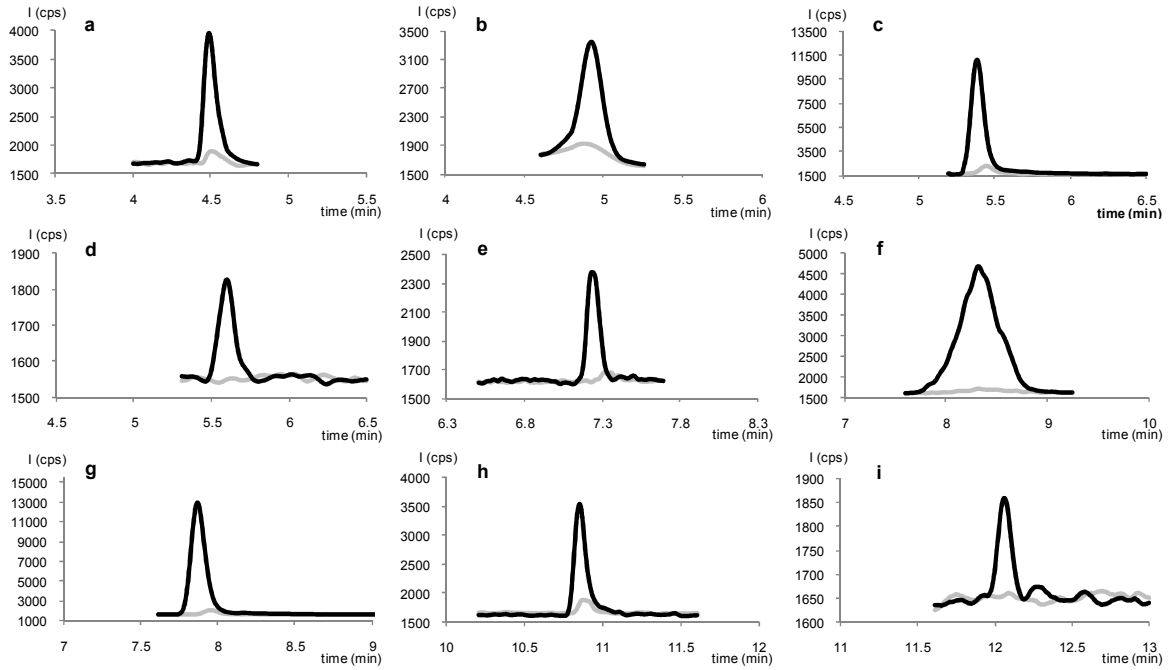


Figure 2.3. MRM windows' chromatographic signals corresponding to a plasma sample spiked with quantitation limit concentrations: (a) atenolol (2 µg/L), (b) enalaprilat (2.5 µg/L), (c) salicylic acid (75 µg/L), (d) hydrochlorothiazide (20 µg/L), (e) chlorthalidone (5 µg/L), (f) enalapril (3.5 µg/L), (g) bisoprolol (1.5 µg/L), (h) valsartan (2 µg/L), and (i) fluvastatin (1 µg/L) with their blank signal (gray lines). LC and MS conditions described in Section 3.1.

2.3.3.4. Accuracy and precision

Plasma samples spiked with low, medium and high concentrations of drugs were prepared and their concentrations were obtained from the interpolation on their respective calibration curves. The intra and inter day accuracy (RE) and precision (RSD) is summarized in Table 2.4. As it can be seen intra-day precision varied between 1.8% and 10.0%, and inter-day precision between 0.7% and

12.7%. Intra-day accuracy varied from 1.4% to 13.0% and inter-day accuracy from 0.9% to 14.0%. Therefore, obtained values agree with the FDA and ICH recommendations.

Table 2.4. Intra- and inter-day accuracy (RE%) and precision (RSD%) values obtained for the analytes at three different concentrations.

		RSD (%)		RE (%)	
Compound	**Concentration** (µg/L)	**Intra-day**	**Inter-day**	**Intra-day**	**Inter-day**
Atenolol	2 (LOQ)	9.0	3.1	9.8	12.9
	100	5.4	2.7	7.8	8.4
	1000	2.4	3.8	11.4	9.0
Enalaprilat	2.5 (LOQ)	10.0	0.7	1.9	1.8
	25	5.1	4.6	3.8	1.9
	250	1.8	2.4	2.0	0.6
Salicylic acid	187.5	1.8	5.9	2.1	4.3
	750	4.0	0.7	10.8	11.4
	7500	3.4	2.1	8.5	9.7
Hydrochlorothiazide	20 (LOQ)	9.7	12.7	7.2	6.0
	200	8.2	6.3	6.6	6.1
	2000	3.2	9.0	5.5	3.6
Chlorthalidone	5 (LOQ)	6.6	3.3	2.3	1.9
	50	3.8	4.5	3.0	0.9
	500	2.9	1.9	5.6	3.7
Enalapril	3.5 (LOQ)	4.4	1.0	1.4	1.6
	35	3.9	3.4	3.4	2.2
	350	2.9	1.6	2.2	2.2
Bisoprolol	1.5 (LOQ)	6.4	2.3	13.1	14.0
	15	3.9	3.7	12.5	10.8
	150	4.0	2.4	9.4	10.0
Valsartan	10	6.2	2.9	3.1	3.5
	100	3.7	1.2	5.1	5.4
	5000	2.7	1.2	11.5	10.3
Fluvastatin	1 (LOQ)	5.0	11.4	3.8	8.0
	50	3.3	7.0	4.7	4.3
	500	4.2	7.3	7.6	8.3

2.3.3.5. Stability

Stability was studied by comparing the corrected area of QC samples with those samples subjected to stability tests. No significant changes in corrected areas were noticed after three freeze-thaw cycles, long term storage or after 24 h in autosampler storage. Therefore, all analytes seem to be stable in those conditions, both at high concentration as well as at low concentration. Working solutions used in sample preparation were also stable for at least one month.

When stability in room conditions (bench top, room temperature) was studied degradation of fluvastatin was observed, surely due to the photodegradation of the molecule reported by Mielcarek et al. [25]. This degradation was not significant during required analysis time, but in order to avoid it, samples light exposure was minimized and amber vials were used.

2.3.4. Application to real samples

The developed method has been applied to plasma samples obtained from 14 patients under cardiovascular treatment with different combinations of atenolol, enalapril, hydrochlorothiazide, aspirin, chlorthalidone, bisoprolol, valsartan or fluvastatin. These patients were also co-administered with other β-blockers (metoprolol), statins (atorvastatin, simvastatin), ARA-II (olmesartan), calcium channel blockers (amlodipine, felodipine), antidiabetic drugs (metformin, glibenclamide) and other drugs not involved in cardiovascular therapy (omeprazole, allopurinol, salmeterol…)

Table 2.5. Plasmatic concentrations of the studied drugs obtained for patients under combined cardiovascular therapy (SA: salicylic acid).

N	Sex	Administered drugs	Time after oral intake	Concentration µg/L (± s)
1	F	Atenolol 50 mg	2 h	225 ± 2
		Aspirin 250 mg (as SA)	20 h	1837 ± 44
2	F	Bisoprolol 10 mg	2 h	18.0 ± 0.3
		Valsartan 160 mg	2 h	152 ± 3
3	M	Enalapril 20 mg	1 h	183 ± 2
		Enalaprilat	-	49.2 ± 0.3
		Chlorthalidone	1 h	364 ± 3
		Aspirin 100 mg (as SA)	20 h	362 ± 10
4	F	Hydrochlorothiazide 12.5 mg	2 h	43 ± 3
		Valsartan 80 mg	2 h	355 ± 6
5	F	Enalapril 50 mg	Unknown	32.1 ± 0.2
		Enalaprilat		62.7 ± 0.4
		Aspirin 100 mg (as SA)	20 h	79 ± 8
6	F	Atenolol 50 mg	Unknown	75.1 ± 0.8
		Enalapril 20 mg	Unknown	121 ± 1
		Enalaprilat		35.5 ± 0.2
7	M	Hydrochlorothiazide 12.5 mg	1 h	94 ± 3
		Bisoprolol 10 mg	1 h	53 ± 1
8	F	Hydrochlorothiazide 25 mg	2 h	174 ± 5
		Valsartan 160 mg	2 h	581 ± 10
		Fluvastatin 80 mg	12 h	15.6 ± 0.2
9	F	Enalapril 20 mg	3 h	66.5 ± 0.5
		Enalaprilat		18.4 ± 0.1
		Chlorthalidone 50 mg	3 h	209 ± 2
10	M	Atenolol 100 mg	2 h	391 ± 4
		Enalapril (5mg)	> 24 h	<LOQ
		Enalaprilat		2.6 ± 0.1
		Chlorthalidone	2 h	148 ± 1
11	F	Hydrochlorothiazide 25 mg	1 h	195 ± 6
		Fluvastatin 80 mg	1 h	46.4 ± 0.5
12	F	Bisoprolol 5 mg	3h	37.6 ± 0.7
		Valsartan 160 mg	3h	2516 ± 43
13	F	Hydrochlorothiazide 25 mg	8 h	446 ± 13
		Valsartan 160 mg	8 h	4019 ± 44
		Fluvastatin 80 mg	2h	6.7 ± 0.1
14	M	Atenolol 100 mg	12 h	310 ± 3
		Chlorthalidone 25 mg	11 h	228 ± 2
		Fluvastatin 80 mg	1 h	28.7 ± 0.3

Real samples were collected early in the morning, 1 to 3 hours after the oral intake (around the time of maximum concentration) for most of the analytes. Statins are usually prescribed to be taken late in the evening, so not to change the habits of the patients samples were collected in the morning, 12 hours after of the oral intake instead of taking them at the time of maximum concentration. The same criterion was applied to the salicylic acid which was administered 20 hours before the blood extraction.

A total of 14 samples were analyzed with the developed method, the plasmatic concentrations obtained are gathered in Table 2.5 and the chromatographic signals obtained for one of them (patient number 3) are shown as example in Figure 2.4

2.4. Conclusions

Even if several methods have been reported for the quantitation of drugs prescribed against hypertension, high cholesterol level or diabetes, very few have been developed for the simultaneous determination of drugs used in combined cardiovascular therapy. In this way, this paper reports a method for the simultaneous determination of two diuretics (chlorthalidone and hydrochlorothiazide), two β-blockers (atenolol and bisoprolol) two ACEI (enalapril and its active metabolite enalaprilat), one statin (fluvastatin), one antiplatelet agent (salicylic acid) and one ARA-II (valsartan).

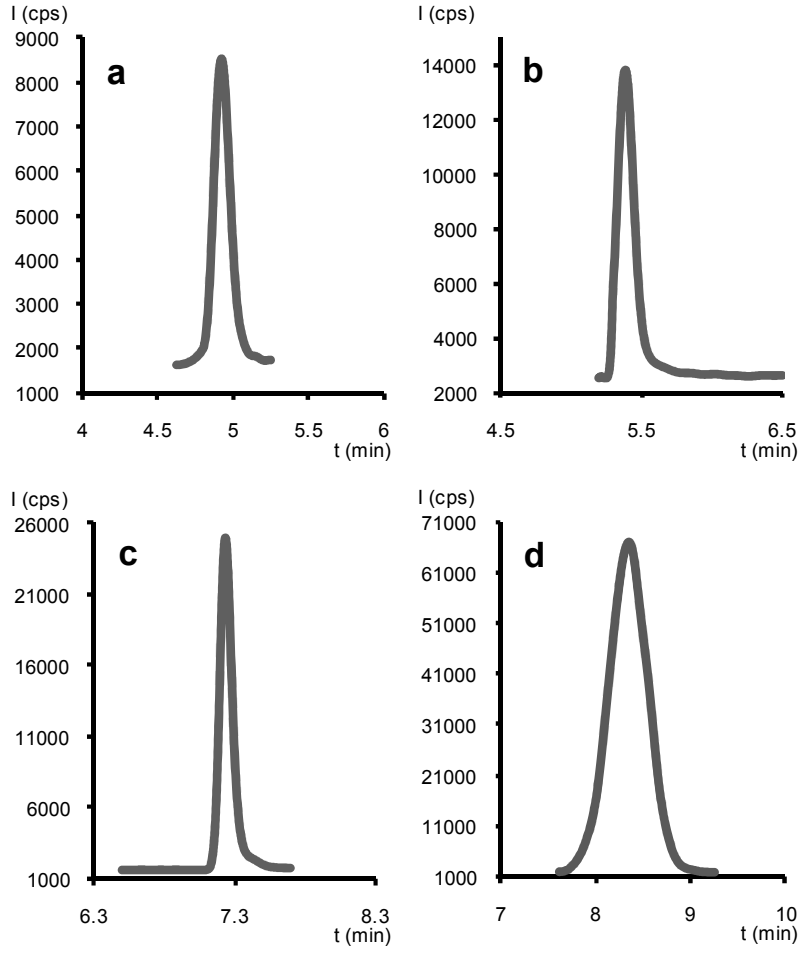


Figure 2.4. MRM windows' chromatographic signals corresponding to a plasma extract from patient number 3: enalaprilat (a), salicylic acid (b), chlorthalidone (c), and enalapril (d). LC and MS conditions described in Section 3.1.

The proposed LC-MS was fully validated and showed an appropriate specificity, linearity, sensitivity and precision for all the analytes studied. It was successfully applied to the determination of these drugs in plasma samples obtained from patients under combined cardiovascular therapy.

The calibration curve of salicylic acid covers a shorter range than other methods previously reported [12,22], but taking into account that the purpose of

this analytical method is the determination of samples from patients under cardiovascular treatment, where salicylic acid is used in lower concentration levels, this range is adequate for the expected plasmatic concentrations.

The developed method appears to be the first direct method for the simultaneous analysis of the studied drugs.

Acknowledgments

The authors thank the University of Basque Country and Ministry of Science and Technology and Ministry of Science and Innovation for financial support (GIU07/02, CTQ2006-11285, CTQ2009-11690) and the pharmaceutical companies for the kind supply of drugs studied. Oskar Gonzalez thanks the University of Basque Country for his predoctoral grant. N.Ferreirós thanks the Government of the Basque Country for the post-doctoral grant. The authors also thank the Health Centre of Vitoria-Gasteiz's old town, belonging to the Basque Health Service-Osakidetza (Basque Government), and specially Mª Mercedes Saez Etxarri, Ana M. Iturri, Javier Nafarrate and Angel Elorriaga for supplying blood samples from patients under cardiovascular treatment.

2.5. References

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APPENDIX I

MASS AND PRODUCT ION SCANS OF THE STUDIED ANALYTES

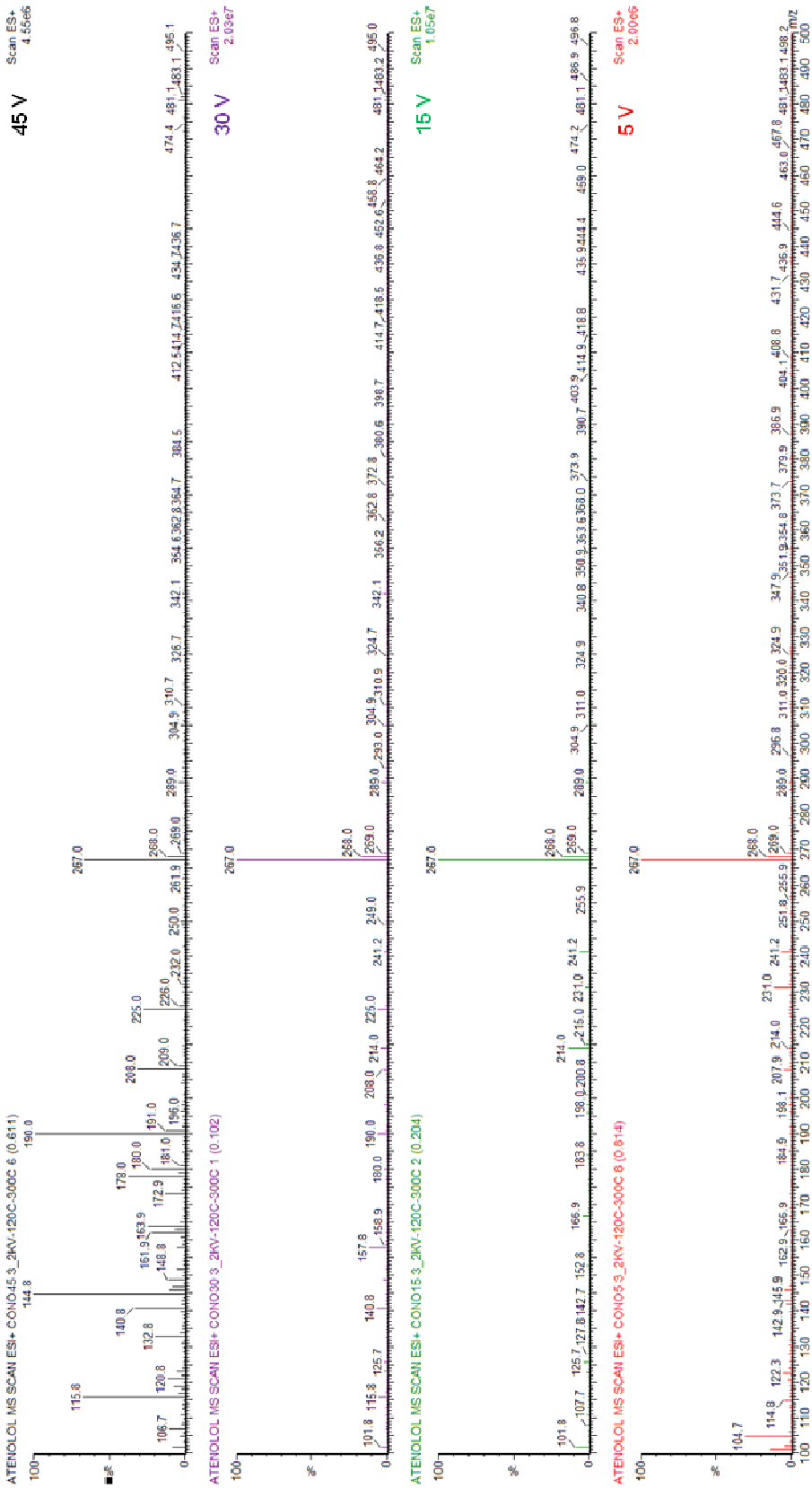

Figure 2.5. Mass scan (ESI+) of Atenolol at different cone voltages under conditions described in section 2.2.5. The most intense molecular ion ($[M+H]^+$=267.0) is obtained using 30 V cone voltage

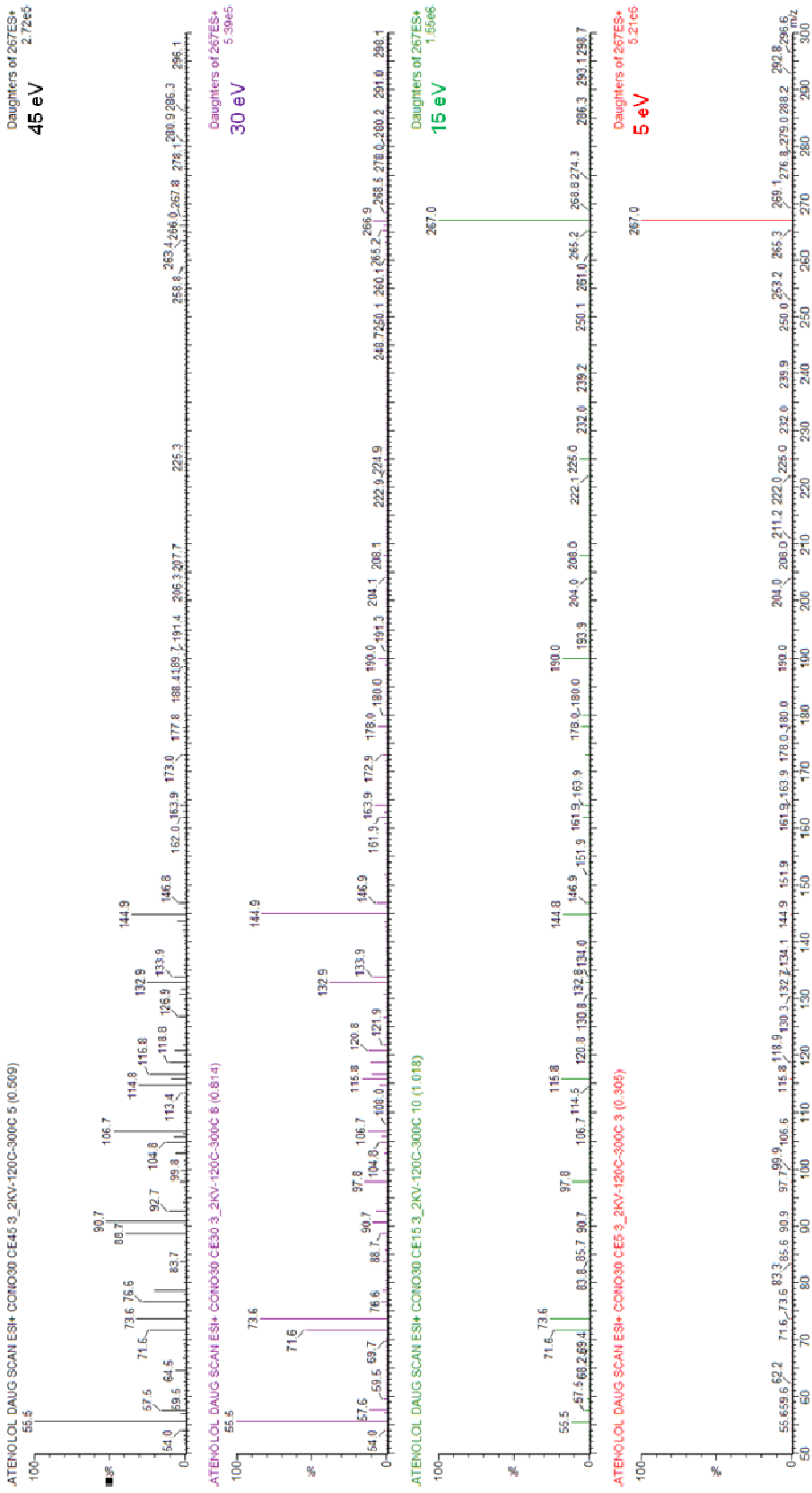

Figure 2.6. Product ion scan (ESI+) of Atenolol ($[M+H]^+$=267.0) using a 30 V cone voltage, at different collision energies under conditions described in section 2.2.5. The chosen fragment ion ($[M+H]^+$=144.9) offers the highest signal with a 30 eV collision energy.

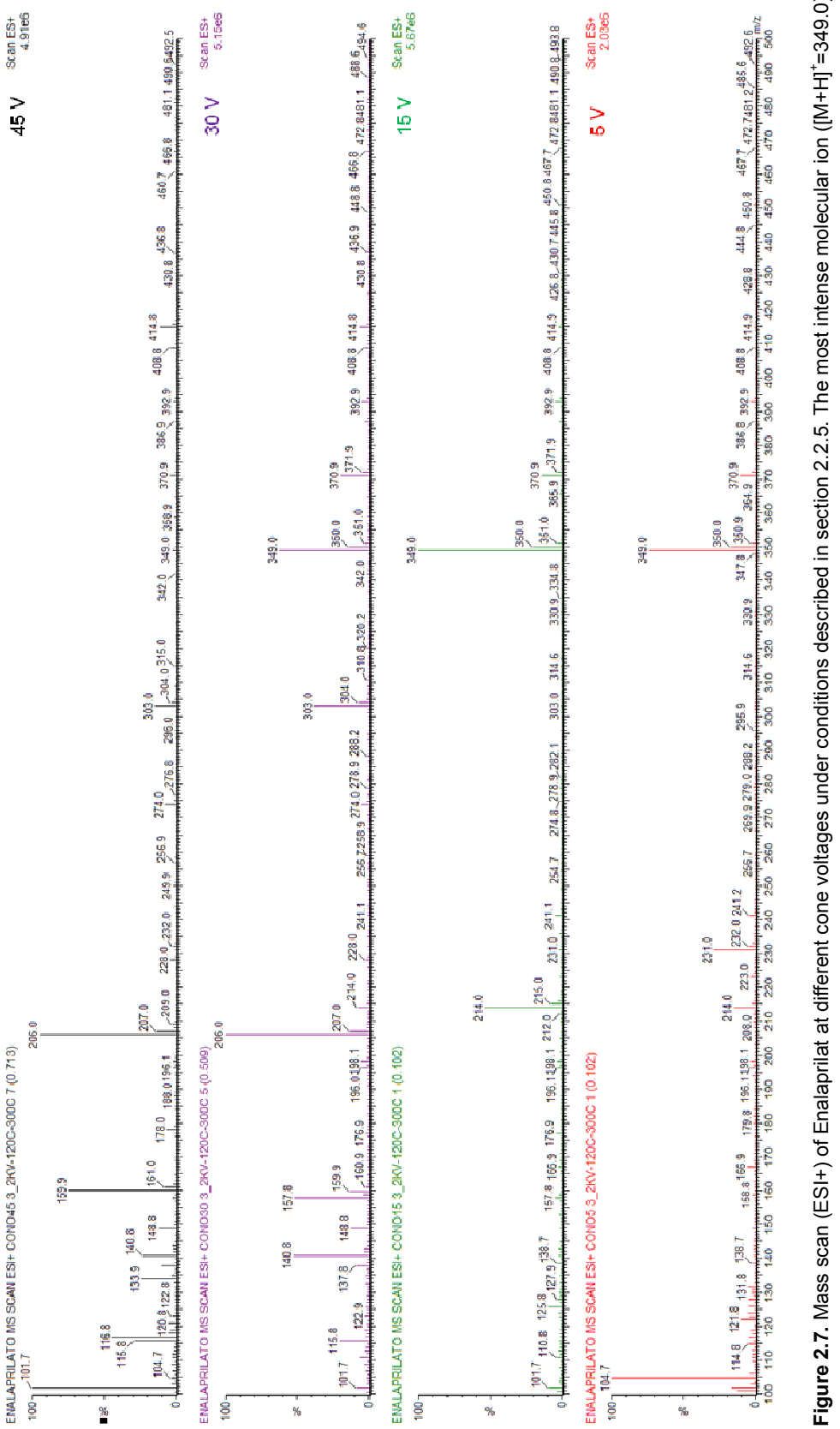

Figure 2.7. Mass scan (ESI+) of Enalaprilat at different cone voltages under conditions described in section 2.2.5. The most intense molecular ion ($[M+H]^+$=349.0) is obtained using 15 V cone voltage

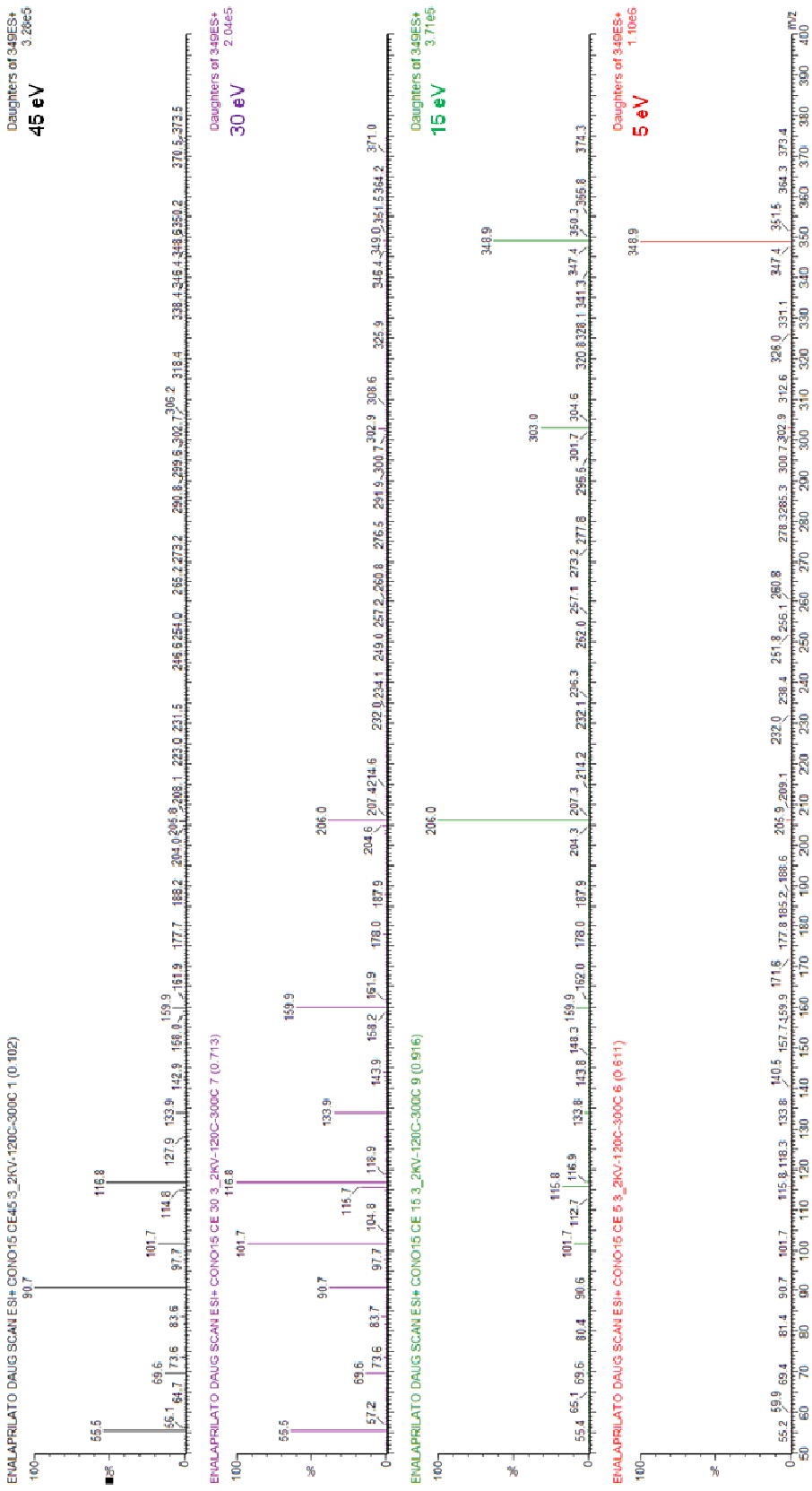

Figure 2.8. Product ion scan (ESI+) of Enalaprilat ($[M+H]^+$=349.0) using a 15 V cone voltage, at different collision energies under conditions described in section 2.2.5. The chosen fragment ion ($[M+H]^+$=206.0) offers the highest signal with a 15 eV collision energy.

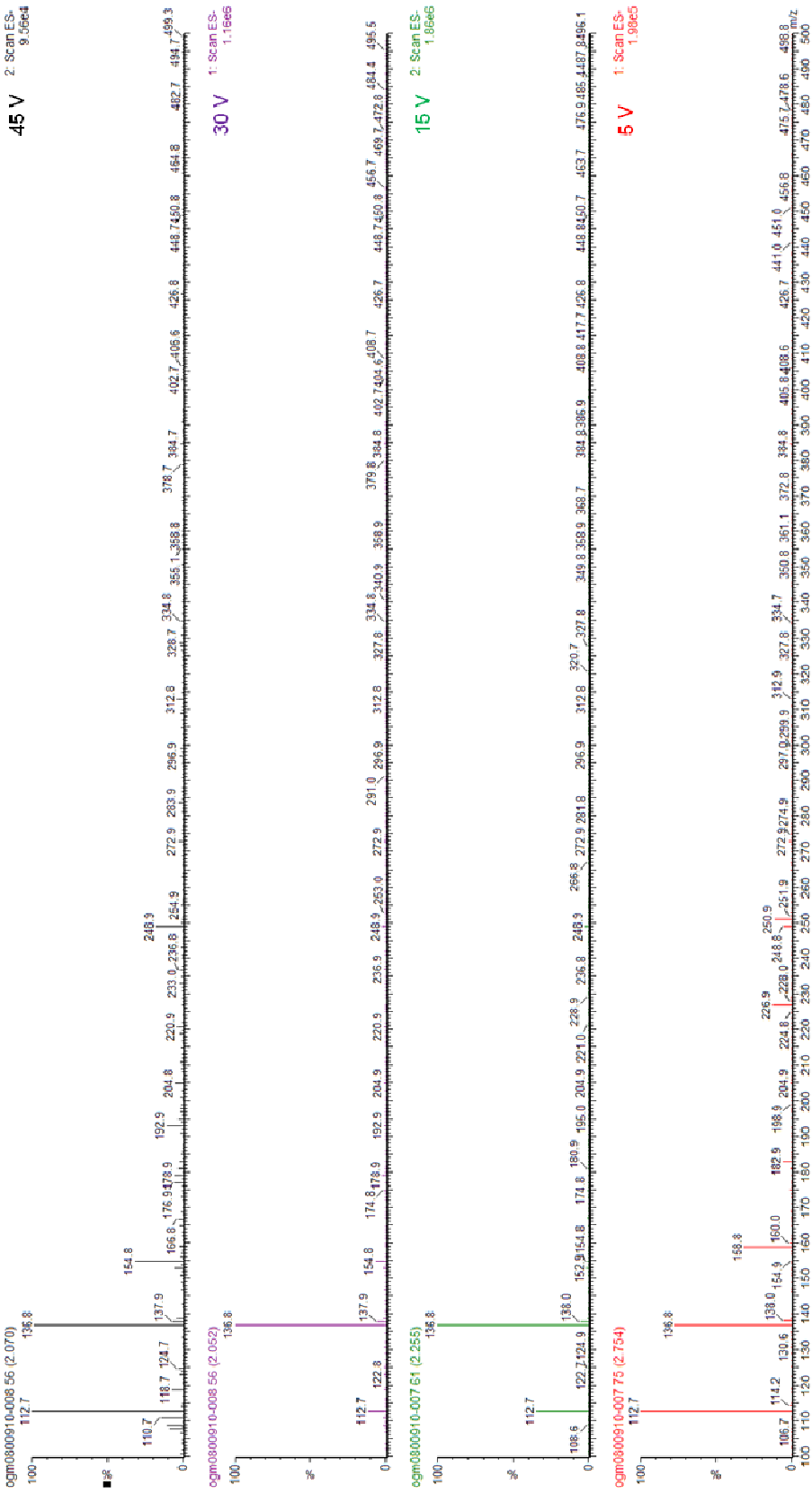

Figure 2.9. Mass scan (ESI-) of Salicylic acid at different cone voltages under conditions described in section 2.2.5. The most intense molecular ion ([M-H]$^-$ =136.8) is obtained using 15 V cone voltage.

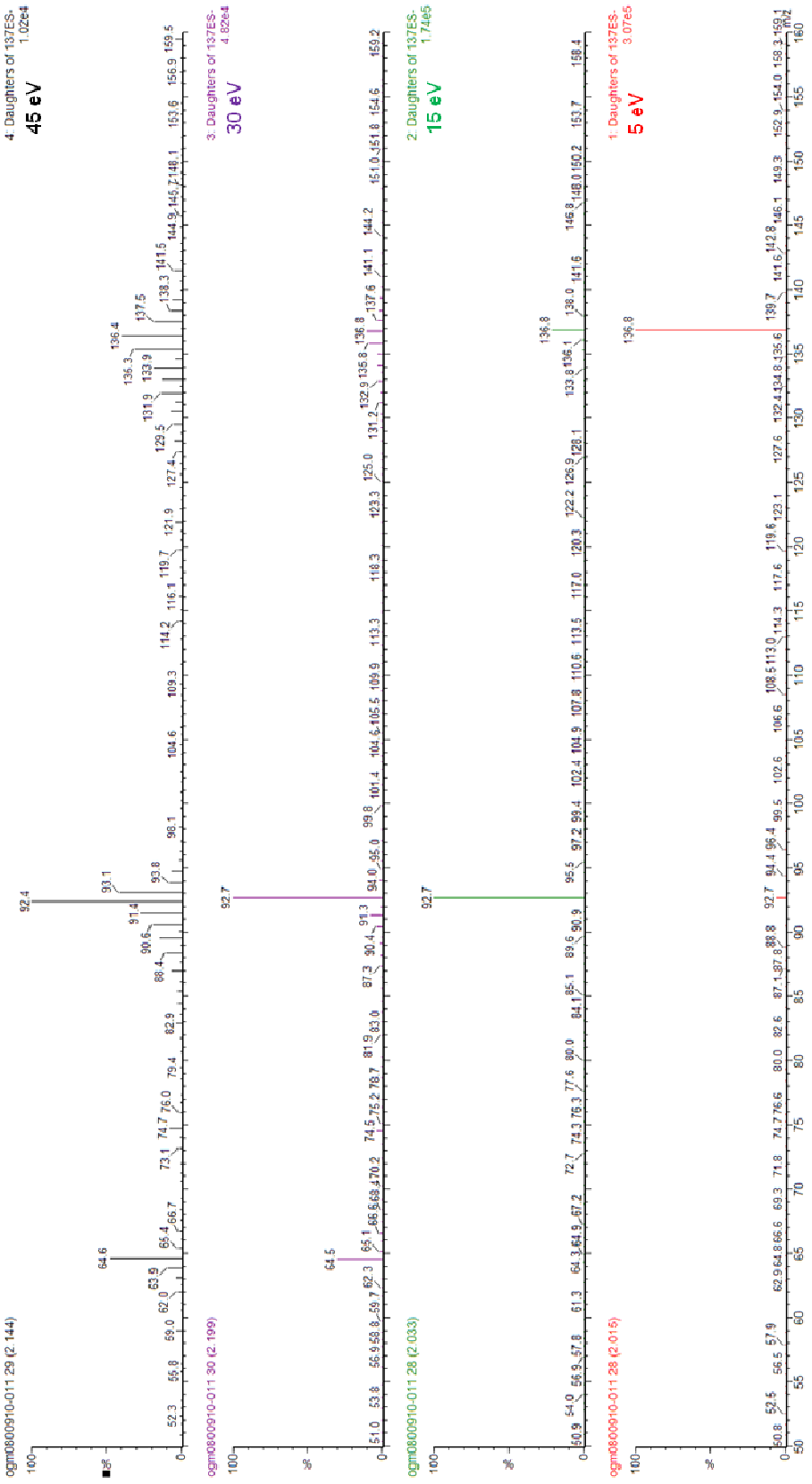

Figure 2.10. Product ion scan (ESI-) of Salicylic acid ([M-H]$^-$=136.8) using a 15 V cone voltage, at different collision energies under conditions described in section 2.2.5. The chosen fragment ion ([M+H]$^-$=92.7) offers the highest signal with a 15 eV collision energy.

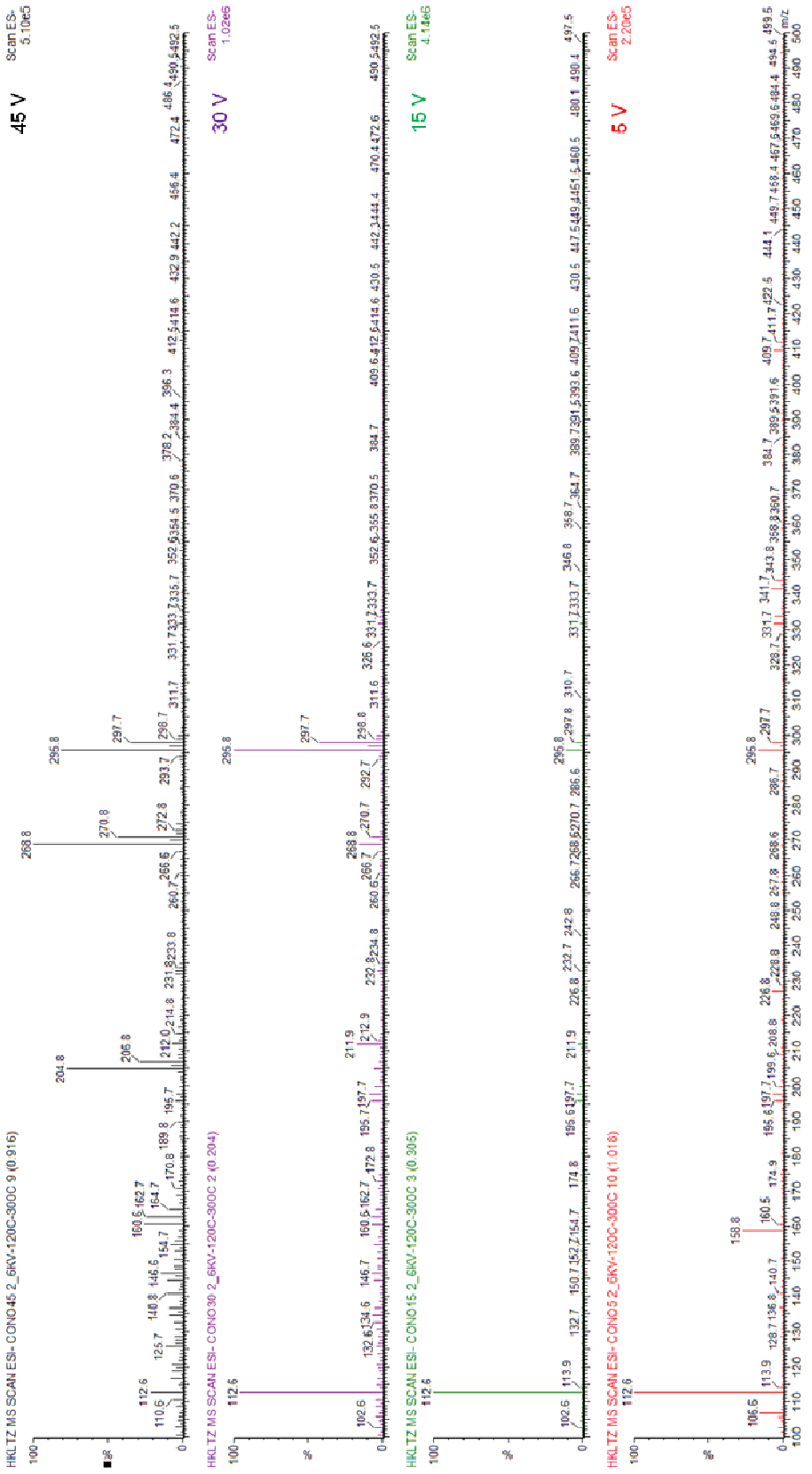

Figure 2.11. Mass scan (ESI-) of Hydrochlorothiazide at different cone voltages under conditions described in section 2.2.5. The most intense molecular ion ($[M-H]^-$=295.8) is obtained using 30 V cone voltage.

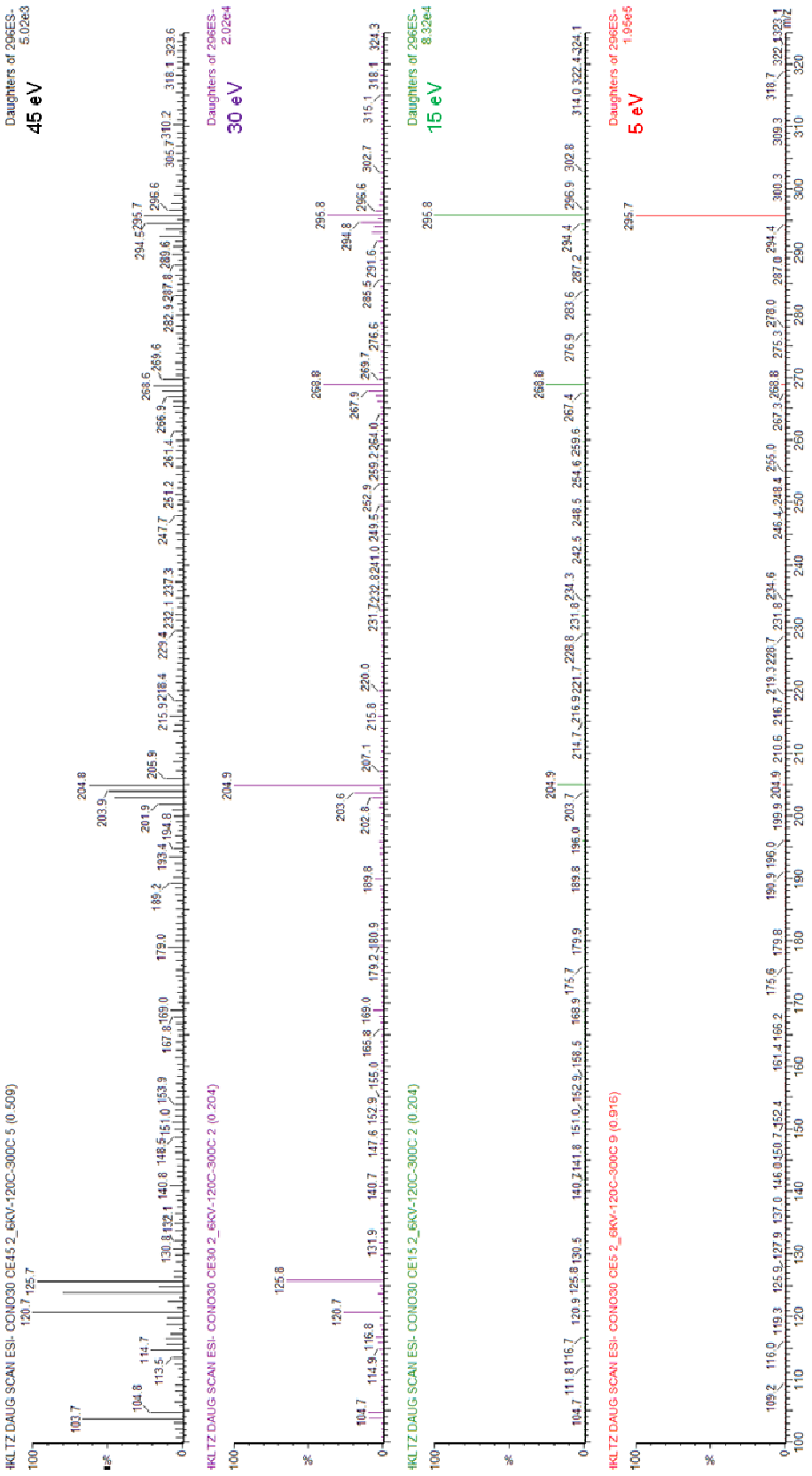

Figure 2.12. Product ion scan (ESI-) of Hydrochlorothiazide ([M-H]$^-$=295.8) using a 30 V cone voltage, at different collision energies under conditions described in section 2.2.5. The chosen fragment ion ([M+H]$^-$=268.8) offers the highest signal with a 30 eV collision energy.

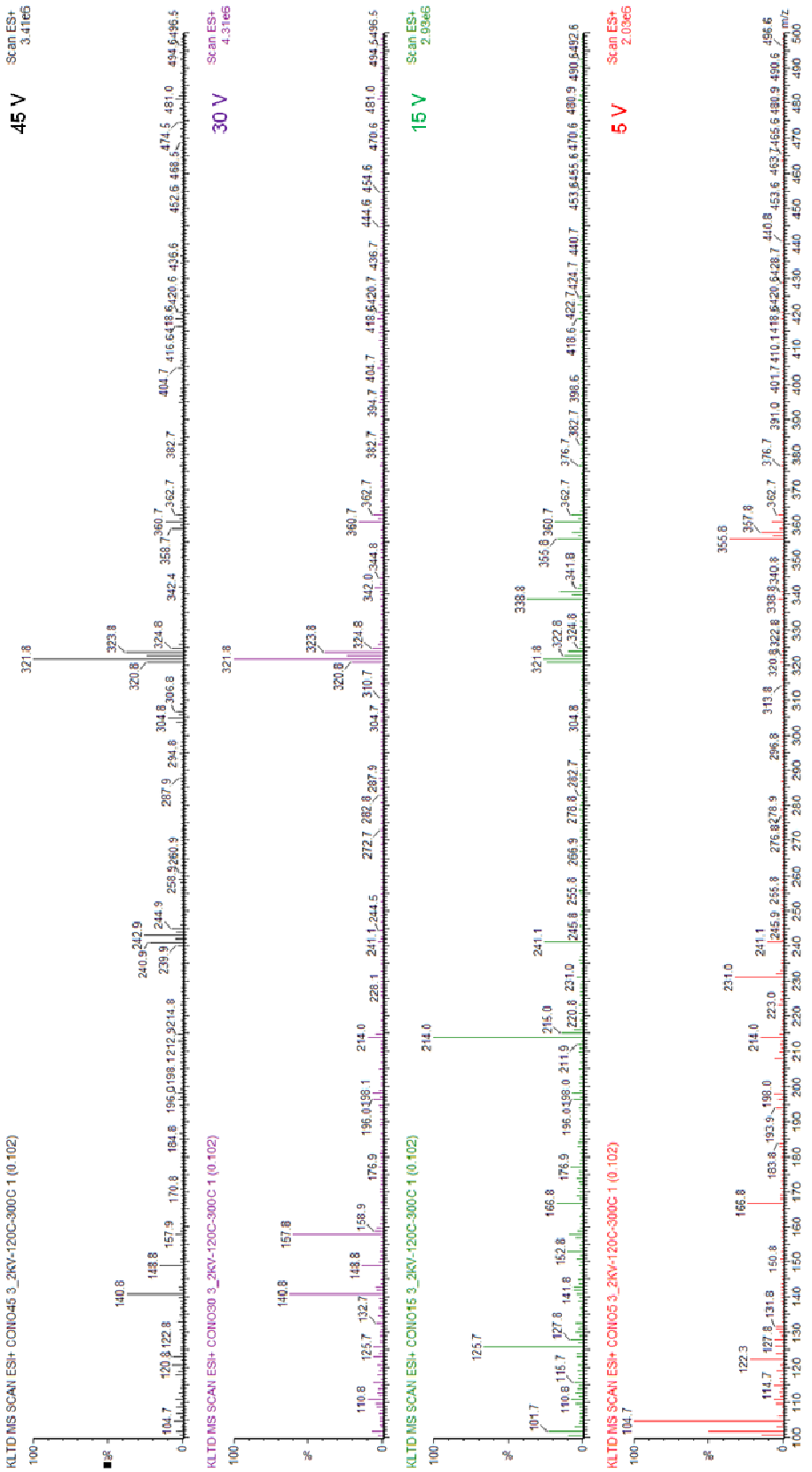

Figure 2.13. Mass scan (ESI+) of Chlorthalidone at different cone voltages under conditions described in section 2.2.5. The most intense molecular ion ($[M+H]^+$=338.8) is obtained using 15 V cone voltage.

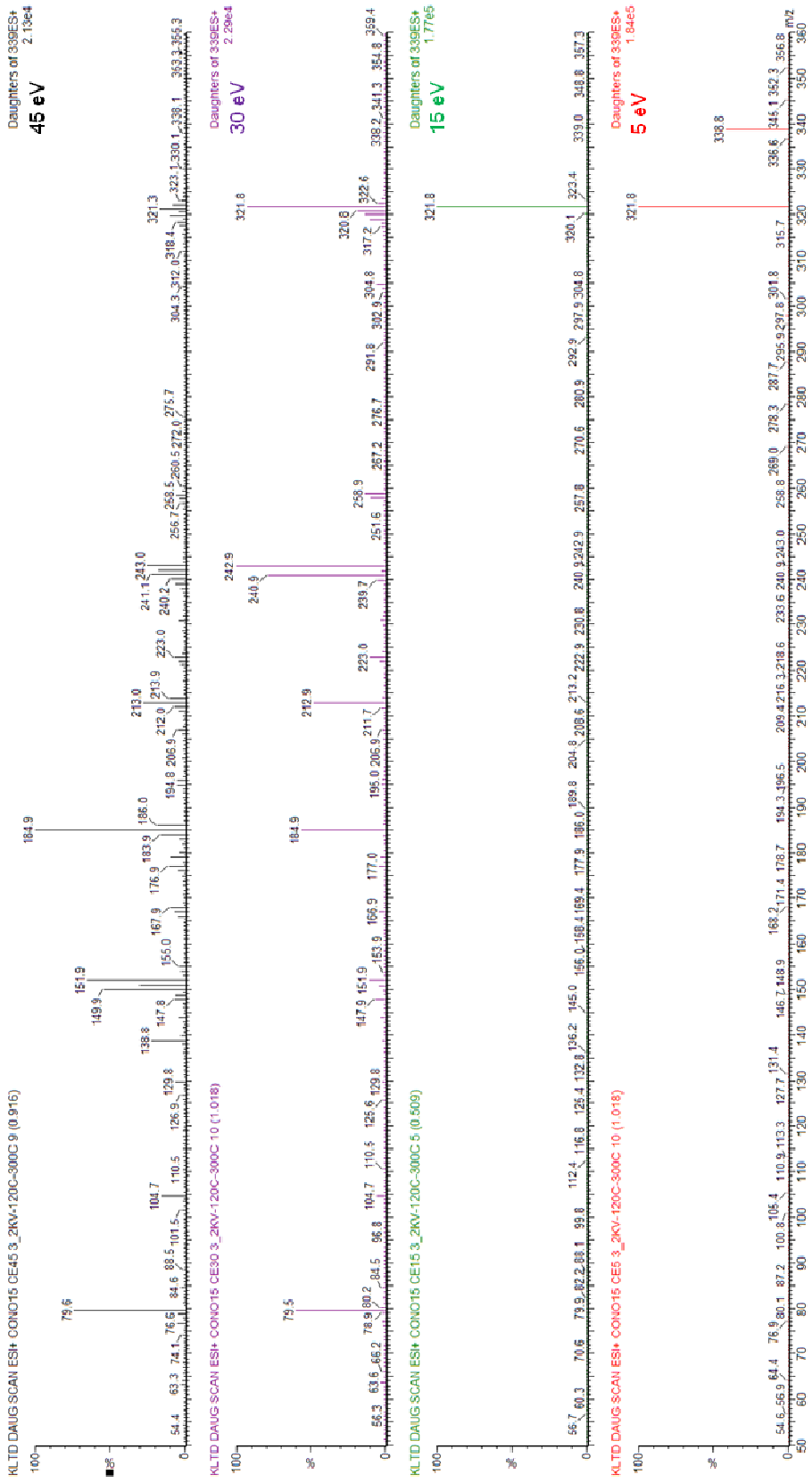

Figure 2.14. Product ion scan (ESI+) of Chlorthalidone ($[M+H]^+$=338.8) using a 15 V cone voltage, at different collision energies under conditions described in section 2.2.5. The chosen fragment ion ($[M+H]^+$=321.8) offers the highest signal with a 15 eV collision energy.

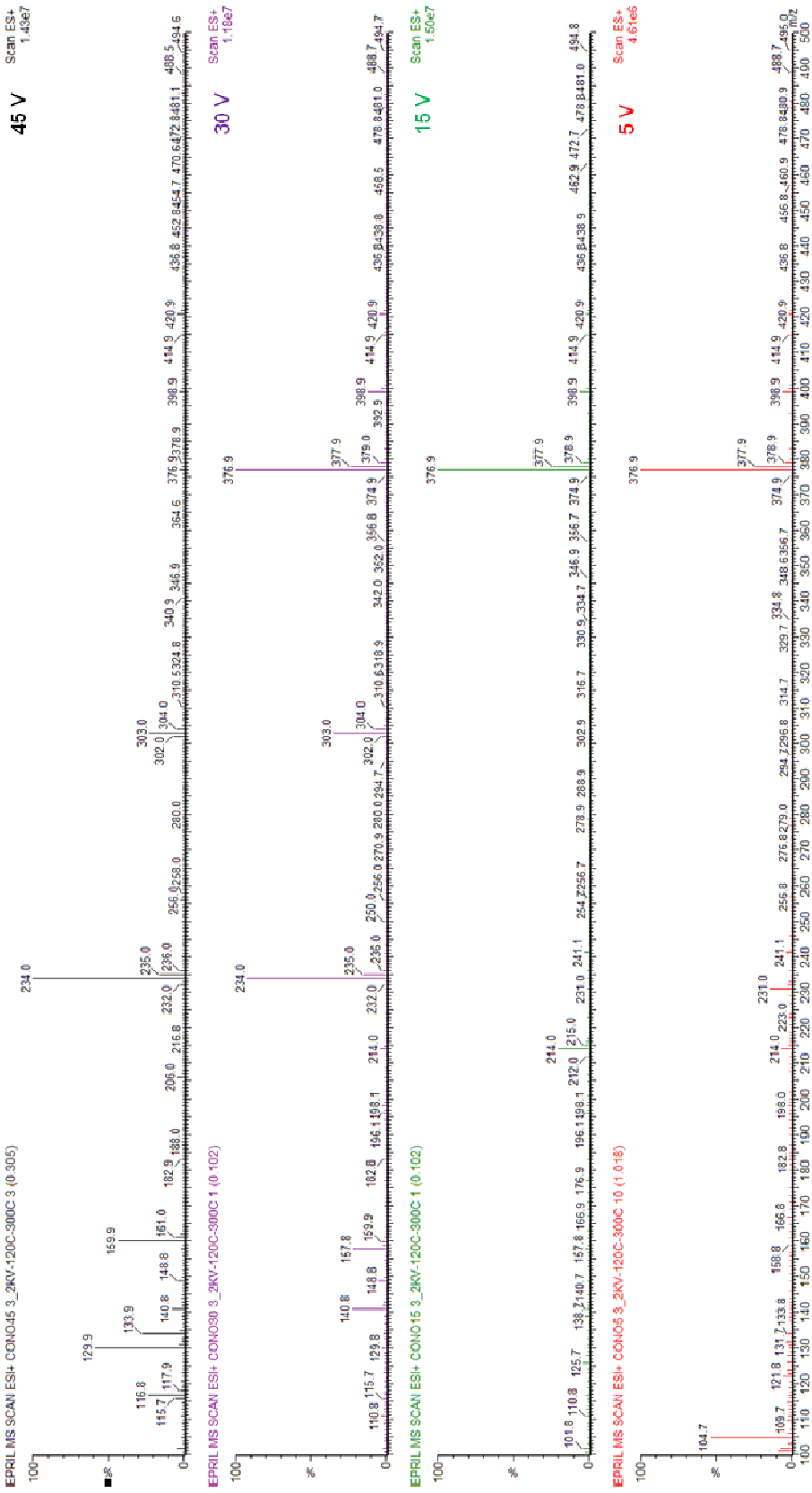

Figure 2.15. Mass scan (ESI+) of Enalapril at different cone voltages under conditions described in section 2.2.5. The most intense molecular ion ($[M+H]^+$=376.9) is obtained using 15 V cone voltage.

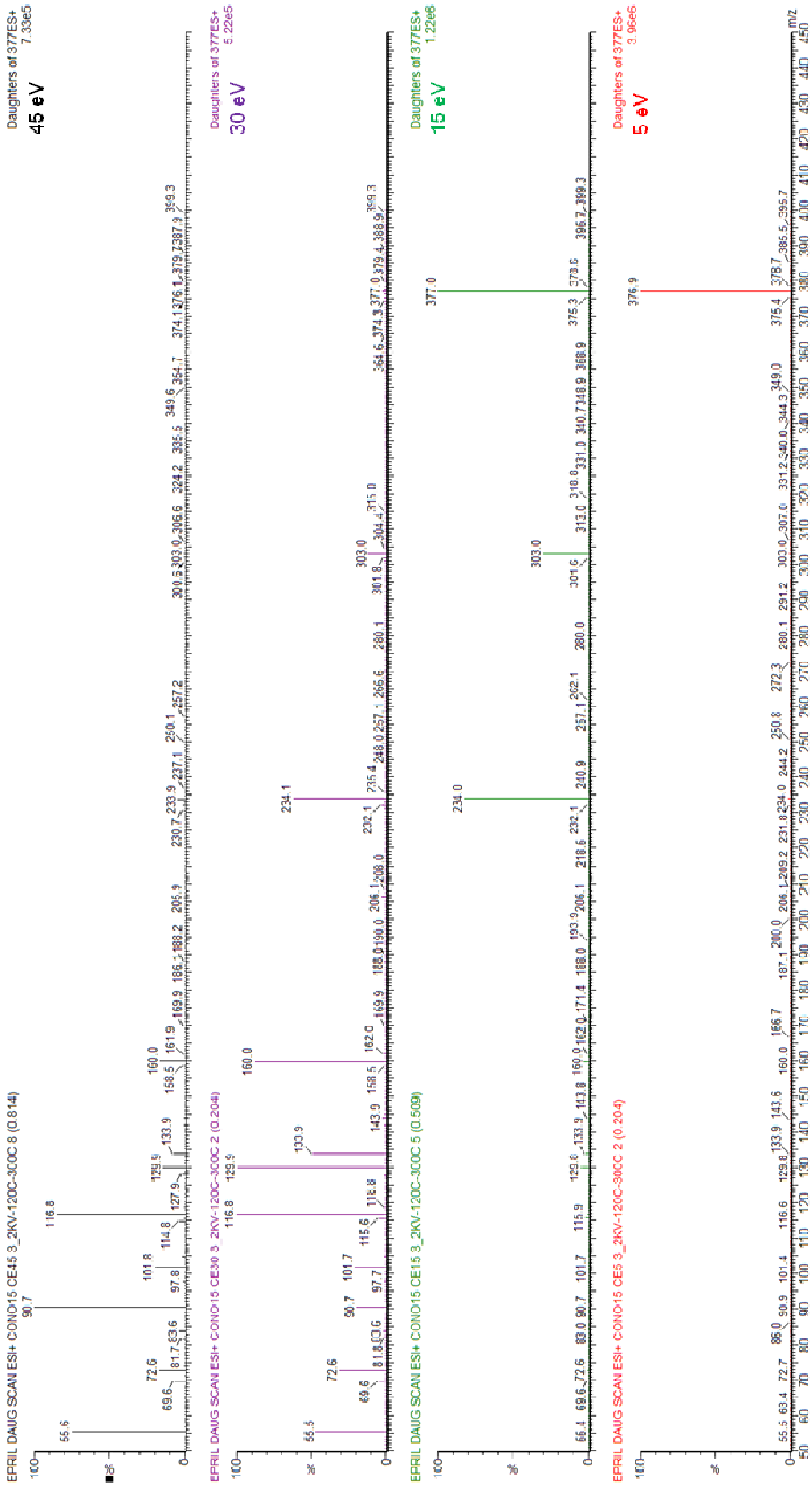

Figure 2.16. Product ion scan (ESI+) of Enalapril ($[M+H]^+$=376.9) using a 15 V cone voltage, at different collision energies under conditions described in section 2.2.5. The chosen fragment ion ($[M+H]^+$=234.0) offers the highest signal with a 15 eV collision energy.

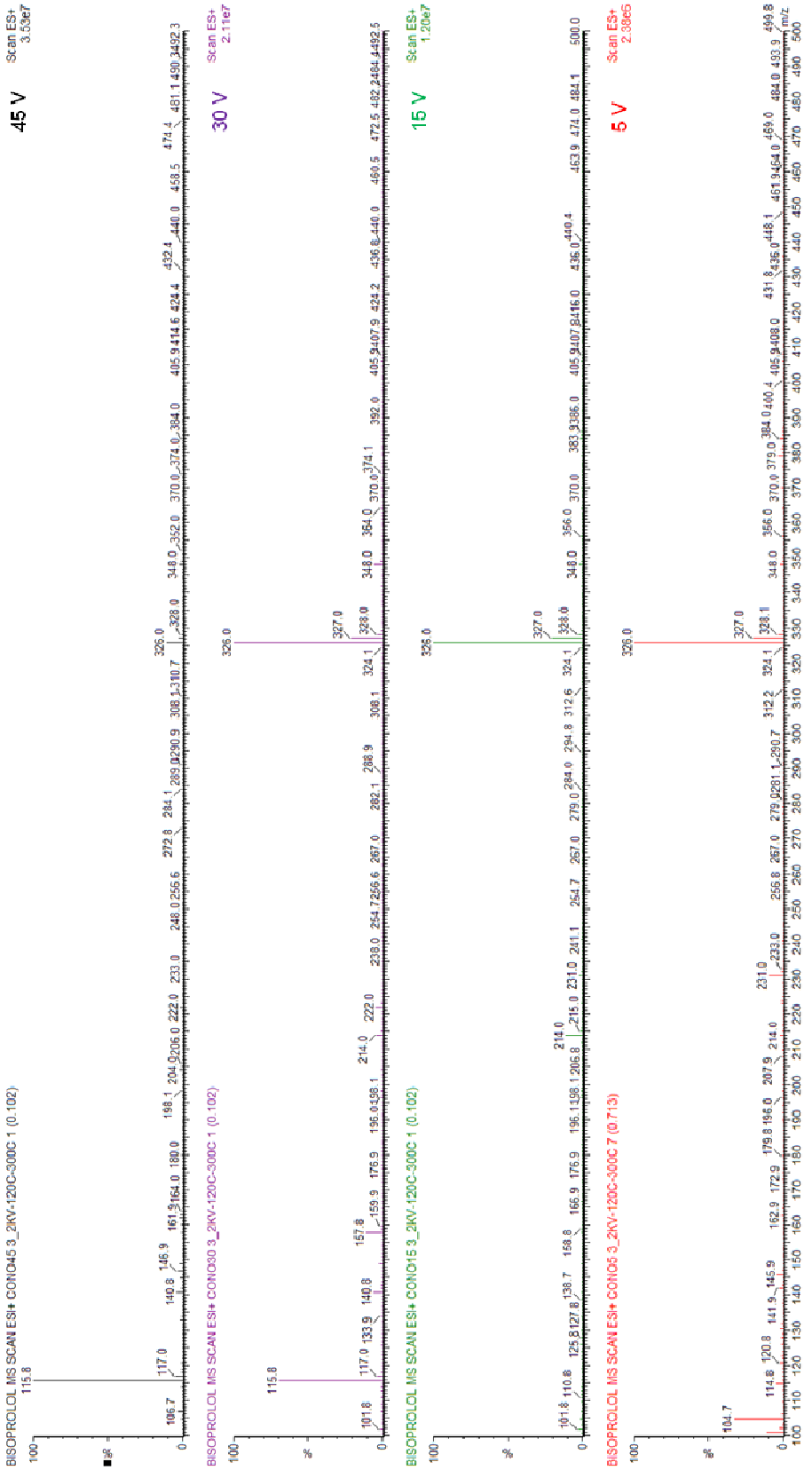

Figure 2.17. Mass scan (ESI+) of Bisoprolol at different cone voltages under conditions described in section 2.2.5. The most intense molecular ion ($[M+H]^+$=326.0) is obtained using 30 V cone voltage.

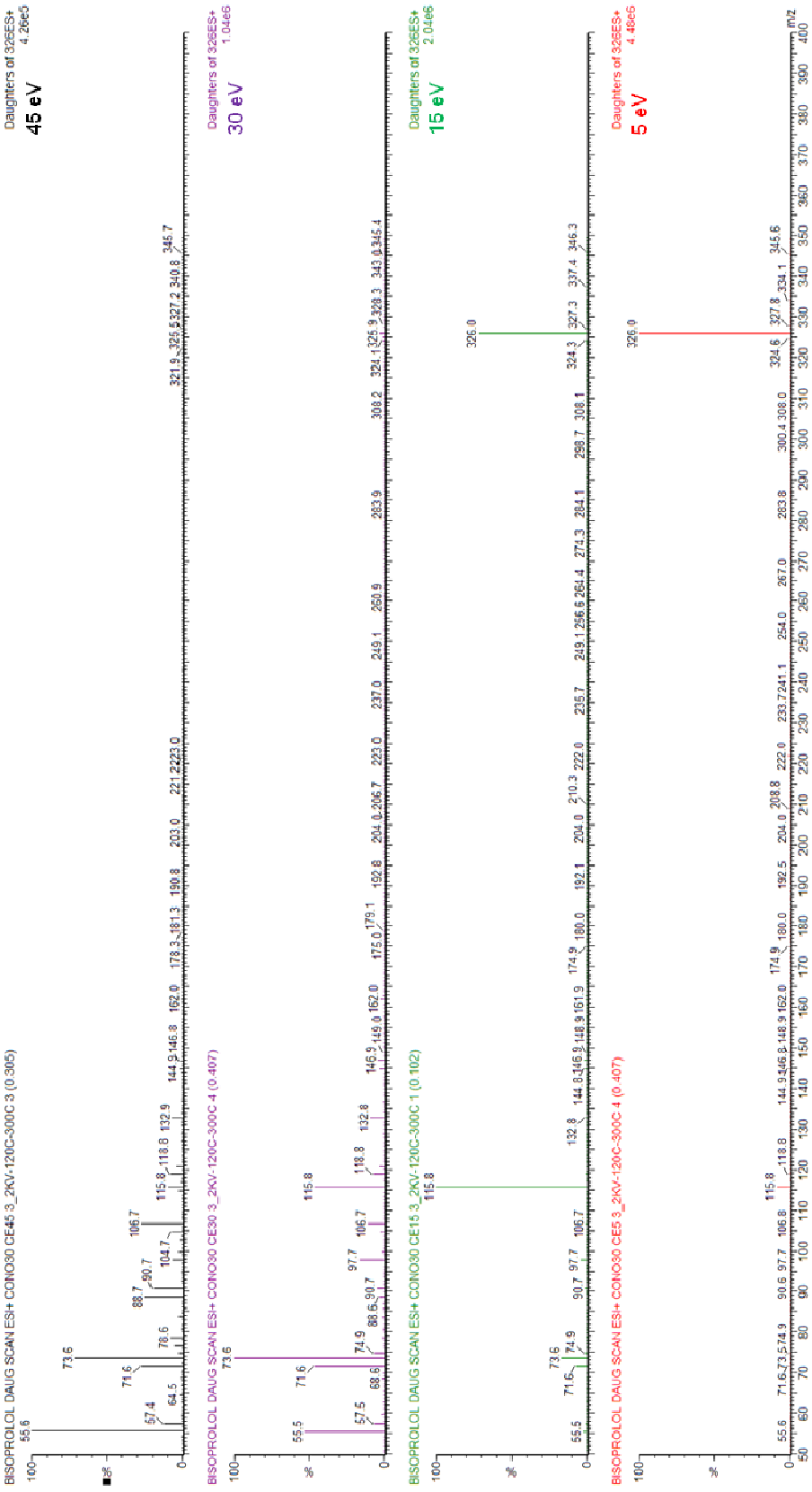

Figure 2.18. Product ion scan (ESI+) of Bisoprolol ($[M+H]^+$=326.0) using a 30 V cone voltage, at different collision energies under conditions described in section 2.2.5. The chosen fragment ion ($[M+H]^+$=115.8) offers the highest signal with a 15 eV collision energy.

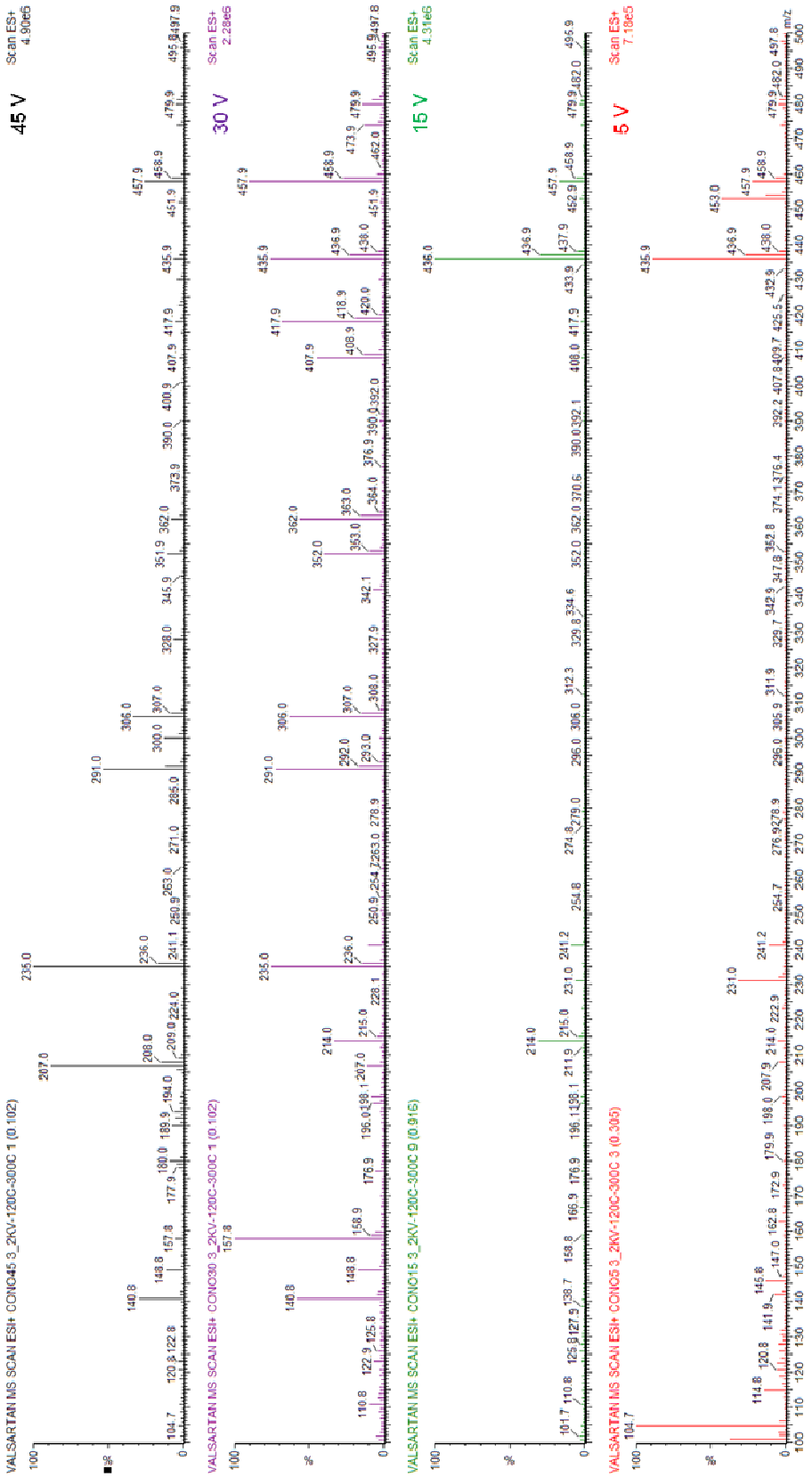

Figure 2.19. Mass scan (ESI+) of Valsartan at different cone voltages under conditions described in section 2.2.5. The most intense molecular ion ($[M+H]^+$=436.0) is obtained using 15 V cone voltage.

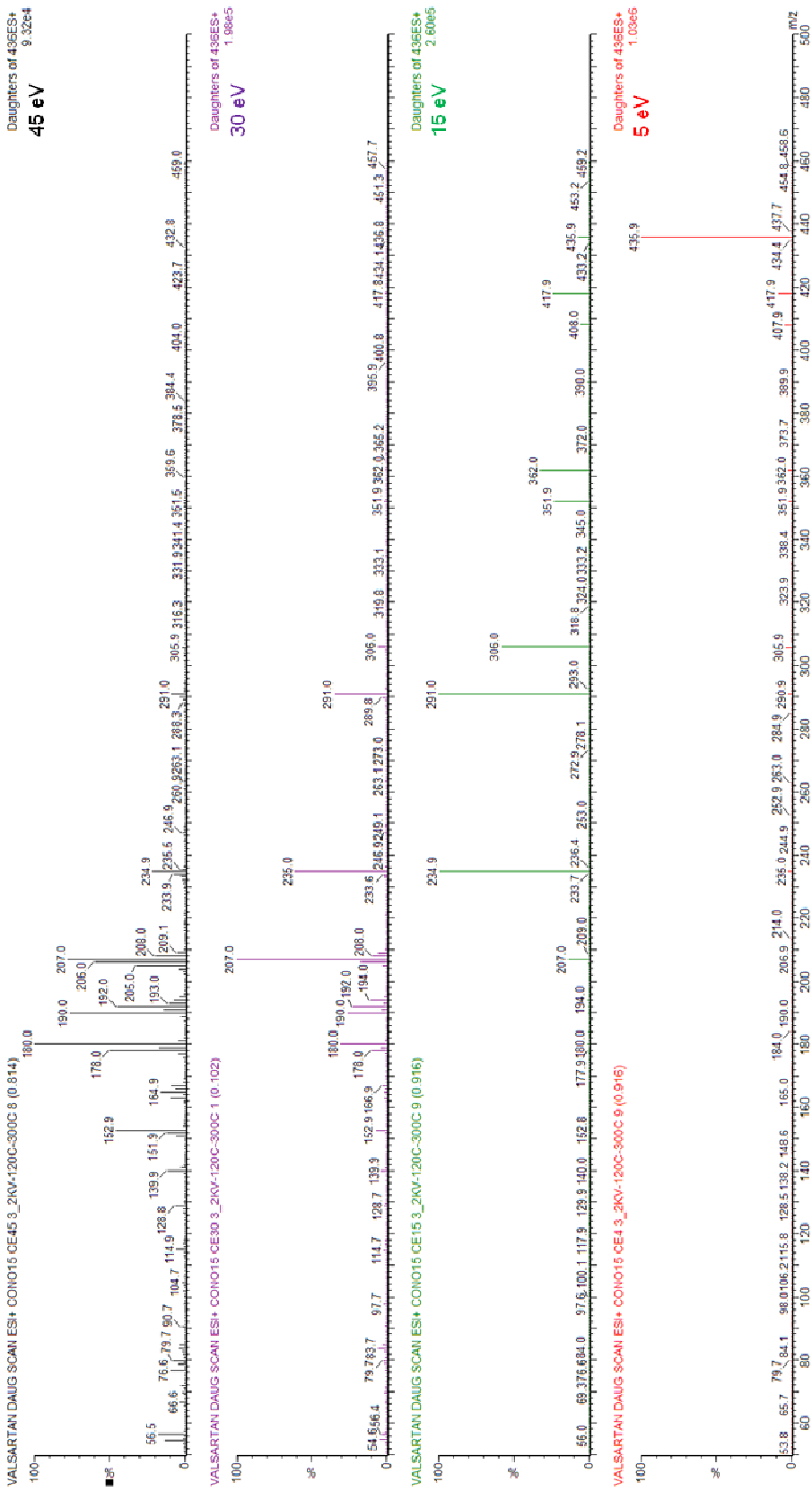

Figure 2.20. Product ion scan (ESI+) of Valsartan ($[M+H]^+$=436.0) using a 30 V cone voltage, at different collision energies under conditions described in section 2.2.5. The chosen fragment ion ($[M+H]^+$=234.9) offers the highest signal with a 15 eV collision energy.

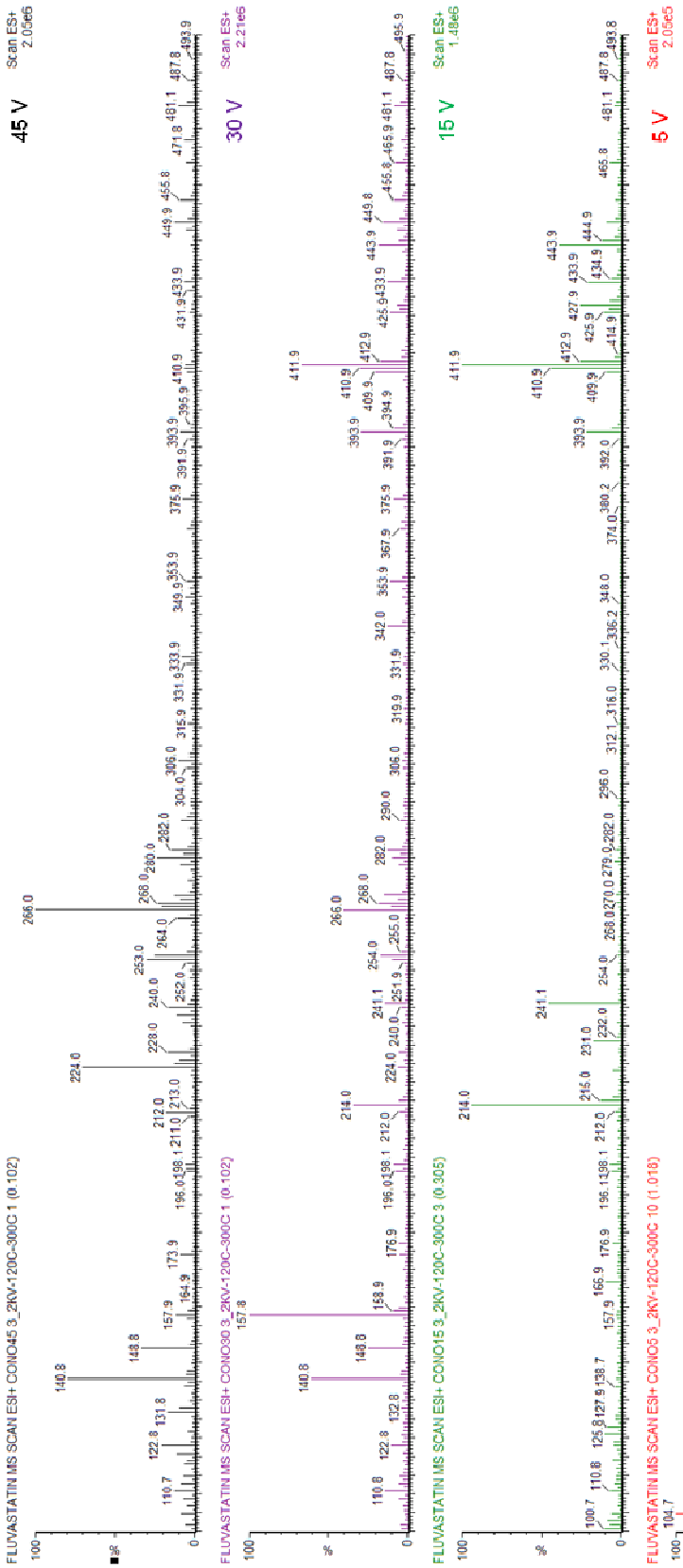

Figure 2.21. Mass scan (ESI+) of Fluvastatin at different cone voltages under conditions described in section 2.2.5. The most intense molecular ion ($[M+H]^+$=411.9) is obtained using 15 V cone voltage.

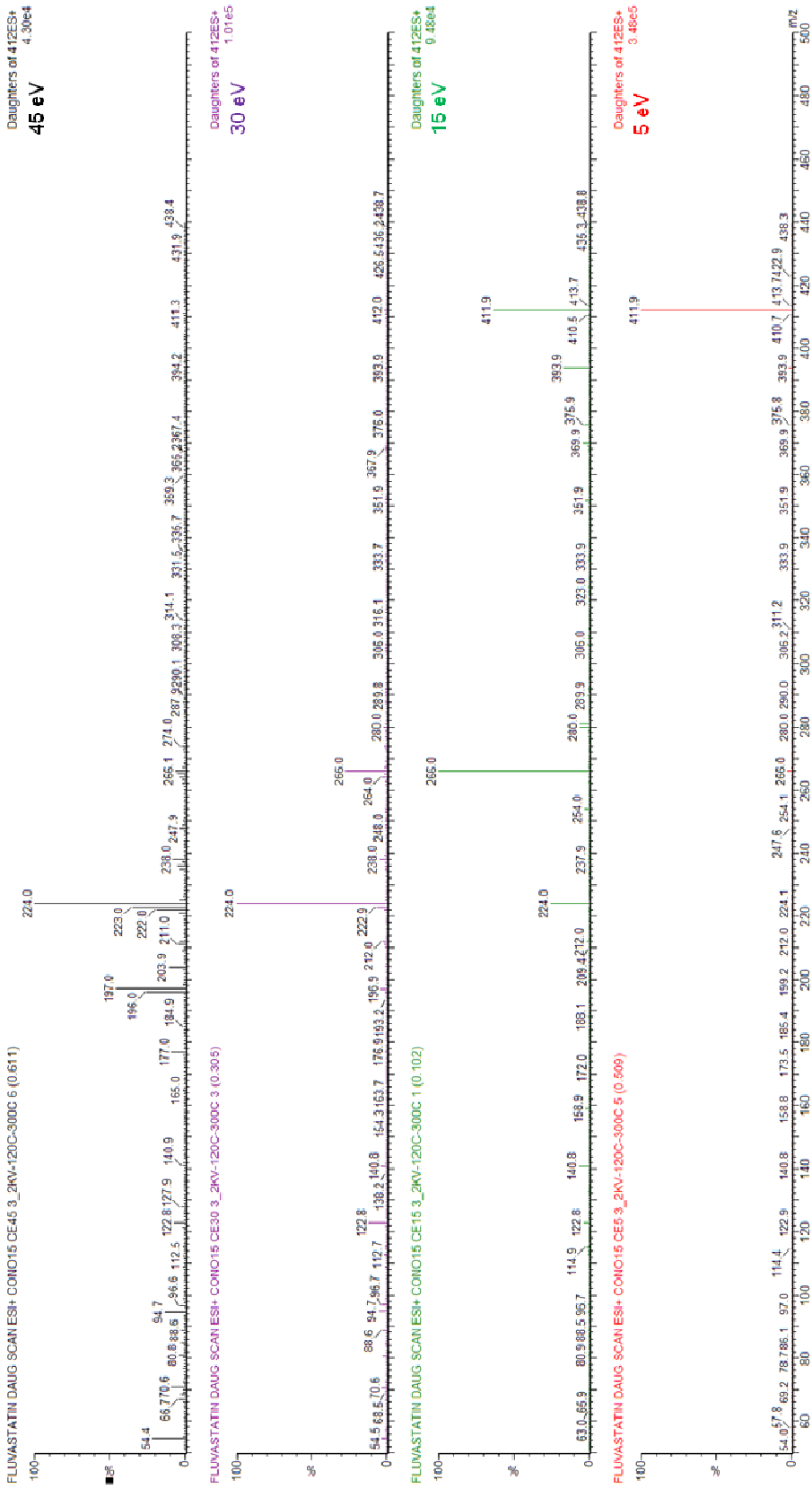

Figure 2.22. Product ion scan (ESI+) of Fluvastatin ($[M+H]^+$=411.9) using a 15V cone voltage, at different collision energies under conditions described in section 2.2.5. The chosen fragment ion ($[M+H]^+$=224.0) offers the highest signal with a 30 eV collision energy.

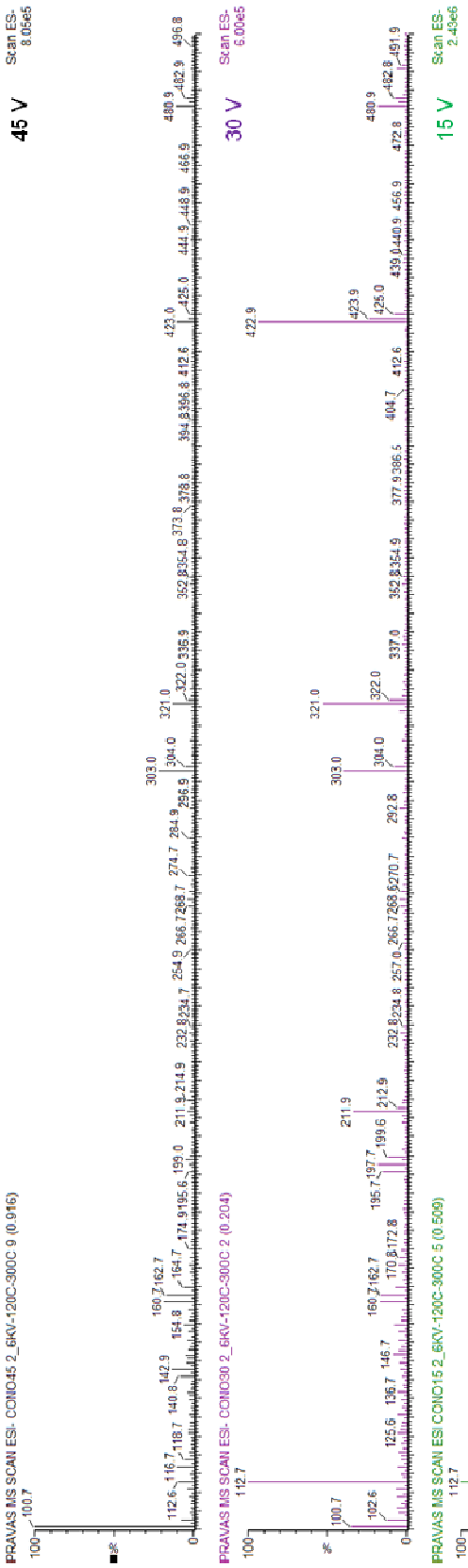

Figure 2.23. Mass scan (ESI-) of Pravastatin (I.S) at different cone voltages under conditions described in section 2.2.5. The most intense molecular ion ([M-H]$^-$ =422.9) is obtained using 30 V cone voltage.

Figure 2.24. Product ion scan (ESI-) of Pravastatin ($[M-H]^-$=422.9) using a 30V cone voltage, at different collision energies under conditions described in section 2.2.5. The chosen fragment ion ($[M-H]^-$=321.0) offers the highest signal with a 15 eV collision energy.

CHAPTER III

DEVELOPMENT OF AN LC–MS/MS METHOD FOR THE QUANTITATION OF 55 COMPOUNDS PRESCRIBED IN COMBINED CARDIOVASCULAR THERAPY

A version of this work published on:

Journal of Chromatography B

Volume 879, Issues 3-4, 1 February 2011, Pages 243-252

Abstract

This paper reports an LC–MS/MS method with positive electrospray ionization for the screening of commonly prescribed cardiovascular drugs in human plasma, including compounds with antihypertensive (57), antidiabetic (12), hypolipemiant (5), anticoagulant (2) and platelet anti-aggregation (2) effects. Sample treatment consisted of a simple protein precipitation with MeOH/0.1 M $ZnSO_4$ (4:1, v/v) solution after the addition of internal standard, followed by evaporation and reconstitution. Analytes separation was performed on a Polar-RP column (150 mm × 2 mm, 4 μm) using a gradient elution of 15 min. The MS system was operated in MRM mode, monitoring one quantitation and one confirmation transition for each analyte. The recovery of the protein precipitation step ranged from 50 to 70% for most of the compounds, while some were considerably affected by matrix effects. Since several analytes fulfilled the linearity, accuracy and precision values required by the ICH guidelines, the method proved to be suitable for their quantitative analysis. The limits of quantitation varied from 0.4 to 9.1 μg/L and the limits of detection from 0.1 to 5.3 μg/L. The method showed to be suitable for the detection of plasma samples of patients under cardiovascular treatment with the studied drugs, and for 55 compounds reliable quantitative results could be obtained.

Keywords: LC–MS/MS; Metabolic syndrome; Matrix effect; Cardiovascular drugs

3.1. Introduction

The probability of suffering from cardiovascular diseases is closely related to several risk factors such as high blood pressure, obesity, high blood levels of cholesterol and triglycerides and insulin resistance. The combination of these medical disorders is known as metabolic syndrome [1-3] and it is the first cause of mortality worldwide, with more than 17 million deaths each year [4]. Therefore, the prevention and treatment of the disorders associated to the metabolic syndrome is one of the main tasks of the pharmacological therapy. The pathology of the disorders implies a combined cardiovascular therapy [5-7] with drugs which have different targets and mechanisms of action, to regulate each factor separately: reducing the high blood pressure (antihypertensive compounds), decreasing the triglyceride and cholesterol levels (lipid lowering drugs), lowering the sugar concentration in blood (antidiabetics) and increasing the fluidity of blood to avoid thrombus formation (anti-platelets, anticoagulants).

Monitoring of blood concentrations of drugs is always crucial to understand their pharmacokinetics and pharmacodynamics. But in the case of several compounds used in combined cardiovascular therapy, it is also important to detect misuse of these drugs, i.e. for doping, or to reveal accidental or intentional intoxications [8-10] or to check the compliance of patients. Moreover, it provides very valuable information about possible interactions and secondary effects derived from the co-administration of several drugs which share metabolic and/or excretion pathways [11-13].

The analysis of multiple substances with different physicochemical properties and physiological behaviour is always a challenge for the analytical chemist, due to the different expected concentrations in biological fluids (from several micrograms to few picograms) and due to the difficulty of developing an extraction process adequate for all the analytes, but selective enough to reduce as much as possible the matrix effects. These factors are responsible for the small number of publications aimed on the simultaneous target screening of a high number of drugs belonging to different compound classes [14-16]. However, several analytical methods have been reported for the simultaneous determination of the frequently prescribed associations [17-21].

Therefore, the aim of this work is the development of an analytical method for the simultaneous screening of the most commonly drugs prescribed in combined cardiovascular therapy in human plasma; including 57 antihypertensive (8 angiotensin converting enzyme inhibitors (ACEI) of which 6 are prodrugs and 2 are active compounds, 6 angiotensin-II-receptor antagonists (ARA-II), 29 β-blockers, 2 diuretics and 12 calcium antagonists), 12 antidiabetic (9 belonging to sulfonylurea class, 1 biguanide, 1 meglitinide, 1 thiazolidinedione), 2 anticoagulant (acenocoumarol and warfarin), 5 lipid lowering (statins) and 2 anti-platelet (ticlopidine, clopidogrel) compounds. Concurrently, a sample preparation procedure has been completed.

Due to the required sensitivity and selectivity, and the convenient sample preparation without derivatization procedures, liquid chromatography–tandem

mass spectrometry (LC–MS/MS) [22] was the selected technique to fulfil the objective of this research work.

3.2. Experimental

3.2.1. Chemicals and reagents

Fluvastatin was kindly supplied by Novartis Pharma AG (Basel, Switzerland). All the other compounds were generously provided by the Institute of Legal Medicine, Humboldt University (Berlin, Germany).

HPLC-grade methanol was obtained from J.T. Baker Mallinckrodt (Deventer, The Netherlands). Deionized water was prepared with a cartridge deionizer from Memtech (Moorenweis, Germany). Formic acid was purchased from Carl Roth GmbH (Karlsruhe, Germany) and ammonium formate and zinc sulfate from Sigma–Aldrich (Steinheim, Germany). Blank plasma samples were obtained from the University Medical Centre of Freiburg (Freiburg, Germany), and were tested by GC/MS for the absence of drugs prior to use.

3.2.2. Instrumentation

The LC–MS/MS system consisted of a 3200 Q TRAP triple-quadrupole linear ion trap mass spectrometer fitted with a TurboIonSpray interface (AB Sciex, Darmstadt, Germany) and a Shimadzu Prominence HPLC system: two LC-20ADsp isocratic pumps, a CTO-20AC column oven, an SIL-20AC autosampler, a DGU-20A3 degasser, and a CBM-20A controller (Shimadzu, Duisburg, Germany). A Polar RP 150 mm × 2 mm, 4 µm column (Phenomenex, Aschaffenburg,

Germany) with a guard column of the same material (4 mm × 2 mm) was used for the chromatographic separation.

Eppendorf centrifuge 5415D (Hamburg, Germany) was used for plasma samples centrifugation after protein precipitation.

3.2.3. Standard solutions and plasma samples

Standard stock solutions of 1000 mg/L were prepared in methanol for each analyte separately. With those solutions a working solution containing the adequate concentrations of all the analytes to spike the plasma samples covering the linearity range (0.12–30 mg/L) was prepared in methanol. A 2.5 mg/L solution containing the three IS (d_3-doxepin, d_5-diazepam and methaqualone) was also prepared in methanol.

Calibration standards were prepared by spiking a pool of drug-free human plasma with the working standard solution and diluting it covering the full studied concentration range. The calibration curves were built from 25 to 5000 µg/L for carbutamide, eprosartan, glibornuride, gliclazide, metformin, tolazamide, tolbutamide, torasemide, valsartan, and warfarin; from 10 to 2000 µg/L for candesartan, glipizide, gliquidone and irbesartan; from 4 to 800 µg/L for atenolol, candesartan cilexetil (cand. cilex.), fluvastatin, glibenclamide, metoprolol, quinaprilat and ticlopidine; from 3 to 600 µg/L for glimepiride; from 2 to 400 µg/L for acenocoumarol, enalapril, enalaprilat, fendiline, nadolol, nicardipine, nifedipine, rosiglitazone, sotalol, telmisartan, tertalolol and toliprolol; from 1 to 200 µg/L for

alprenolol, amiloride, betaxolol, carteolol, celiprolol, cilazapril, gallopamil, imidapril, lisinopril, lovastatin, nimodipine, nisoldipine, nitrendipine, oxprenolol, penbutolol, perindopril, pindolol, propranolol, repaglinide, simvastatin, talinolol, timolol and verapamil; from 0.5 to 100 µg/L for acebutolol, amlodipine, atorvastatin, befunolol, bisoprolol, bopindolol, bunitrolol, bupranolol, carazolol, carvedilol, cerivastatin, esmolol, felodipine, isradipine, labetalol, lercanidipine, levobunolol, mepindolol, practolol and ramipril and from 0.5 to 20 µg/L for clopidogrel. All calibration curves consisted of seven calibration points.

3.2.4. Chromatographic and MS conditions

The mobile phase consisted of solvent A (0.1% formic acid (v/v) with 1 mmol/L ammonium formate) and solvent B (methanol: 0.1% formic acid (v/v) with 1 mmol/L ammonium formate). The gradient applied was the following: 0–1 min, 0% B; 1–5 min, 0–65% B; 5–8 min, 65–95% B, 8–11 min 95% B, 11–12.5 min 95–0% B, 12.5–15.5 min 0% B. Flow started at 0.2 mL/min and linearly increased to 0.4 mL/min during the first minute, then it decreased again to 0.2 mL/min from minute 11 to 12.5. During the chromatographic analysis the column was thermostated at 40 °C and samples were kept at 10 ± 1 °C in the autosampler.

The TurboIonSpray source was operated in positive mode at 500 °C with an ionization voltage of 5500 V. Nitrogen was used as curtain gas (10 psi), gas 1 (80 psi), gas 2 (70 psi) and collision gas (6 psi). Analysis was performed by multiple reaction monitoring mode (MRM), using the precursor ions and the

corresponding product ions. Two transitions were monitored for each analyte, one for quantitation and the other one for confirmation. The choice of these transitions and the optimization of the parameters related to their sensitivity are explained at the end of this chapter. In order to obtain a minimum of 10 data points for each chromatographic peak scheduled MRM was used. The cycle time and the retention time window were fixed at 1.8 s and 90 s, respectively, which determines a minimum dwell time of 17 ms.

3.2.5. Sample treatment

100 µL of human plasma sample were transferred to a 1.5 mL Eppendorf cup and spiked with 10 µL of a 2.5 mg/L IS solution (d5-diazepam, d3-doxepin and methaqualone). Protein precipitation was carried out using 200 µL of MeOH/0.1 M $ZnSO_4$ (4:1, v/v) solution. After vortex mixing and centrifugation for 5 min at 16,100 × g, the supernatant was transferred to a 2 mL glass vial and evaporated to dryness under N_2 stream at 60 °C. The residue was then reconstituted with 100 µL of mobile phase (A/B, 60:40 (v/v)) and vortex mixed. 30 µL of aliquots were injected into the LC system for analysis.

3.2.6. Method validation

Recovery and matrix effect for each analyte were determined at three different concentrations (low, middle and high) following the strategies reported by Matuszewski et al. [23]. Three sets of samples were used for this aim: standards prepared in neat solvent (A, n = 5), plasma samples spiked after the protein

precipitation (PPT) but before the evaporation step (B, n = 5) and plasma samples spiked before PPT (C, n = 5). Recovery was calculated by comparing the areas of B and C samples (Rec (%) = $C_{area}/B_{area} \times 100$) and matrix effect by comparing the areas of A and B samples (M.E. (%) = $B_{area}/A_{area} \times 100$). A matrix effect value higher than 100% indicates enhancement, whereas a lower one indicates suppression effects.

The matrix effect was also qualitatively studied using the post-column infusion technique reported by Bonfiglio et al. [24]. For this purpose, a methanolic solution of the studied compounds (120 µg/L) was infused post-column via a mixing tee at a flow rate of 25 µL/min while the analysis of a pretreated blank plasma sample was carried out.

The selectivity of the method was tested by analyzing blank human plasma samples from 6 different sources under optimized chromatographic conditions, and by comparing them with spiked plasma samples at a concentration close to the lower limit of quantitation (LLOQ).

LLOQ was calculated according to criteria of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [25]: a specific calibration curve containing the analytes in the range of the LLOQ was studied and the value of the LLOQ was determined based on the standard deviation of the regression curve. Signal to noise ratio corresponding to the LLOQ was required to be higher than 10. Limit of detection

(LOD) was calculated following the same approach using the confirmation transition with a signal to noise ratio of 3.

Considering the large concentration ranges for several analytes, an $1/x^2$ statistical weight was applied in order to obtain the most reliable calibration curves [26]. Calibration curves (n = 7) were built by plotting the corrected areas (analyte area/IS area) for each concentration level versus the nominal concentration of each calibration standard.

Three samples, corresponding to low, medium and high concentration levels, were assessed in sets of five replicates in order to evaluate the intra- and interday accuracy and precision. This procedure was repeated at three different days. The deviation of the mean from the true value, expressed as relative error (RE), served to measure the accuracy. In the same way relative standard deviation (RSD) was used to express the precision.

3.2.7. Application to real samples

Blood samples were collected from 19 different patients under treatment with a combination of the studied drugs between 1 and 20 h after the oral intake of the drugs. Blood samples were immediately transferred into tubes containing ethylenediaminetetraacetic acid (EDTA) and gently mixed. Then, they were centrifuged at 1.301 × g for 10 min at 4 °C. The plasma supernatant was carefully separated from blood cells and collected in polypropylene tubes to be frozen at −20 °C until analysis.

3.3. Results

3.3.1. Matrix effect and recovery

The chromatographic separation obtained for a spiked sample of 50 µg/L is shown in Figure 3.1. The blue line corresponds to the total ion current as the sum of all MRM signals of the analytes during a post-column infusion of a 120 µg/L solution containing all the studied analytes.

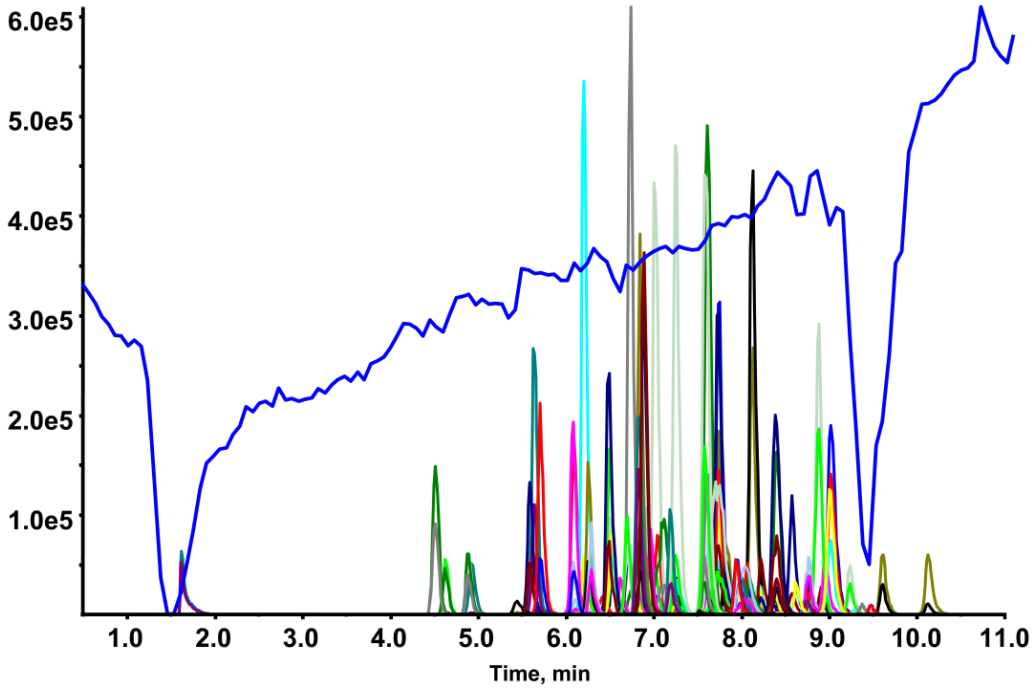

Figure 3.1. Chromatographic separation obtained for a plasma sample spiked with all the studied analytes (50 µg/L) together with the TIC corresponding to a post-column infused 120 µg/mL solution (red line).

Considerable ion suppression at the elution peak retention time (1–2 min) and around the last part of the gradient elution (9–10 min) can be observed. The ion suppression at the beginning of the chromatographic separation is attributed to

Table 3.1. Average matrix effect (M.E.) and recovery (Rec.) values obtained from three different concentrations (values showed as average ± standard deviation, *n* = 5).

Analyte	M.E.(%)	Rec (%)	Analyte	M.E.(%)	Rec (%)
Acebutolol	90.3 ± 2.5	62.8 ± 0.5	Isradipine	65.8 ± 4.3	65.4 ± 5.0
Acenocoumarol	64.9 ± 5.4	67.9 ± 3.4	Labetalol	116.8 ± 16.1	57.9 ± 2.8
Alprenolol	94.8 ± 3.9	65.3 ± 4.0	Lercanidipine	68.6 ± 4.6	56.1 ± 3.4
Amiloride	78.2 ± 1.9	52.3 ± 3.0	Levobunolol	105.2 ± 7.2	68.0 ± 4.1
Amlodipine	77.7 ± 3.5	56.8 ± 2.2	Lisinopril	178.7 ± 14.4	23.0 ± 1.0
Atenolol	66.9 ± 6.0	65.8 ± 3.5	Lovastatin	16.6 ± 2.1	59.1 ± 1.9
Atorvastatin	75.0 ± 1.0	62.6 ± 2.7	Mepindolol	82.0 ± 6.2	64.1 ± 2.6
Befunolol	104.1 ± 5.8	64.2 ± 4.2	Metformin	8.0 ± 1.5	65.1 ± 4.6
Betaxolol	96.9 ± 2.0	65.0 ± 3.4	Metoprolol	96.1 ± 3.3	66.5 ± 3.5
Bisoprolol	103.5 ± 4.6	67.3 ± 3.8	Nadolol	109.1 ± 6.1	65.0 ± 2.4
Bopindolol	90.1 ± 4.8	57.8 ± 0.3	Nicardipine	90.6 ± 1.3	64.0 ± 3.0
Bunitrolol	84.8 ± 0.8	64.4 ± 3.8	Nifedipine	73.5 ± 3.2	68.5 ± 2.9
Bupranolol	88.1 ± 1.0	63.1 ±3.5	Nimodipine	71.7 ± 5.4	65.9 ± 2.2
Candesartan	100.3 ± 6.5	51.9 ± 3.2	Nisoldipine	55.1 ± 2.0	68.4 ± 2.1
Cand Cilex	104.6 ± 6.1	48.7 ± 2.3	Nitrendipine	59.9 ± 3.5	67.2 ±1.9
Carazolol	101.1 ± 4.5	62.5 ± 6.2	Oxprenolol	95.1 ± 1.4	67.0 ± 2.8
Carbutamide	103.1 ± 14.9	66.6 ± 1.3	Penbutolol	88.7 ± 4.4	64.7 ± 4.2
Carteolol	90.4 ± 5.2	64.9 ± 3.5	Perindopril	113.3 ± 10.9	67.2 ± 3.3
Carvedilol	96.3 ± 1.2	55.5 ± 3.2	Pindolol	91.6 ± 4.1	63.0 ± 2.1
Celiprolol	104.7 ± 6.6	65.2 ± 1.4	Practolol	84.4 ± 4.2	65.0 ± 3.0
Cerivastatin	78.8 ± 5.9	61.2 ± 1.8	Propranolol	94.8 ± 3.6	63.2 ± 3.9
Cilazapril	142.0 ± 8.6	65.5 ± 3.4	Quinaprilat	112.3 ± 13.5	29.9 ± 0.8
Clopidogrel	58.0 ± 4.0	60.5 ± 4.9	Ramipril	117. 3 ± 6.4	68.5 ± 3.2
Enalapril	129.3 ± 11.6	64.6 ± 2.7	Repaglinide	98.2 ± 11.7	58.7 ± 3.1
Enalaprilat	104 ± 9.6	42.6 ± 2.9	Rosiglitazone	91.6 ± 4.2	57.5 ± 6.0
Eprosartan	103.3 ± 7.1	44.9 ± 3.0	Simvastatin	23.3 ± 0.3	59.2 ± 3.2
Esmolol	103.1 ± 7.5	65.8 ± 2.2	Sotalol	73.1 ± 8.6	66.5 ± 3.0
Felodipine	81.4 ± 5.5	63.5 ± 3.1	Talinolol	114.6 ± 5.9	65.0 ± 3.3
Fendiline	74.9 ± 3.5	66.9 ± 2.4	Telmisartan	86.6 ± 7.8	56.1 ± 3.1
Fluvastatin	112.9 ± 13.0	55.6 ± 1.8	Tertatolol	93.2 ± 2.6	66.2 ± 3.5
Gallopamil	91.6 ± 1.9	65.1 ± 1.8	Ticlopidine	81.6 ± 2.0	51.4 ± 4.2
Glibenclamide	115.2 ± 8.3	62.0 ± 2.1	Timolol	99.9 ± 5.7	67.6 ± 3.8
Glibornuride	89.9 ± 5.3	63.6 ± 6.4	Tolazamide	101.3 ± 6.5	47.3 ± 2.6
Gliclazide	98.2 ± 8.6	39.0 ± 6.2	Tolbutamide	92.4 ± 4.7	65.8 ± 3.2
Glimepiride	63.9 ± 3.2	61.8 ± 1.8	Toliprolol	90.1 ± 4.6	65.6 ± 3.3
Glipizide	97.6 ± 9.7	74.5 ± 9.9	Torasemide	107.5 ± 14.5	58.9 ± 4.9
Gliquidone	48.2 ± 4.5	56.1 ± 2.0	Valsartan	103.9 ± 14.2	53.3 ± 3.5
Imidapril	111.2 ± 8.7	61.9 ± 4.0	Verapamil	90.7 ± 1.3	64.5 ± 1.9
Irbesartan	98.4 ± 7.1	64.5 ± 3.6	Warfarin	83.5 ± 6.8	70.7 ± 8.0

the elution of polar compounds present in plasma, whereas the suppression at the final part is associated with the elution of non-polar phospholipids [27,28].

Consequently, the analytes eluting in those ranges of retention time suffer from high signal suppression as it can be observed in Table 3.1. Metformin, coeluting with the injection peak, suffers a suppression of more than ten times in its intensity and lovastatin, simvastatin and gliquidone, which all co-elute with phospholipids also show significant signal suppression. Matrix effects were studied quantitatively for all the analytes and it showed to be comparable at the three different studied concentrations (n = 5). In Table 3.1, the average values obtained for the three concentrations are shown.

Recovery of the analytes, also gathered in Table 3.1, ranges from 29.9% to 74.5%. For most of the analytes, the recovery value was higher than 50%. Only candesartan cilexetil, enalaprilat, eprosartan, gliclazide, lisinopril, quinaprilat and tolazemide did not fulfil this criterion.

3.3.2. Selectivity

Selectivity has been studied by analyzing 6 blank plasma samples from different healthy volunteers. The chromatograms did not show interfering signals within the retention time windows of the chromatographic peaks of the analytes and the internal standards, which could be misinterpreted as the target compounds or could affect the bias of the method.

The selectivity of the isobaric transitions has also been tested. From the 156 transitions only atenolol and practolol shared an isobaric transition (267.2 → 190.2). In the case of atenolol the transition (267.2 → 145.1) was used for quantitation, because it was the most intensive; whereas for practolol the transition (267.2 → 148.2) was used, in order to avoid possible interferences between the analytes. Nevertheless, as it can be seen in Figure 3.2, both analytes are separated to baseline, which confirms the suitability of the isobaric transition for confirmation or quantitation purposes.

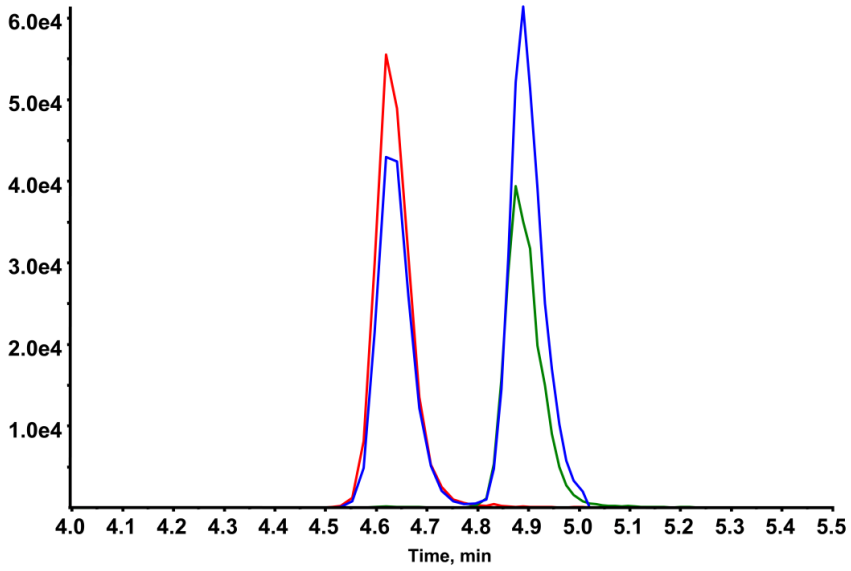

Figure 3.2. Chromatogram with the isobaric analytes atenolol (left) and practolol (right). Blue line belongs to the isobaric transition (267.2 → 190.2).

3.3.3. LOD, LLOQ and linearity

LOD and LLOQ, calculated following the ICH criteria, are shown in Table 3.2.

Table 3.2. LOD, LLOQ, precision (as average inter and intra-RSD for three different concentrations) and accuracy (as average inter an intra RE for three different concentrations) for each analyte with the corresponding IS. (d_5-diaz: d_5-diazepam; d_3-dox: d_3-doxepin; Meth: methaqualone; Ext: external calibration; Screen: only for qualitative purposes)

Analyte	I.S.	LOD (μg/L)	LLOQ (μg/L)	RSD Inter (%)	RSD Intra (%)	RE Inter (%)	RE Intra (%)
Acebutolol	d_5-diaz	0.18	0.50	5.6±3.3	6.7±0.5	5.1±3.0	5.7±2.0
Acenocoumarol	d_5-diaz	0.65	1.02	4.1±1.8	6.2±2.7	2.7±3.3	3.8±1.9
Alprenolol	d_3-dox	0.16	0.60	3.6±0.6	4.8±1.6	7.1±5.6	7.1±4.7
Amiloride	d_5-diaz	0.24	0.60	5.6±1.6	9.1±2.3	5.3±5.3	6.8±2.8
Amlodipine	d_3-dox	0.17	0.50	7.9±6.1	8.1±0.9	4.9±4.1	7.3±0.5
Atenolol	d_3-dox	0.22	0.80	5.5±4.8	6.0±0.6	4.7±4.4	6.5±3.5
Befunolol	d_3-dox	0.16	0.50	4.7±1.5	5.7±2.4	5.0±1.1	4.8±1.0
Betaxolol	d_3-dox	0.40	0.47	5.0±1.7	5.0±1.5	8.4±1.2	7.6±1.5
Bopindolol	d_5-diaz	0.27	0.50	6.5±2.3	6.5±1.0	2.1±1.4	4.3±1.8
Bunitrolol	d_3-dox	0.17	0.50	3.0±0.4	5.0±1.6	7.2±3.3	6.3±3.0
Bupranolol	d_3-dox	0.16	0.48	2.7±0.7	4.6±1.6	5.4±5.7	5.5±4.0
Candesartan	d_3-dox	0.34	1.12	11.7±2.6	9.3±1.7	1.3±0.9	8.6±1.6
Carazolol	d_3-dox	0.17	0.43	3.1±0.6	5.0±2.1	8.4±4.6	8.3±3.6
Carteolol	d_3-dox	0.48	0.81	2.8±1.4	4.7±0.9	8.2±7.0	8.1±6.1
Carvedilol	d_3-dox	0.16	0.50	7.2±0.9	6.9±1.7	8.0±0.9	8.0±1.3
Celiprolol	d_3-dox	0.14	0.52	6.8±1.3	5.8±0.2	4.0±4.8	6.1±2.1
Cilazapril	d_3-dox	0.17	0.53	5.5±2.0	6.3±1.2	4.6±3.0	5.6±3.0
Clopidogrel	d_5-diaz	0.13	0.49	7.6±4.1	5.1±2.0	9.1±3.6	8.7±4.3
Enalapril	d_5-diaz	0.18	0.55	7.2±3.7	8.4±2.2	7.0±5.8	8.7±3.1
Eprosartan	d_3-dox	5.34	9.10	5.5±6.0	8.0±2.5	6.9±3.7	8.6±3.7
Esmolol	d_3-dox	0.21	0.50	6.6±1.1	5.4±2.2	2.7±1.5	5.2±1.6
Felodipine	d_5-diaz	0.43	0.50	8.1±0.5	8.7±2.8	4.1±0.6	6.1±1.0
Fendiline	d_3-dox	0.32	0.61	5.7±2.9	7.9±1.7	6.7±4.0	6.9±3.7
Gallopamil	d_3-dox	0.17	0.48	6.5±0.9	6.3±1.5	8.4±2.7	8.2±2.2
Glibenclamide	d_5-diaz	0.18	0.62	5.1±1.1	7.7±3.1	4.4±4.5	5.5±2.1
Glibornuride	Meth	0.25	0.48	10.5±3.2	6.7±2.0	2.0±2.3	7.8±2.4
Gliclazide	d_3-dox	0.61	1.46	2.9±0.3	7.4±2.0	9.9±1.8	9.1±1.6
Glipizide	d_3-dox	0.24	0.69	7.5±4.0	6.1±3.5	2.5±3.1	5.7±2.2
Gliquidone	d_5-diaz	0.39	0.61	6.1±5.8	6.9±0.6	3.9±2.4	6.2±4.3
Irbesartan	d_3-dox	0.37	1.25	3.7±2.6	5.6±1.5	8.5±4.0	7.9±3.3
Lercanidipine	d_3-dox	0.19	0.50	4.4±1.6	5.1±1.8	3.6±2.6	3.8±1.4
Levobunolol	d_3-dox	0.12	0.42	1.8±0.8	4.7±1.8	8.8±7.0	8.7±6.0
Mepindolol	d_5-diaz	0.50	0.79	9.2±2.7	8.4±1.7	2.5±3.6	7.1±2.9
Metformin	d_5-diaz	0.76	0.84	5.1±2.3	7.9±2.5	1.4±1.6	4.5±2.0
Nadolol	d_3-dox	0.30	1.00	5.0±2.6	5.5±1.8	7.6±3.6	7.5±3.4
Nicardipine	d_3-dox	0.15	0.51	4.5±0.9	4.1±1.2	7.7±3.2	6.8±3.5
Penbutolol	d_3-dox	0.20	0.42	9.9±0.8	5.5±2.0	1.8±0.6	7.4±1.0
Perindopril	d_3-dox	0.61	1.14	7.0±1.5	5.9±3.3	4.4±1.1	6.2±1.7
Pindolol	d_3-dox	0.26	0.74	3.0±0.4	5.4±0.8	5.1±1.1	4.5±1.0
Practolol	d_5-diaz	0.22	0.50	4.7±3.4	10.2±2.1	2.2±0.8	3.3±1.9

Table 3.2. Continuation

Analyte	I.S.	LOD (μg/L)	LLOQ (μg/L)	RSD Inter (%)	RSD Intra (%)	RE Inter (%)	RE Intra (%)
Propranolol	d_3-dox	0.13	0.38	2.4±1.9	4.1±1.0	7.4±5.1	7.5±4.0
Ramipril	d_3-dox	0.20	0.50	3.3±2.6	5.6±1.8	6.9±6.1	7.1±4.7
Repaglinide	d_5-diaz	0.20	0.73	4.1±1.6	6.2±0.2	3.3±2.9	3.7±1.9
Rosiglitazone	d_3-dox	0.20	0.77	3.1±1.5	5.2±1.4	8.4±6.3	8.6±4.8
Sotalol	d_3-dox	0.20	0.60	5.4±0.8	6.2±0.6	3.7±1.6	4.2±0.9
Talinolol	d_3-dox	0.13	0.43	8.6±5.0	5.2±2.3	2.5±1.3	6.8±5.1
Telmisartan	d_5-diaz	0.14	0.62	8.4±2.9	4.6±1.5	4.0±4.4	7.5±1.4
Tertatolol	d_3-dox	0.15	0.58	3.2±2.2	5.0±2.0	4.7±4.8	5.6±2.8
Ticlopidine	d_3-dox	0.20	0.68	5.1±5.2	5.3±0.5	7.6±2.6	8.4±1.6
Tolbutamide	d_3-dox	0.57	2.52	8.0±4.3	6.4±1.6	2.9±2.4	6.8±4.4
Toliprolol	d_3-dox	0.52	0.52	3.6±1.1	4.6±1.0	8.6±5.6	8.5±4.7
Torasemide	d_3-dox	0.19	0.77	7.4±5.2	6.8±1.4	5.5±4.6	7.9±1.5
Valsartan	d_3-dox	0.30	1.00	8.2±4.3	7.3±2.5	3.8±5.6	7.7±1.3
Verapamil	d_3-dox	0.20	0.69	6.1±5.0	5.1±1.7	5.6±4.8	6.8±3.6
Warfarin	d_3-dox	0.31	1.52	1.6±1.4	7.3±2.5	8.4±4.5	8.3±3.7
Bisoprolol*	d_3-dox	0.15	0.51	3.1±3.6	4.5±2.0	11.4±3.1	11.4±2.2
Carbutamide*	d_3-dox	0.32	1.13	3.2±1.0	5.7±3.1	6.3±7.0	7.3±4.8
Labetalol*	d_3-dox	0.50	0.50	2.5±1.1	4.9±3.7	14.3±0.6	14.2±0.3
Metoprolol*	d_3-dox	0.18	0.66	3.6±0.8	5.5±3.3	7.9±6.8	7.9±5.8
Oxprenolol*	d_3-dox	0.17	0.65	1.4±0.6	3.9±1.1	10.2±6.2	9.5±6.2
Timolol*	d_3-dox	0.18	0.54	4.2±2.5	5.3±1.9	6.0±7.2	7.3±4.4
Tolazamide*	d_3-dox	0.47	1.20	3.0±1.5	8.0±2.3	7.2±7.6	7.7±5.9
Atorvastatin	Ext	0.24	0.48	9.0±1.4	4.9±3.2	4.8±1.8	7.9±1.6
Cand Cilex	Ext	0.47	1.10	12.1±4.6	7.1±3.7	2.7±1.0	9.1±3.6
Cerivastatin	Ext	0.20	0.42	10.6±1.2	8.0±4.6	2.8±2.3	8.1±1.9
Enalaprilat	Ext	0.25	0.78	9.6±3.4	4.2±1.9	3.5±0.8	6.7±3.2
Fluvastatin	Ext	0.18	1.42	6.8±1.0	5.0±1.4	1.6±1.0	5.3±1.1
Glimepiride	Ext	0.20	0.60	8.0±1.5	6.1±3.7	5.6±0.8	7.4±0.4
Imidapril	Ext	0.30	0.81	10.1±6.6	4.3±1.6	1.7±1.7	7.4±5.1
Lisinopril	Ext	0.34	1.00	7.5±2.5	5.0±1.4	5.8±2.9	6.8±3.0
Lovastatin	Ext	1.00	1.50	5.3±3.0	7.9±3.3	5.7±1.9	7.0±1.3
Quinaprilat	Ext	0.79	4.45	11.0±4.5	4.0±0.2	3.6±2.8	8.9±3.2
Simvastatin	Ext	0.25	1.25	10.5±1.6	5.1±1.6	2.6±2.6	7.2±0.9
Isradipine	Screen	0.39	1.35	-	-	-	-
Nifedipine	Screen	0.56	2.00	-	-	-	-
Nimodipine	Screen	0.21	0.68	-	-	-	-
Nisoldipine	Screen	0.44	1.37	-	-	-	-
Nitrendipine	Screen	0.17	1.21	-	-	-	-

*RSD and RE values of the high concetrations of these analytes were not taken into account for the calculation of the average RSD and RE values due to the lack of linearity of the last calibration points

For calibration purposes, the most suitable IS was chosen by studying the relative error values of the calibration standard concentrations and the linearity of the calibration curves. For some analytes (atorvastatin, candesartan cilexetil, cerivastatin, enalaprilat, fluvastatin, glimepiride, imidapril, lisinopril, lovastatin, quinaprilat and simvastatin) better results were obtained with an external calibration compared to a calibration using the reported IS. The applicability of external calibration using electrospray as ionization source is debatable due to its associated matrix effects. Moreover, only the use of isotopically marked IS can correct the matrix effects caused by the ionization technique, which is not feasible with these types of screening methods. Therefore, for the above mentioned substances, other IS should be tested for quantitation purposes [29].

Some calcium channel blockers (isradipine, nifedipine, nimodipine, nisoldipine, and nitrendipine) did not fit conveniently to a calibration curve with any IS and showed high imprecision among their replicas, which is probably due to the fact that these analytes degrade under light exposure [30]. Thus, even the method is not acceptable for the quantitative analysis of these drugs; it can be used to detect the presence of these calcium channel blockers in plasma samples.

Seven analytes (bisoprolol, carbutamide, labetalol, metoprolol, oxprenolol, timolol and tolazemide) lost their linearity in the higher range of the calibration curve, which may indicate a saturation of the detector due to the high intensity of the response. Problems of ion suppression were rejected since the quantitative values obtained for matrix effect showed no difference between high and low

concentrations. In order to evaluate the suitable calibration range the highest calibration standard was removed from the calibration curves of metoprolol and oxprenolol, which improved linearity of both compounds. For the other five analytes the last two calibration standards were rejected to obtain satisfying calibration curves. Therefore the calibration range for the quantitative analysis of these compounds was fixed as follows: from 0.5 to 38 µg/L for bisoprolol and labetalol; from 1 to 75 µg/L for timolol; from 1 to 125 µg/L for oxprenolol; from 4 to 500 µg/L for metoprolol and from 25 to 1880 µg/L for carbutamide and tolazemide. Dilution of plasma samples might be necessary in cases the concentrations of the analytes exceed the calibration range.

3.3.4. Precision and accuracy

Plasma samples spiked with low, medium and high concentrations of drugs were processed and analyzed. Their concentrations were obtained from the interpolation of the corrected area of each analyte on its corresponding calibration curve. Results obtained for the intra and inter day accuracy (RE) and precision (RSD) are summarized in Table 3.2 as the average values and their corresponding standard deviation obtained for the three different concentration levels (the complete validation data for precision and accuracy is gathered in Appendix V). Among the studied compounds 65 fulfil the precision (RSD < 15%) and accuracy (RE < 15%) criteria, including the compounds that fit better to an external calibration. Isradipine, nifedipine, nimodipine, nisoldipine and nitrendipine showed high RSD values and the calibration curves were not adequate, thus, the method

is only suitable for their qualitative analysis. Considering the lack of linearity of bisoprolol, carbutamide, labetalol, metoprolol, oxprenolol, timolol and tolazemide calibration curves, RE and RSD values corresponding to the high concentration of these analytes were not determined.

3.3.5. Application to real samples

Plasma samples obtained from patients under treatment with acenocoumarol, amlodipine, atenolol, atorvastatin, bisoprolol, enalapril, enalaprilat, felodipine, fluvastatin, lisinopril, metformin, metoprolol, quinaprilat, repaglinide, simvastatin, valsartan or a combination of them were analyzed using the described methodology. Besides studying quantitation and confirmation transitions, their ratio was measured and compared with the ratio obtained for a standard solution. All of these drugs were detected in the plasma samples except simvastatin, which was only detected in three plasma samples of the five samples of patients treated with this drug. Only in one sample simvastatin could be quantified.

Probable reasons for the lack of detection are its low therapeutic concentration range and the high ion suppression it suffers. In Table 4.3 the plasmatic concentration levels for 13 plasma samples obtained from patients under treatment with a combination of the studied compounds are reported. The chromatogram corresponding to patient 2 containing atorvastatin, bisoprolol and valsartan and spiked with the three different IS is shown in Figure 4.3.

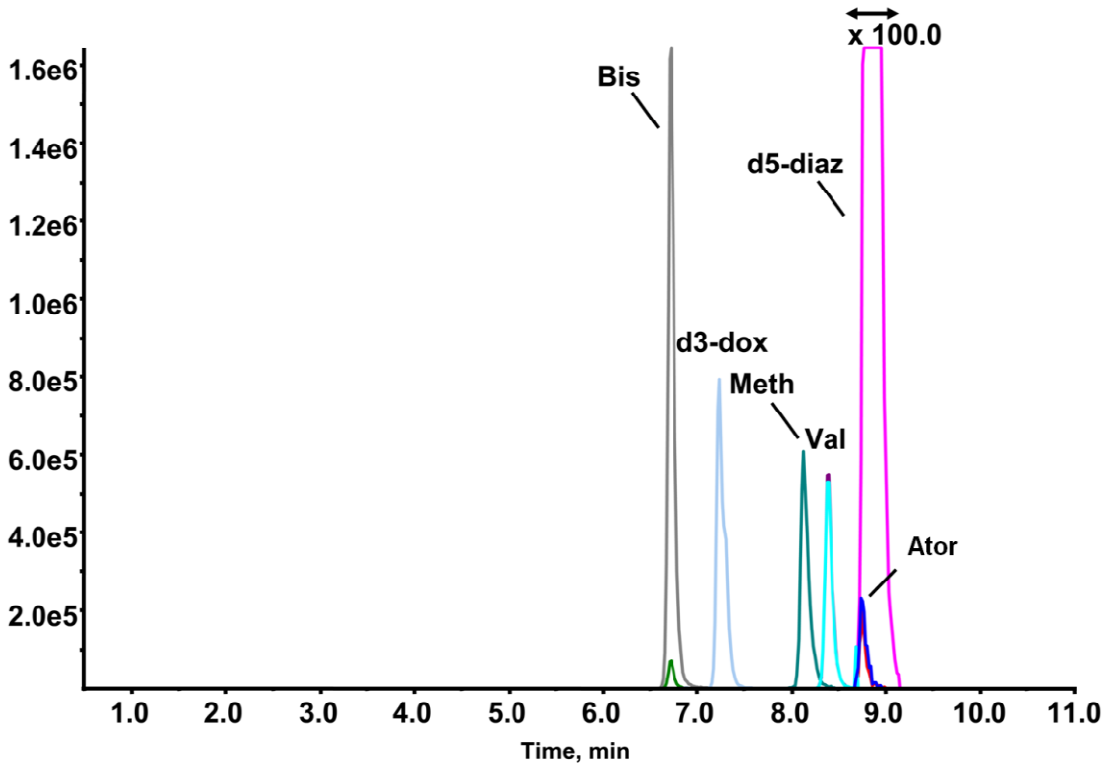

Figure 3.3. Chromatogram corresponding to a plasma sample of a patient (no. 2) under treatment with atorvastatin (Ator), bisoprolol (Bis) and valsartan (Vals) with the internal standards: d_5-diazepam (d_5-diaz), d_3-doxepin (d_3-dox) and methaqualone (meth).

3.4. Discussion and conclusions

We present a comprehensive approach for simultaneous analysis of many compounds usually prescribed in cardiovascular combined therapy. The proposed LC–MS/MS method is adequate for the screening of 78 drugs and showed an appropriate specificity, precision and accuracy for the quantitative determination of 55 compounds, using the internal standard approach. It was successfully applied to the detection and quantitation of several of the studied analytes in plasma samples obtained from patients under treatment with these drugs.

Table 3.3. Concentration values for plasma samples obtained from patients under cardiovascular treatment with a combination of the studied drugs (results reported with a 95% confidence level)

Patient	Administered Drug	Time after oral intake (h)	Dose (mg)	Concentration (μg/L) ±ts
1	Amlodipine	Unknown	Unknown	126.5 ± 22.0
	Fluvastatin	12	40	4.7 ± 0.3
2	Atorvastatin	12	5	1.0 ± 0.1
	Bisoprolol	3	160	2.4 ± 0.6
	Valsartan	3	10	732.9 ± 42.7
3	Amlodipine	12	10	128.4 ± 22.4
	Bisoprolol	12	10	2.8 ± 0.7
	Quinaprilat	1	12.5	363.9 ± 33.0
	Simvastatin	12	5	< LLOQ
4	Felodipine	12	5	1.4 ± 0.2
	Fluvastatin	12	80	20.0 ± 1.3
	Metoprolol	12	50	16.2 ± 2.2
	Valsartan	3	160	94.7 ± 5.5
5	Atorvastatin	12	8	0.9 ± 0.1
	Bisoprolol	2	10	< LLOQ
6	Atenolol	2	50	53.8 ± 5.2
	Enalapril	2	20	71.0 ± 6.2
	Enalaprilat	-	-	25.0 ± 2.5
	Simvastatin	12	40	< LLOQ
7	Atorvastatin	12	20	6.7 ± 0.5
	Enalapril	3	5	16.0 ± 1.4
	Enalaprilat	-	-	26.5 ± 2.6
	Metformin	3	850	755.5 ± 13.6
	Repaglinide	3	1	10.4 ± 1.0
8	Amlodipine	12	5	1.5 ± 0.2
	Atorvastatin	12	40	9.7 ± 0.7
	Metformin	18	425	57.3 ± 1.0
	Quinaprilat	2	20	6.1 ± 0.56
9	Felodipine	12	5	1.5 ± 0.2
	Fluvastatin	12	80	7.5 ± 0.5
	Metoprolol	12	50	14.4 ± 1.9
	Valsartan	3	160	1039.9 ± 60.6
10	Atorvastatin	12	5	0.9 ± 0.1
	Valsartan	2	180	68.36 ± 4.0

Table 3.3. Continuation

Patient	Administered Drug	Time after oral intake (h)	Dose (mg)	Concentration (μg/L) ±ts
11	Enalapril	3	20	103.3 ± 9.0
	Enalaprilat	-	20	15.1 ± 1.5
	Simvastatin	12	20	<LOD
12	Amlodipine	12	5	60.8 ± 10.6
	Atenolol	2	50	88.7 ± 8.6
	Lisinopril	2	20	43.5 ± 6.5
	Simvastatin	22	10	1.9 ± 0.2
13	Valsartan	3	160	87.9 ± 5.12
	Simvastatin	12	10	<LOD

Some of the compounds of interest fulfilled the precision and accuracy requirements only for external calibration. This kind of calibration is not suitable for the analysis of biological matrices with electrospray ionization, thus, different IS should be assessed to find a suitable one. If it is possible, the authors recommend the use of isotopic marked internal standards. A standard addition method could be an adequate alternative as well, since matrix effects are constant within the same plasma sample, but this method is very time consuming and a higher volume of sample is needed.

Isradipine, nifedipine, nimodipine, nisoldipine, and nitrendipine showed a very high variability probably due to their photodegradation. In order to avoid the degradation of these compounds light exposure of the sample should be minimized and amber vials used.

The recovery range is very wide (29.9–74.5%), probably due to the different chemical properties of the compounds, and for some analytes is lower

than 50%. However, the sensitivity of the method allows the analysis of those compounds in their therapeutic range, although some compounds showed a high matrix effect. Since protein precipitation does not remove endogenous plasma compounds (such as phospholipids), they cause high ion suppression to some of the studied analytes. This was the case for simvastatin, for which detection and quantitation limits were too high to cover the therapeutic range, as it was observed in the analysis of this drug in plasma samples. The same problem is expected to occur to lovastatin, taking into account the similar therapeutic range and quantitation limit. Alternative sample treatments such as solid phase extraction might reduce the matrix effects and improve the sensitivity, but the sample preparation process would be more time consuming and acceptable recoveries for all analytes included into this method would probably not be feasible with one extraction method.

The simple and non-selective pre-treatment procedure allows adding further analytes in case of addition of new compounds of the studied substance class, new drug families and new associations to maintain the applicability of the method for pharmacological therapies of the metabolic syndrome.

However, the suitability of using the method for quantitation purposes must be studied for each single compound, assuring the usefulness of the respective internal standard and including a validation for each analyte.

Acknowledgements

The authors thank the University of Basque Country and Ministry of Science and Innovation and Ministry of Science and Technology for financial support (GIU07/02, CTQ2009-11690, CTQ2006-11285) and Prof. Dr. Fritz Pragst from the Institute of Legal Medicine, Humboldt University, Berlin, Germany for the kind supply of drugs studied. Oskar Gonzalez thanks the University of Basque Country for his predoctoral grant and Nerea Ferreirós thanks the Government of the Basque Country for her post-doctoral grant. The authors also thank the Health Centre of Vitoria-Gasteiz old town for supplying blood samples from patients under cardiovascular treatment.

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APPENDIX II

OPTIMIZATION OF THE MRM (MULTIPLE REACTION MONITORING) CONDITIONS FOR THE STUDIED DRUGS.

Mass spectrometer arrangement

A simple diagram of the triple-quadrupole linear ion trap mass spectrometer used during this work is shown in Figure 3.4. When a multiple reaction monitoring (MRM) experiment is performed a precursor ion is selected on the first quadrupole (Q1). This ion suffers a fragmentation on the collision cell (CE or Q2) and then, the desired product ion is selected on the third quadrupole (Q3) to be monitored. In this way, MRM allows a sensitive and specific quantitation even in complex matrices.

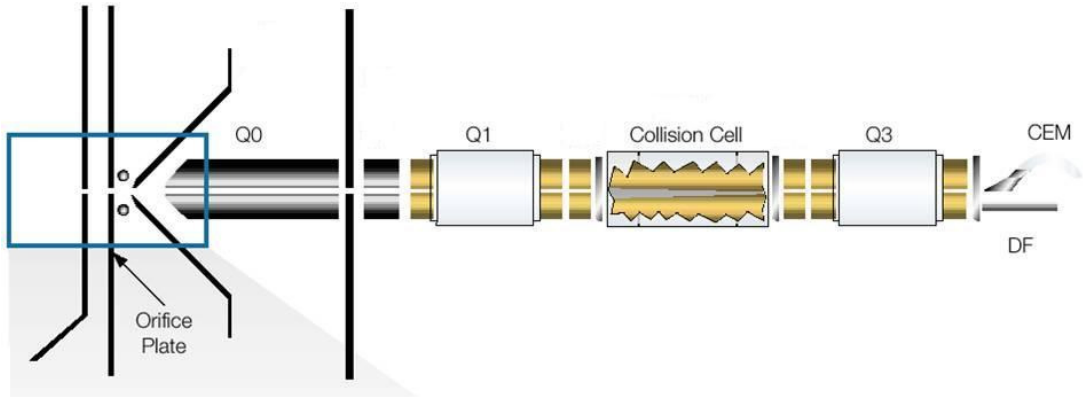

Figure 3.4. Diagram of the triple quadrupole mass analyser. Q0, Q1 and Q3 are zero, first and third quadrupole respectively.

MRM is a complex process where several factors can affect the intensity of the analytical signal: Declustering potential, collision energy… Therefore, in order to obtain the most sensitive signal a thorough optimization process must be followed.

Optimization of the mass transitions for the MRM analysis

The choice of the mass transitions used for the MRM method was carried out following the same protocol for each analyte. For this aim a solution of 1 mg/L of the analyte in methanol was infused directly on the mass spectrometer with a flow rate of 10 µL/min while mobile phase (A:B, 50:50) was being injected with a 200 µL/min flow rate. The followed procedure can be explained as follows, taking as an example the optimization for the β-blocker carvedilol MRM transitions:

1. A Mass Scan in ESI+ mode was carried out in order to identify the molecular ion $[M+H]^+$ (407.2 m/z for carvedilol).

2. Once the molecular ion was known, Enhanced Product Ion scans (EPI) at different collision energies (20, 35 and 50 V) were carried out to select the candidate product ions for the MRM transitions (Figure 3.5). In the case of carvedilol 407.2/283.2, 407.2/222.2, 407.2/194.2 and 407.2/100.1 transitions were chosen.

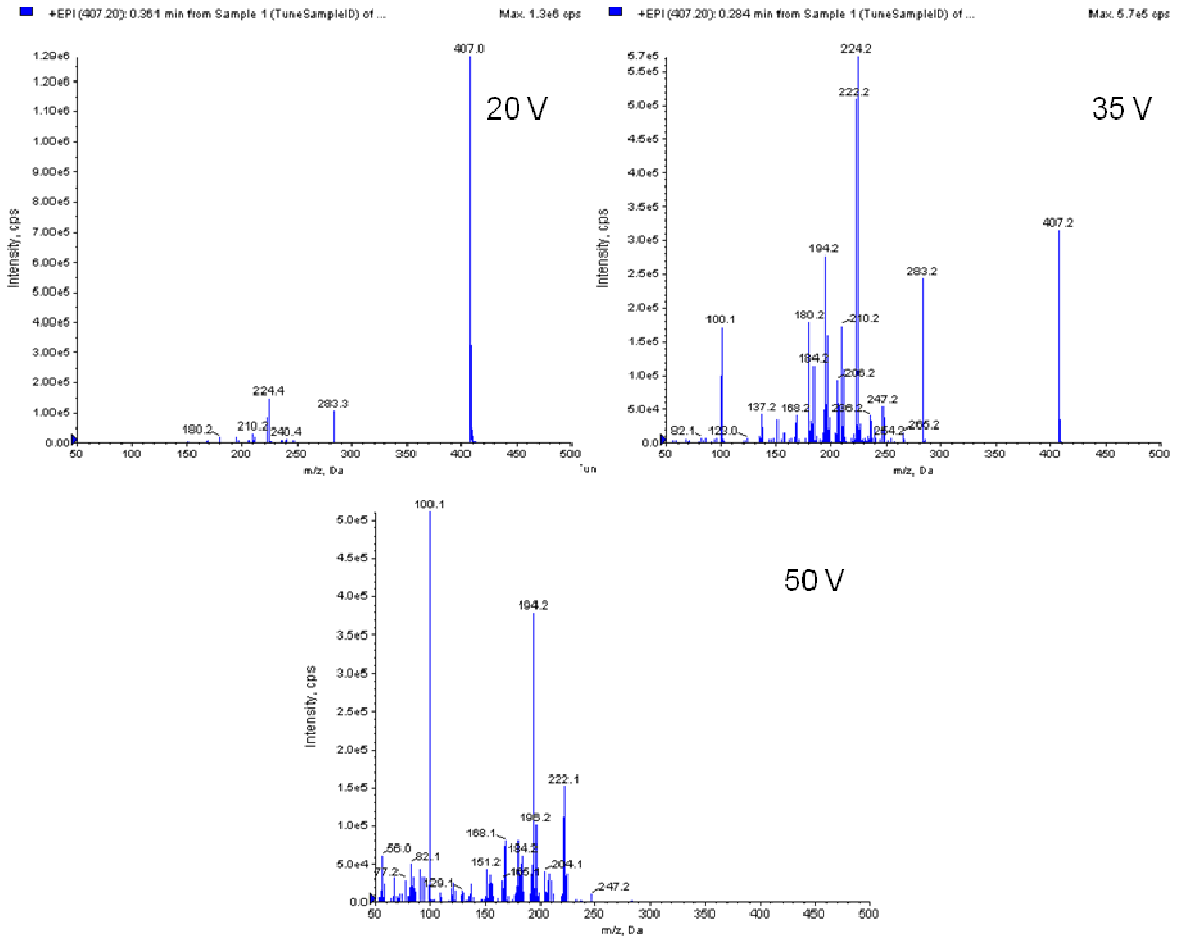

Figure 3.5. Carvedilol's EPI scans for 20, 35 and 50 V collision energies.

3. The best declustering potentials (DP) were found doing a screening from 0 to 100 V for all the chosen transitions at the same time(Figure 3.6). The DP controls the voltage of the orifice and it is used to minimize the solvent clusters. The higher it is, the higher energy the ions will receive. The potential that offered the highest intensity was fixed for the following parameter's optimization. 60 V declustering potential was chosen for carvedilol.

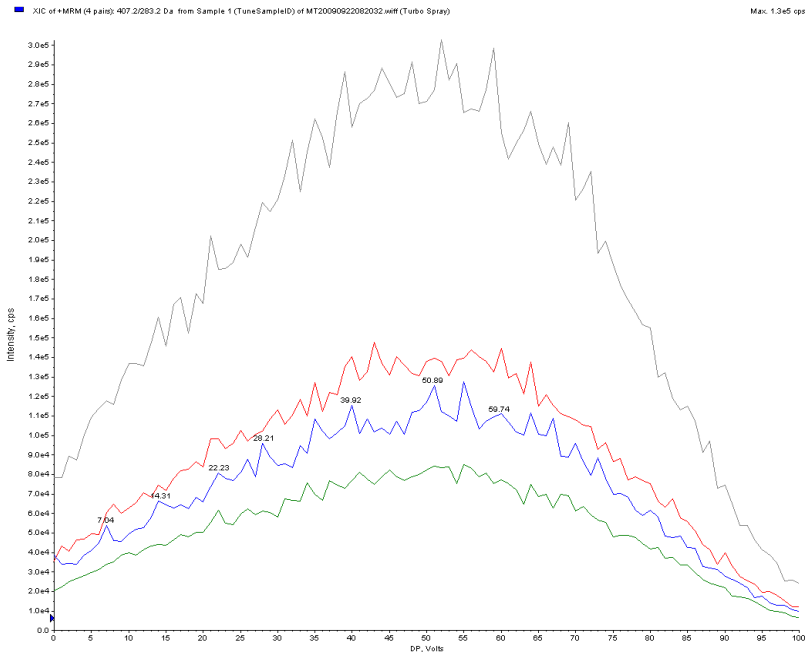

Figure 3.6. Declustering potential scan for the carvedilol transitions 407.2→283.2 (blue), 407.2→222.2 (red), 407.2→194.2 (green) and 407.2→100.1 (gray)

4. Afterwards, Entrance Potential (EP) scan was carried out from 1 to 12 V. This is the potential difference between Q0 and ground and guides the ions through the Q0. In Figure 3.7, it can be observed that EP keeps more or less constant from 4 V on. EP for carvedilol was fixed at 5 V.

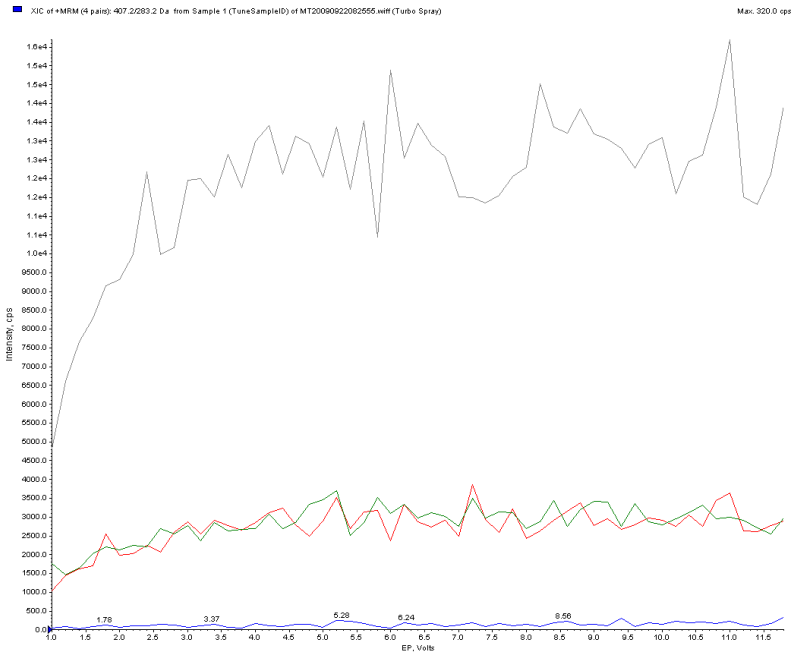

Figure 3.7. Entrance potential scan for the carvedilol transitions 407.2→283.2 (blue), 407.2→222.2 (red), 407.2→194.2 (green) and 407.2→100.1 (gray)

5. The next parameter to be optimized was Collision Energy (CE). For this aim a scan from 5 to 100 V was performed. Since CE is the amount of energy the precursor ion receives in the collision cell, each transition has a different optimal collision value. Usually, the smaller the product ion, the higher the optimal collision energy. In Figure 3.8 the CE scans for the four carvedilol transitions are shown. The chosen energy values were 27 V (407.2/283.2), 35 V (407.2/222.2), 53 V (407.2/194.2) and 44 V (407.2/100.1).

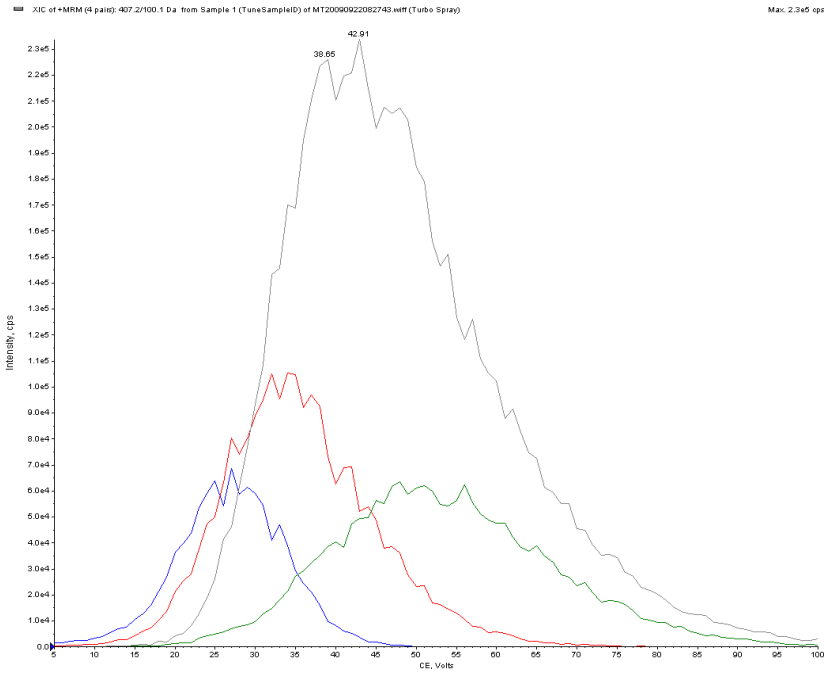

Figure 3.8. Collision Energy scan for the carvedilol transitions 407.2→283.2(blue), 407.2→222.2(red), 407.2→194.2 (green) and 407.2→100.1 (gray).

6. Once the different CE were fixed a Collision Cell Entrance Potential (CEP) scan was carried out from 0 to 40 V. This is the potential difference between Q0 and Q2 that focuses the ions into the collision cell. In Figure 3.9 the CEP scan for carvedilol transitions is shown. The intensity reaches a maximum around 15 V and then it maintains constant or slightly decreases. Therefore 15V CEP was fixed for MRM analysis. The value of this parameter can affect the optimal collision energy values. Thus, CE maximum values should be checked again as described in point 5. If the values vary, which was not the case of carvedilol, they are to be changed in the method.

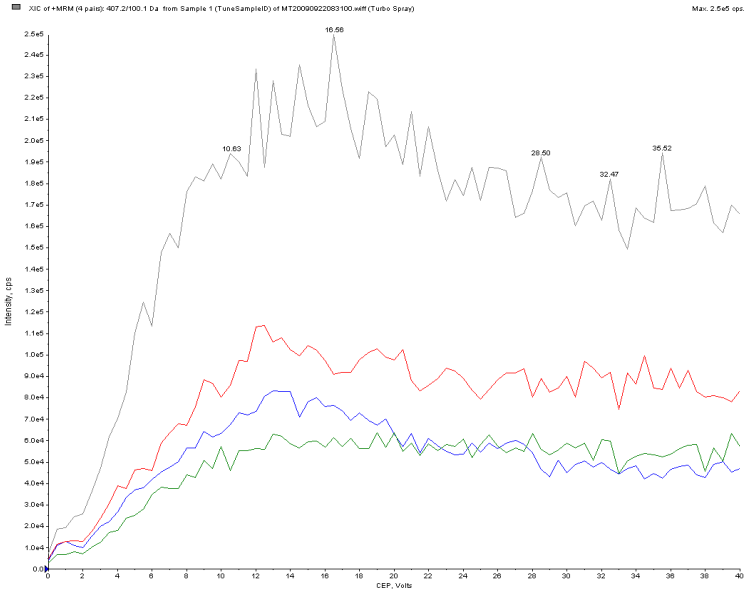

Figure 3.9. CEP scan for the carvedilol transitions 407.2→283.2(blue), 407.2→222.2(red), 407.2→194.2 (green) and 407.2→100.1 (gray).

7. The last parameter to set is the collision exit potential (CXP), which is the potential that transmits the ion into Q3. It is not possible to optimize this parameter at the same time for all the transition, thus each one must be analyzed independently. In Figure 3.10 the CXP scan for the 407.2/283.2 transition can be observed. An intensity peak is obtained around 3 V, which is selected as CXP for this transition.

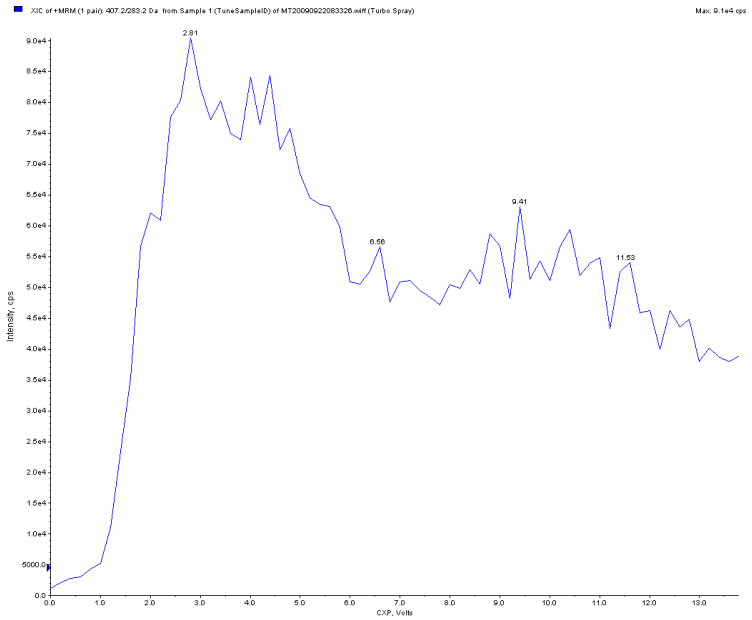

Figure 3.10. CXP scan corresponding to the 407.2→283.2 transition of carvediolol

8. Once all the parameters have been set, the intensity of all the transitions is monitored. The most intense one is chosen as quantitation transition and the second one as confirmation transition. Afterwards, the Ion Spray (IS) voltage is changed from 5000 V to 5500 V, then to 4000 V, to 3000 V, to 2000 V and finally to 1500 V. In Figure 3.11, the change of intensity can be observed for the four transitions of carvedilol where 407.2/100.1 was chosen as quantitation transition and 407.2/222.2 as confirmation transition. The intensity maintains constant from 5500 V to 3000V, but there are two great decays when the voltage changes to 2000 V (around minute 1.7) and to 1500 V (around minute 2.3). The IS cannot be changed during the analysis, thus, a suitable IS voltage for the monitorization of the transitions of the different analytes must be chosen. In the reported case, the IS voltage was set at 5500 V.

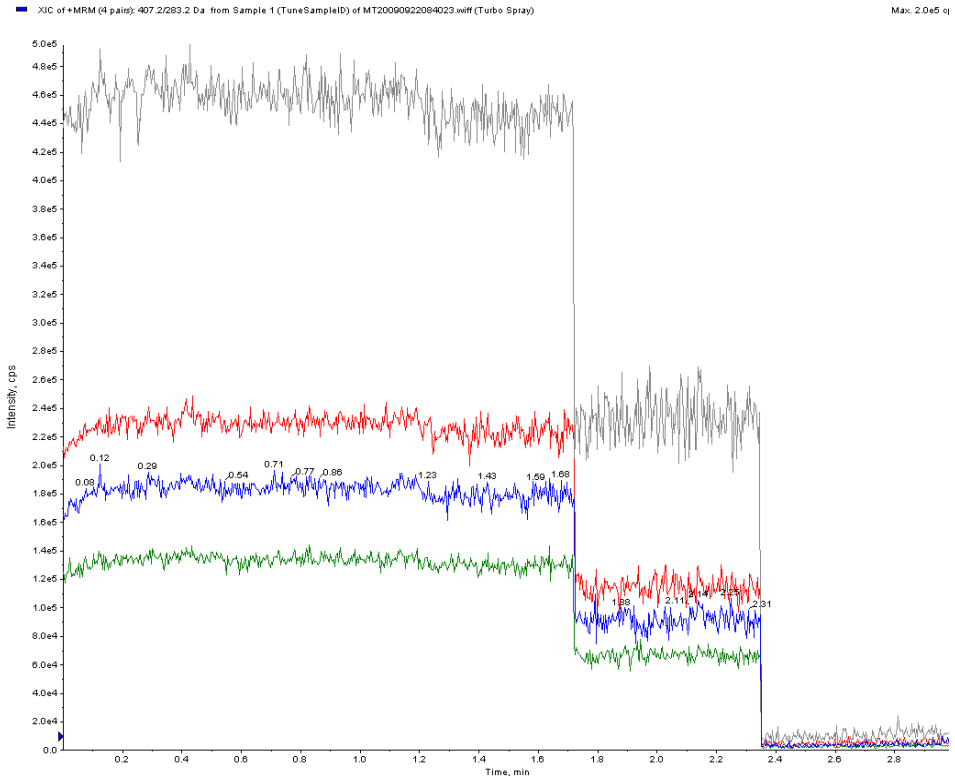

Figure 3.11. Intensity obtained for each transition under fixed conditions for carvedilol (407.2→283.2 (blue), 407.2→222.2 (red), 407.2→194.2 (green) and 407.2→100.1 (gray)). The signal decay around minute 1.7 coincides with the change of the IS to 2000V and the second decay with the change to 1500 V.

Dwell time and time windows for MRM analysis

A large number of transitions must be measured for the analysis of all the compounds, thus, scheduled MRM was used to maximize the number of points obtained for each chromatographic peak. This means that each transition was monitored only in the time window corresponding to the retention time of the analyte. This time window was fixed to 90 s in order to compensate for a possible change of the retention time. Once this value was set, a graph was built plotting the retention time versus the transition number (considering the time window as retention time error). As it can be observed in Figure 3.12 the maximum peak overlaying takes place around the minute 7 of the chromatographic separation,

when 83 transitions are measured at the same time. Taking into consideration that the length of the broadest chromatographic peak is 18 s, the cycle time (time needed to measure all the transitions one time) was fixed to 1.8 s in order to collect at least 10 points for this peak. In addition, the mass spectrometer has a pause time of 5 ms, which is the necessary time to change from one transition to the next one. Taking all of this into account, the minimum dwell time (the time the instrument measures each transition) is 17 ms, which is supposed to guarantee a suitable analysis.

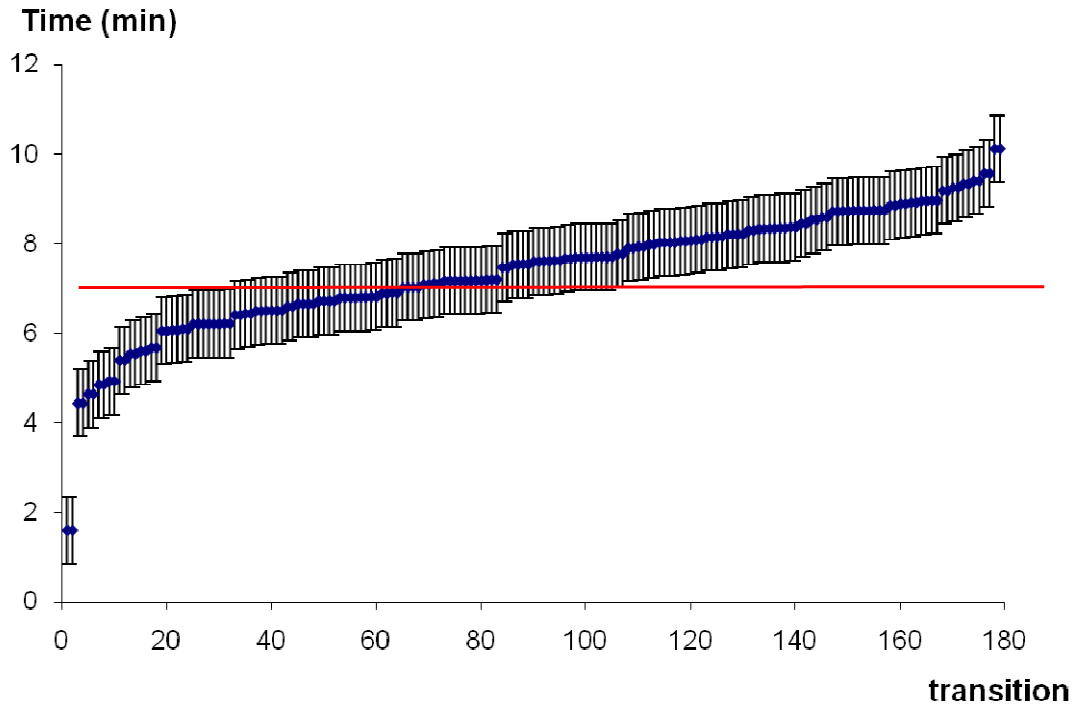

Figure 3.12. Retention time versus transition number. Y error lines show the time window and the red line belongs to the time when a maximum number of measures coincide.

In order to confirm that 17 ms dwell time was enough to allow a correct analysis two MRM methods were developed. The first one included 16 transitions of different analytes with a dwell time of 15 ms. The second one was set up with

the same transitions but it included an equilibration transition of 100 ms before each MRM transition to condition the mass spectrometer. The aim of this equilibration transition is to prepare the instrument for the measurement of the analysis transition and consequently be sure that it acquires the maximum number of counts. A sample containing these 16 analytes was analyzed with both methods and comparing the obtained analytical signals (as peak areas) no significant differences were observed. Therefore, it can be assured that the method developed without equilibration transition is able to measure the MRM signals correctly.

The quantitation and confirmation transitions that were used in the MRM method for each analyte are shown in Table 1 together with the optimal values for DP, EP, CEP, CE and CXP.

Table 4. MRM analysis conditions

Legend: **Q1:** Precursor Ion (m/z), **Q3:** Fragment Ion (m/z), **DP:** Declustering Potential (V), **EP:** Entrance Potential (V), **CEP:** Cell Entrance Potential (V), **CE:** Collision Energy (V), **CXP:** Cell Exit Potential (V), **t_r:** Retention Time (min). Quantitation transitions are shown in bold.

Analyte	Q1	Q3	DP	EP	CEP	CE	CXP	t_r
Acebutolol	337.2	319.2	53	5	11	22	3.5	**6.07**
Acebutolol	**337.2**	**116.1**	**53**	**5**	**11**	**32**	**2.0**	
Acenocoumarol	354.1	296.1	33	5	16	25	3.5	**8.75**
Acenocoumarol	**354.1**	**163.1**	**33**	**5**	**16**	**24**	**2.5**	
Alprenolol	**250.2**	**116.1**	**47**	**4**	**9**	**24**	**2.0**	**6.88**
Alprenolol	250.2	91.1	47	4	9	56	2.0	
Amiloride	**230.1**	**171.1**	**41**	**5**	**9**	**24**	**2.5**	**4.92**
Amiloride	230.1	161.1	41	5	9	35	2.5	
Amlodipine	**409.2**	**294.1**	**28**	**4**	**16**	**17**	**3.5**	**7.61**
Amlodipine	409.2	238.1	28	4	16	19	3.5	
Atenolol	267.2	190.2	43	5	10	26	3.0	**4.63**
Atenolol	**267.2**	**145.1**	**43**	**5**	**10**	**36**	**2.5**	
Atorvastatin	**559.3**	**440.2**	**53**	**5**	**17**	**32**	**4.0**	**8.74**
Atorvastatin	559.3	250.2	53	5	17	61	3.0	
Befunolol	**292.2**	**250.2**	**50**	**5**	**11**	**24**	**3.0**	**6.50**
Befunolol	292.2	203.2	50	5	11	30	3.0	
Betaxolol	308.2	133.1	40	5	10	37	2.0	**7.02**
Betaxolol	**308.2**	**116.1**	**40**	**5**	**10**	**28**	**2.0**	
Bisoprolol	326.2	222.2	53	4	10	19	3.0	**6.72**
Bisoprolol	**326.2**	**116.1**	**53**	**4**	**10**	**26**	**2.0**	
Bopindolol	**381.2**	**186.1**	**51**	**5**	**13**	**31**	**2.5**	**7.62**
Bopindolol	381.2	158.1	51	5	13	49	2.5	
Bunitrolol	**249.2**	**193.2**	**31**	**5**	**9**	**20**	**2.5**	**6.20**
Bunitrolol	249.2	175.2	31	5	9	24	2.5	
Bupranolol	**272.1**	**216.1**	**41**	**5**	**9**	**20**	**3.0**	**7.02**
Bupranolol	272.1	155.1	41	5	9	34	2.5	
Candesartan	441.2	423.2	37	5	15	18	4.0	**8.34**
Candesartan	**441.2**	**263.2**	**37**	**5**	**15**	**18**	**3.0**	
Cand. Cilex.	**611.3**	**423.2**	**39**	**5**	**22**	**18**	**4.0**	**10.12**
Cand. Cilex.	611.3	207.2	39	5	58	18	2.5	
Carazolol	299.2	222.2	43	5	10	27	3.0	**6.80**
Carazolol	**299.2**	**116.2**	**43**	**5**	**10**	**29**	**2.5**	
Carbutamide	**272.2**	**156.1**	**32**	**5**	**11**	**22**	**2.5**	**6.59**
Carbutamide	272.2	92.1	32	5	11	42	1.5	

Table 4. MRM analysis conditions (continuation)

Analyte	Q1	Q3	DP	EP	CEP	CE	CXP	t_r
Carteolol	**293.2**	**237.2**	**36**	**5**	**10**	**21**	**3.0**	**5.60**
Carteolol	293.2	202.2	36	5	10	30	2.5	
Carvedilol	407.2	222.2	60	5	13	35	3.0	**7.70**
Carvedilol	**407.2**	**100.1**	**60**	**5**	**13**	**44**	**2.0**	
Celiprolol	380.2	307.2	50	5	12	25	3.5	**6.49**
Celiprolol	**380.2**	**251.2**	**50**	**5**	**12**	**31**	**3.0**	
Cerivastatin	**460.2**	**356.2**	**78**	**5**	**15**	**50**	**3.5**	**8.54**
Cerivastatin	460.2	324.2	78	5	15	67	3.5	
Cilazapril	**418.2**	**211.2**	**34**	**5**	**15**	**28**	**3.0**	**7.70**
Cilazapril	418.2	70	34	5	15	70	1.5	
Clopidogrel	**322.1**	**212.1**	**33**	**5**	**12**	**23**	**3.0**	**8.97**
Clopidogrel	322.1	184.1	33	5	12	30	3.0	
Enalapril	**377.2**	**234.2**	**45**	**5**	**12**	**27**	**3.5**	**7.08**
Enalapril	377.2	117.1	45	5	12	58	2.5	
Enalaprilat	**349.2**	**206.2**	**46**	**5**	**12**	**26**	**3.0**	**6.10**
Enalaprilat	349.2	117.1	46	5	12	49	2.0	
Eprosartan	425.2	207.2	65	5	16	33	3.0	**7.17**
Eprosartan	**425.2**	**107**	**65**	**5**	**16**	**75**	**2.0**	
Esmolol	296.2	219.2	53	5	10	27	3.0	**6.50**
Esmolol	**296.2**	**145.1**	**53**	**5**	**10**	**38**	**2.0**	
Etacrynic acid	303	303	30	4	10	5	4.0	**8.14**
Etacrynic acid	**303**	**257**	**30**	**4**	**10**	**13**	**3.0**	
Felodipine	384.1	352.1	25	4	12	17	3.5	**8.89**
Felodipine	**384.1**	**338.1**	**25**	**4**	**12**	**17**	**3.5**	
Fendiline	316.2	212.2	40	5	13	19	3.0	**8.09**
Fendiline	**316.2**	**105.1**	**40**	**5**	**13**	**40**	**2.0**	
Fluvastatin	**412.2**	**266.2**	**46**	**5**	**13**	**26**	**3.5**	**8.74**
Fluvastatin	412.2	224.2	46	5	13	44	3.0	
Gallopamil	**485.3**	**165.1**	**68**	**5**	**17**	**42**	**2.5**	**7.60**
Gallopamil	485.3	150.1	68	5	17	63	2.5	
Glibenclamide	**494.2**	**369.1**	**25**	**5**	**19**	**21**	**3.5**	**8.92**
Glibenclamide	494.2	169.1	25	5	19	50	2.5	
Glibornuride	367.2	170.2	43	5	13	27	3.0	**8.20**
Glibornuride	**367.2**	**152.2**	**43**	**5**	**13**	**27**	**2.5**	
Gliclazide	**324.2**	**127.1**	**47**	**5**	**13**	**27**	**2.5**	**8.15**
Gliclazide	324.2	110.1	47	5	13	42	2.0	

Table 4. MRM analysis conditions (continuation)

Analyte	Q1	Q3	DP	EP	CEP	CE	CXP	t_r
Glimepiride	**491.2**	**352.2**	**38**	**5**	**15**	**20**	**4.0**	**9.19**
Glimepiride	491.2	126.1	38	5	15	47	2.5	
Glipizide	446.2	347.2	34	5	15	20	3.5	**8.21**
Glipizide	**446.2**	**321.1**	**34**	**5**	**15**	**20**	**3.5**	
Gliquidone	**528.2**	**403.2**	**40**	**5**	**16**	**18**	**3.5**	**9.57**
Gliquidone	528.2	386.2	40	5	16	30	3.5	
Imidapril	406.2	332.2	51	5	14	26	3.5	**7.17**
Imidapril	**406.2**	**234.2**	**51**	**5**	**14**	**28**	**3.0**	
Irbesartan	429.2	207.1	54	5	17	34	3.0	**8.37**
Irbesartan	**429.2**	**180.1**	**54**	**5**	**17**	**60**	**2.5**	
Isradipine	372.2	340.2	21	4	13	17	3.5	**8.45**
Isradipine	**372.2**	**312.2**	**21**	**4**	**13**	**17**	**3.0**	
Labetalol	**329.2**	**311.3**	**30**	**5**	**13**	**18**	**3.5**	**6.65**
Labetalol	329.2	91.1	30	5	13	65	2.0	
Lercanidipine	**612.3**	**280.2**	**50**	**10**	**22**	**32**	**3.5**	**8.85**
Lercanidipine	612.3	100.1	50	10	22	52	2.5	
Levobunolol	**292.2**	**236.2**	**45**	**5**	**11**	**22**	**3.5**	**6.43**
Levobunolol	292.2	201.2	45	5	11	28	3.0	
Lisinopril	406.2	246.2	50	5	15	34	3.0	**5.39**
Lisinopril	**406.2**	**84.1**	**50**	**5**	**15**	**59**	**2.0**	
Lovastatin	**405.3**	**285.3**	**28**	**4**	**12**	**16**	**3.0**	**9.34**
Lovastatin	405.3	199.2	28	4	12	27	2.5	
Mepindolol	263.2	186.2	39	5	10	24	2.5	**6.05**
Mepindolol	**263.2**	**116.1**	**39**	**5**	**10**	**27**	**2.0**	
Metformin	**130.1**	**71**	**33**	**4**	**7**	**32**	**2.0**	**1.58**
Metformin	130.1	60	33	4	7	19	2.0	
Metipranolol	310.2	233.2	55	5	12	25	2.5	**6.72**
Metipranolol	**310.2**	**191.2**	**55**	**5**	**12**	**31**	**2.5**	
Metoprolol	268.2	191.2	50	5	10	25	3.0	**6.20**
Metoprolol	**268.2**	**133.1**	**50**	**5**	**10**	**36**	**2.5**	
Nadolol	310.2	254.2	48	5	10	24	3.0	**5.53**
Nadolol	**310.2**	**201.2**	**48**	**5**	**10**	**32**	**3.0**	
Nifedipine	**347.1**	**315.1**	**15**	**4.5**	**13**	**12**	**3.5**	**7.69**
Nifedipine	347.1	254.2	15	4.5	13	27	3.5	
Nimodipine	**419.2**	**343.2**	**24**	**4**	**15**	**18**	**3.5**	**8.06**
Nimodipine	419.2	301.1	24	4	15	32	3.5	

Table 4. MRM analysis conditions (continuation)

Analyte	Q1	Q3	DP	EP	CEP	CE	CXP	t_r
Nicardipine	**480.2**	**315.2**	**52**	**5**	**17**	**33**	**3.5**	**8.73**
Nicardipine	480.2	166.2	52	5	17	25	2.5	
Nisoldipine	**389.2**	**357.2**	**30**	**4**	**14**	**14**	**3.5**	**8.72**
Nisoldipine	389.2	315.2	30	4	14	14	3.5	
Nitrendipine	361.1	329.2	29	4	13	18	3.5	**8.59**
Nitrendipine	**361.1**	**315.1**	**29**	**4**	**13**	**18**	**3.5**	
Oxprenolol	266.2	225.2	33	5	13	18	3.0	**6.65**
Oxprenolol	**266.2**	**116.1**	**33**	**5**	**13**	**24**	**2.5**	
Penbutolol	**292.2**	**236.2**	**47**	**5**	**10**	**23**	**3.0**	**7.69**
Penbutolol	292.2	133.1	47	5	10	34	2.5	
Perindopril	369.2	295.2	49	5	11	24	3.0	**7.17**
Perindopril	**369.2**	**172.2**	**49**	**5**	**11**	**28**	**2.5**	
Pindolol	249.2	172.2	40	5	12	23	2.5	**5.67**
Pindolol	**249.2**	**116.1**	**40**	**5**	**12**	**24**	**2.0**	
Practolol	267.2	190.2	40	5	10	25	3.0	**4.84**
Practolol	**267.2**	**148.2**	**40**	**5**	**10**	**31**	**2.5**	
Propranolol	**260.2**	**183.2**	**49**	**4.5**	**10**	**26**	**3.0**	**6.90**
Propranolol	260.2	116	49	4.5	10	26	2.5	
Quinaprilat	**411.2**	**206.2**	**37**	**5**	**15**	**28**	**2.5**	**7.20**
Quinaprilat	411.2	117.1	37	5	15	55	2.0	
Ramipril	**417.2**	**234.2**	**52**	**4.5**	**15**	**30**	**3.0**	**7.67**
Ramipril	417.2	117.1	52	4.5	15	57	2.5	
Repaglinide	**453.2**	**230.2**	**54**	**5**	**18**	**39**	**3.0**	**8.33**
Repaglinide	453.2	162.2	54	5	18	30	2.5	
Rosiglitazone	**358.1**	**135.1**	**60**	**4**	**13**	**38**	**2.5**	**6.79**
Rosiglitazone	358.1	107.1	60	4	13	55	2.0	
Simvastatin	**419.3**	**285.3**	**36**	**5**	**14**	**18**	**3.0**	**9.41**
Simvastatin	419.3	199.2	36	5	14	28	2.5	
Sotalol	**273.2**	**255.2**	**28**	**4**	**10**	**16**	**3.0**	**4.43**
Sotalol	273.2	133.1	28	4	10	38	2.5	
Talinolol	364.2	308.2	54	5	12	25	3.5	**6.79**
Talinolol	**364.2**	**100.1**	**54**	**5**	**12**	**33**	**2.0**	
Telmisartan	515.2	497.3	83	5	16	47	4.0	**8.96**
Telmisartan	**515.2**	**276.2**	**83**	**5**	**16**	**65**	**3.0**	
Tertalolol	296.2	240.2	45	5	11	23	3.0	**7.18**
Tertalolol	**296.2**	**222.2**	**45**	**5**	**11**	**27**	**3.0**	

Table 4. MRM analysis conditions (continuation)

Analyte	Q1	Q3	DP	EP	CEP	CE	CXP	t_r
Ticlopidine	**264.1**	**154.1**	**41**	**5**	**12**	**24**	**2.5**	**6.81**
Ticlopidine	264.1	125.0	41	5	12	44	2.5	
Timolol	**317.2**	**261.2**	**45**	**5**	**11**	**23**	**3.5**	**6.20**
Timolol	317.2	244.2	45	5	11	30	3.5	
Tolazemide	**312.1**	**115.1**	**43**	**5**	**12**	**27**	**2.0**	**7.90**
Tolazemide	312.1	91.1	43	5	12	57	2.0	
Tolbutamide	**271.1**	**155.1**	**35**	**5**	**9**	**24**	**2.5**	**7.53**
Tolbutamide	271.1	91.1	35	5	9	49	2.0	
Toliprolol	**224.2**	**147.1**	**45**	**5**	**9**	**24**	**2.5**	**6.21**
Toliprolol	224.2	119.1	45	5	9	31	2.0	
Torasemide	**349.1**	**264.1**	**38**	**5**	**11**	**25**	**3.0**	**7.11**
Torasemide	349.1	168.1	38	5	11	66	2.5	
Valsartan	**436.2**	**235.2**	**37**	**5**	**15**	**27**	**3.0**	**8.30**
Valsartan	436.2	207.1	37	5	15	40	3.0	
Verapamil	**455.3**	**165.1**	**60**	**5**	**16**	**40**	**3.0**	**7.54**
Verapamil	455.3	150.1	60	5	16	59	2.5	
Warfarin	309.1	251.1	27	5	12	26	3.0	**8.35**
Warfarin	**309.1**	**163.1**	**27**	**5**	**12**	**22**	**2.5**	
d3-doxepin	283.1	107.1	40	10	18	35	4	7.19
d5-diazepam	290.1	154.1	40	10	18	35	4	8.75
methaqualone	251.1	91.1	40	10	21	50	4	8.07

CHAPTER IV

SYSTEMATIC APPROACH FOR THE OPTIMIZATION OF AN ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

ABSTRACT

This work reports a systematic approach for the optimization of an UPLC method for the chromatographic separation of drugs of different families prescribed in cardiovascular therapy: acenocoumarol, amiloride, bisoprolol, fluvastatin, furosemide, glibenclamide, hydrochlorothiazide, rosiglitazone, valsartan, verapamil and warfarin. Detection of the analytes was carried out by means of a photodiode array detector (PDA) and a fluorescence (FLUO) detector. Initially, the most significant variables in liquid chromatography were studied: pH of the mobile phase, nature of the organic modifier and stationary phase type. The pH range was fixed between 3.5 and 5.5; the most commonly used methanol and acetonitrile solvents were tested as organic modifiers and two different stationary phases, C18 and pentafluorophenyl (PFP), were assessed. Taking into account the resolution obtained for the chromatographic separations, methanol, C18 phase and pH 4.5 were chosen as organic modifier, stationary phase and mobile phase pH, respectively. Once the previous parameters were fixed gradient slope, column temperature and flow rate were tested. The best chromatographic separation was achieved working at 30 ºC using gradient elution mode with a flow rate of 0.4 mL/min. Finally, robustness of this separation was studied by experimental design. The reported method allows the separation of the 11 analytes in less than six minutes and it is suitable for the analysis of these analytes in spiked human plasma samples.

4.1. Introduction

Since M.S. Tswett carried out the first chromatographic experiments in the early 1900s, this technique has been in a continuous development [1]. In the late 60s the high performance liquid chromatography (HPLC) emerged as a versatile separation technique and in a few years it became a leading analytical tool in pharmaceutical industry. From that moment onward significant progress was made, but it was not until the start of this century, that the Ultra-high Performance Liquid Chromatography (UHPLC) appeared [2].

UHPLC instruments are able to work at higher pressures than conventional HPLC instruments allowing the use of particles of smaller size in the column packing. This increases the efficiency, reduces solvent consumption and minimizes the analysis time. Anyway, as any separation technique it requires a correct optimization to achieve the best performance. Optimization of chromatographic separations can be a tedious and arduous task, especially if the detection is not very selective. Thus, in many research laboratories optimization processes are based on previously developed methods or are carried out following One Variable At a Time (OVAT) strategies. Both options can be very time-consuming and what is worse, the obtained results are seldom the optimum, due to the fact that interactions among variables are not considered [3].

An alternative to these optimization techniques is to follow a systematic approach that takes into account the most important variables at the same time. These variables are usually the pH of the mobile phase, the nature of the organic

modifier and the stationary phase of the chromatographic column. Once these parameters have been chosen less significant variables can be adjusted until a suitable chromatographic separation is achieved.

This work reports an UPLC optimization, following the mentioned approach, for the analysis of several drugs prescribed in combined cardiovascular therapy: acenocoumarol (anticoagulant), amiloride (potasium-sparing diuretic), bisoprolol (β-blocker antihypertensive), fluvastatin (lipid-lowering drug), furosemide (loop diuretic), glibenclamide (sulfonyluric antidiabetic), hydrochlorothiazide (thiazidic diuretic), rosiglitazone (thiazolidinedionic antidiabetic), valsartan (ARA-II antihypertensive), verapamil (calcium channel blocker antihypertensive) and warfarin (anticoagulant). These drugs have very different physicochemical properties, thus, they are adequate to study the suitability of the followed strategy. The detection was carried out by Photodiode-array (PDA) detection and Fluorescence (FLUO) detection.

4.2. Experimental procedure

4.2.1. Instrumentation

Analyses were performed on an Acquity UPLC system (Waters, Milford, USA), coupled to a PDA detector and FLUO detector working with two different channels. The data sampling rate for the PDA detector was set to 20 points/s and for the FLUO detector to 2 points/s. System control, data collection and data processing were accomplished using Empower 2 software.

The chromatographic columns used to perform the separation were BEH C18, 1.7 µm, 50×2.1 mm (Waters, Milford, USA) and Kinetex PFP 1.7 µm, 50×2.1 mm (Phenomenex, California, USA). The pre-columns recommended by the respective companies were coupled prior to the analytical column.

The pH was measured with a Crison GPL 22 pH-meter (Barcelona, Spain) using a Crison glass-combined electrode model 5209 with a reference system Ag/AgCl and KCl 3 M saturated in AgCl as electrolyte.

4.2.2. Chemical and reagents

Acenocoumarol, amiloride hydrochloride, furosemide, rosiglitazone maleate, verapamil hydrochloride and warfarin were generously provided by the Insitute of Forensic Medicine in Freiburg (Germany). Sodium fluvastatin and valsartan were kindly supplied by Novartis (Basel, Switzerland) and bisoprolol by Merck (Darmstad, Germany). Hydrochlorothiazide, metformin hydrochloride and glibenclamide were purchased from Sigma Aldrich (MO, USA). 1000 mg/L methanol stock solutions were prepared for each compound.

Acetic acid, formic acid, ammonium acetate and ammonium formate from Sigma Aldrich were used in the preparation of the buffer solutions. Gradient quality methanol (Romil, Cambridge, USA) and acetonitrile (Baker, Deventer, Holland) were used as organic modifiers. Purified water from a Milli-Q system (Millipore, MA, USA) was used in the preparation of buffer and reagent solutions.

Drug-free human plasma samples were purchased from the Blood Bank of Galdakao Hospital (Biscay, Basque Country) and collected in polypropylene tubes to be frozen at -20 ºC.

4.2.3. Photometric and fluorimetric study

In order to optimize the analysis conditions, the photometric and fluorimetric properties of the analytes were studied. Each analyte was injected separately into the chromatographic system. A 3 minutes linear gradient was performed using methanol as organic modifier (from 10% to 95%). The effect of the pH in the absorption and fluorimetric spectra was studied using four different buffer solutions as aqueous mobile phases: pH 2.5 (formic/formate, 5 mM), pH 4.5 (acetic/acetate, 5 mM), pH 6.5 (citric/citrate, 5 mM) and pH 8.5 (ammonium/ammonia, 5mM).

Absorption spectra were collected during the whole separation using the PDA detector with an acquisition range from 190 to 400 nm at a sampling rate of 80 points/s

The optimum wavelengths for the fluorimetric detection were obtained using the tool the fluorescence detector offers to acquire excitation and emission spectra during a chromatographic separation. First of all, and taking into account the absorption spectrum, the excitation wavelength (λ_{ex}) was set and the emission spectra acquired in a wavelength range of 70 nm. Once the optimum emission wavelength (λ_{em}) was found it was fixed and the excitation spectrum acquired. In

this way, the optimum excitation and emission wavelengths for fluorimetric detection were obtained.

4.2.4. Chromatographic optimization

4.2.4.1. Choice of mobile phase pH, organic solvent and stationary phase

The most significant variables in a chromatographic separation are the pH of the mobile phase, the stationary phase of the column and the nature of the organic modifier [4]. In order to find the best separation two chromatographic columns, two organic modifiers and mobile phases buffered at three different pH were tested. All the aqueous mobile phases had a 10% of the corresponding organic modifier in order to avoid bacterial growth. Due to the lack of fluorescence of some analytes depending on pH, its range was limited. Thus, the pH of the aqueous mobile phases assessed were 3.5, 4.5 and 5.5. The influence of the stationary phase was studied using a common C18 BEH column with a retention mechanism based on hydrophobic interactions and a PFP (pentafluorophenyl) column with a more complex retention mechanism (hydrophobic, aromatic, dipole-dipole and hydrogen bonding interactions). The organic modifiers tested were the most widely used methanol and acetonitrile.

All these variables were systematically studied carrying out the 12 possible combinations of experiments. For this aim, 5 µL of a sample containing 5 µg/mL of all the analytes was repeatedly injected using a linear gradient of 5 minutes. When methanol was used as organic modifier the gradient increased from 0% to 95% in

organic phase, whereas when acetonitrile was used the gradient increased only to 90% in order to compensate the higher eluotropic strength of this solvent. The highest proportion in organic solvent was held for 1 minute before returning to initial conditions.

Once all these experiments were carried out the most suitable combination of organic modifier, stationary phase and pH was chosen, using as selection criteria the peak resolution (Eq. 1) of the adjacent peaks.

$$R_s = \frac{2 \times (t_{R_b} - t_{R_a})}{w_b - w_a} \qquad \begin{matrix} t_R = retention time \\ w = peak\ width \end{matrix} \qquad \text{Eq. 1}$$

4.2.4.2. Fine-tune of the method

Once the most significant variables were fixed, less important factors were studied in order to find the most suitable chromatographic separation. First, different slope gradients were tested, then the flow rate was changed between 0.4 and 0.45 mL/min and finally three different column temperatures were assessed: 27 ºC, 30 ºC and 35 ºC.

Moreover, the best reconstitution solution and injection volume for further analysis of treated samples were studied. For this aim, spiked samples containing 1 µg of each compound were evaporated to dryness under N_2 stream and reconstituted in 200 µL of solutions containing different aqueous phase:methanol proportions (from 100:0, to 0:100). 2.5, 5.0 and 7.5 µL of each one of these samples were injected to test the effect of the injection volume.

4.2.4.3. Choice of IS for further analysis

Once the separation method was fully developed different drugs were injected in order to find possible internal standards (IS) for the future determination of the analytes in human matrices. These compounds tested as IS belonged to the drug families of the studied compounds and were all of them fluorescence: alprenolol, atenolol, betaxolol, bumetanide, candesartan, candesartan cilexetyl, irbesartan, levobunolol, losartan, metoprolol, nadolol, propanolol and telmisartan. 5 µL of 5 µg/mL solutions of each analyte were separately injected to check if they overlapped with the studied analytes.

4.2.5. Robustness of the method

A chromatographic separation can be affected by several factors as it has been observed during method development: pH, temperature… When a chromatographic method is performed these factors can slightly vary from one day to another, thus, these variations should not have influence in the separation. According to ICH definition [5] robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

In order to check the robustness of the developed method experimental design was used to study the effect of the variables that could have a significant influence on the separation: pH of the aqueous mobile phase, temperature of the column, flow rate, reconstitution solution composition and percentage of organic

modifier in aqueous mobile phase. For this aim a fractional factorial design (resolution V) was carried out using *The Unscrambler* software for data treatment [3].

The low and high levels within the variables effect was studied were fixed according to the uncertainty of these variables given by the pH meter, column oven, pumping system and volumetric material. Applying a coefficient for extreme levels (k) all the expected range of variation was covered [6-8]. k=5 is proposed as default value and k≥3 is at least needed to define extreme levels. In this case, this minimum value was applied to the column temperature taking into account the low temperature drift of the column oven. The coefficient applied to the methanol percentage in aqueous phase was considerably higher to compensate for the small uncertainty. The coefficients for extreme levels and the working ranges are gathered in Table 4.1.

Table 4.1. Uncertainty of the varialbes and studied range

Variable	Optimum	Uncertainty	k	Range
pH	4.5	±0.01	5	4.5±0.05
Column Tª (ºC)	30	±1	3	30±3
Flow rate (mL/min)	0.40	±1%	5	0.40±0.02
MeOH in reconstitution (%)	45.00	±0.25*	5	45.00±1.25
MeOH in aqueous phase (%)	10.00	±0.01*	50	10.0±0.5

*by error propagation

Once the variables' ranges were fixed, the responses to be analyzed were chosen. pH of the mobile phase, column temperature, flow rate and percentage of methanol in aqueous phase can affect the retention time of the analytes. Anyway, as long as it does not suppose a peak overlapping it would not be an

inconvenience. Therefore, the responses chosen in this context were the peak resolutions of the most critical separations: rosiglitazone-propanolol, verapamil-rosiglitazone, acenocoumarol-valsartan and valsartan-warfarin. On the other hand, reconstitution solution composition can affect the shape of the early eluting analytes peaks and the dissolution of the most apolar analytes. In order to check this effect, peak width of hydrochlorothiazide and amiloride (early eluting analytes) and area of glibenclamide and fluvastatin (more apolar analytes) were studied.

Taking this into account an eight experiment (2^{5-2}) fractional factorial design with three central samples was built (Table 4.2). The responses obtained from these experiments were statistically treated by analysis of variance (ANOVA) to check which of the studied variables have a significant effect.

Table 4.2. Fractional factorial design experiments carried out to check the effect of 5 variables. A:pH; B:column temperature (ºC), C:flow rate (mL/min), D: MeOH in reconstitution solution (%MeOH); E: MeOH in aqueous phase (%MeOH)

	A	B	C	D	E
Cube 001	4.45	27	0.38	46.25	10.5
Cube 002	4.55	27	0.38	43.75	9.5
Cube 003	4.45	33	0.38	43.75	10.5
Cube 004	4.55	33	0.38	46.25	9.5
Cube 005	4.45	27	0.42	46.25	9.5
Cube 006	4.55	27	0.42	43.75	10.5
Cube 007	4.45	33	0.42	43.75	9.5
Cube 008	4.55	33	0.42	46.25	10.5
Center a	4.50	30	0.40	45.00	10
Center b	4.50	30	0.40	45.00	10
Center c	4.50	30	0.40	45.00	10

4.2.6 Application to plasma samples

The main objective of a chromatographic separation for drug analysis is usually to be applied to the determination of the analytes in a human matrix, such as plasma or urine. In order to check the applicability of the method developed, a plasma sample spiked with all the analytes and a blank plasma sample underwent a simple solid phase extraction prior to injection in the chromatographic system.

Human plasma samples were spiked to achieve a 2 µg/mL concentration of each analyte and the IS candidates. 500 µL of plasma were taken and 750 µL of acetonitrile added for protein precipitation in an Eppendorf cup. After vortex mixing and centrifugation for 5 minutes at 10000 rpm and 20 ºC, the supernatant was transferred to a 6 mL glass tube and the excess or organic solvent was evaporated under N_2 stream at 60 ºC for 5 minutes. 500 µL of formic acid solution (2%) were added to the resulting solution and after vortex-mixing it was transferred to an Oasis HLB cartridge (Wates, Milford, USA) previously activated and conditioned with 1 mL methanol and 1 mL water respectively. The cartridge was washed with 1 mL water:methanol (95:5) solution and finally the analytes were eluted with 1mL methanol. The eluent was collected on a glass tube and evaporated to dryness under N_2 stream at 60 ºC. The residue was reconstituted with 200 µL aqueous phase:methanol (55:45) solution, vortex mixed, filtered with GHP filters (hydrophilic polypropylene, 0.2 µm, 13 mm Ø) supplied by PALL (Ann Arbor, MI, USA) transferred to amber vials and injected into the UPLC system for analysis.

4.3. Results

4.3.1. Photometric and fluorimetric study

The absorption spectra for each analyte are shown in Figure 4.1 and 4.2, none of the studied compounds showed a significant absorption dependence on pH. During the fluorimetric analysis, instead, it was observed that the fluorescence of some analytes depended on pH: furosemide and valsartan were not fluorescent neither at pH 6.5 or 8.5, while rosiglitazone was not fluorescent at pH 2.5. Acenocoumarol, hydrochlorothiazide and metformin are not fluorescent at any pH, thus, they can be only analyzed photometrically. Moreover, glibenclamide fluorescence is weak and PDA detection seems to be more sensitive. The optimum excitation and emission wavelengths for fluorimetric analysis together with the absorption maxima for photometric analysis are gathered in Table 4.3.

Table 4.3. Optimum absorption (Abs), excitation (λ_{ex}) and emission (λ_{em}) wavelengths for the chromatographic analysis. In bold the absorption wavelengths chosen for the PDA analysis.

Analyte	Abs (nm)	Excitation (nm)	Emission (nm)
Acenocumarol	**285**, 304	-	-
Amiloride	212, 256, **362**	363	415
Bisoprolol	**225**, 272	225	305
Fluvastatin	235, **305**	307	390
Furosemide	230, **276**, 332	235	398
Glibenclamide	**230**, 301	305	354
Hydrochlorothiazide	224, **271**, 317	-	-
Metformin	**230**	-	-
Rosiglitazone	241, **315**	309	365
Valsartan	**254**	237	371
Verapamil	230, **279**	226	307
Warfarin	**282**, 306	309	378

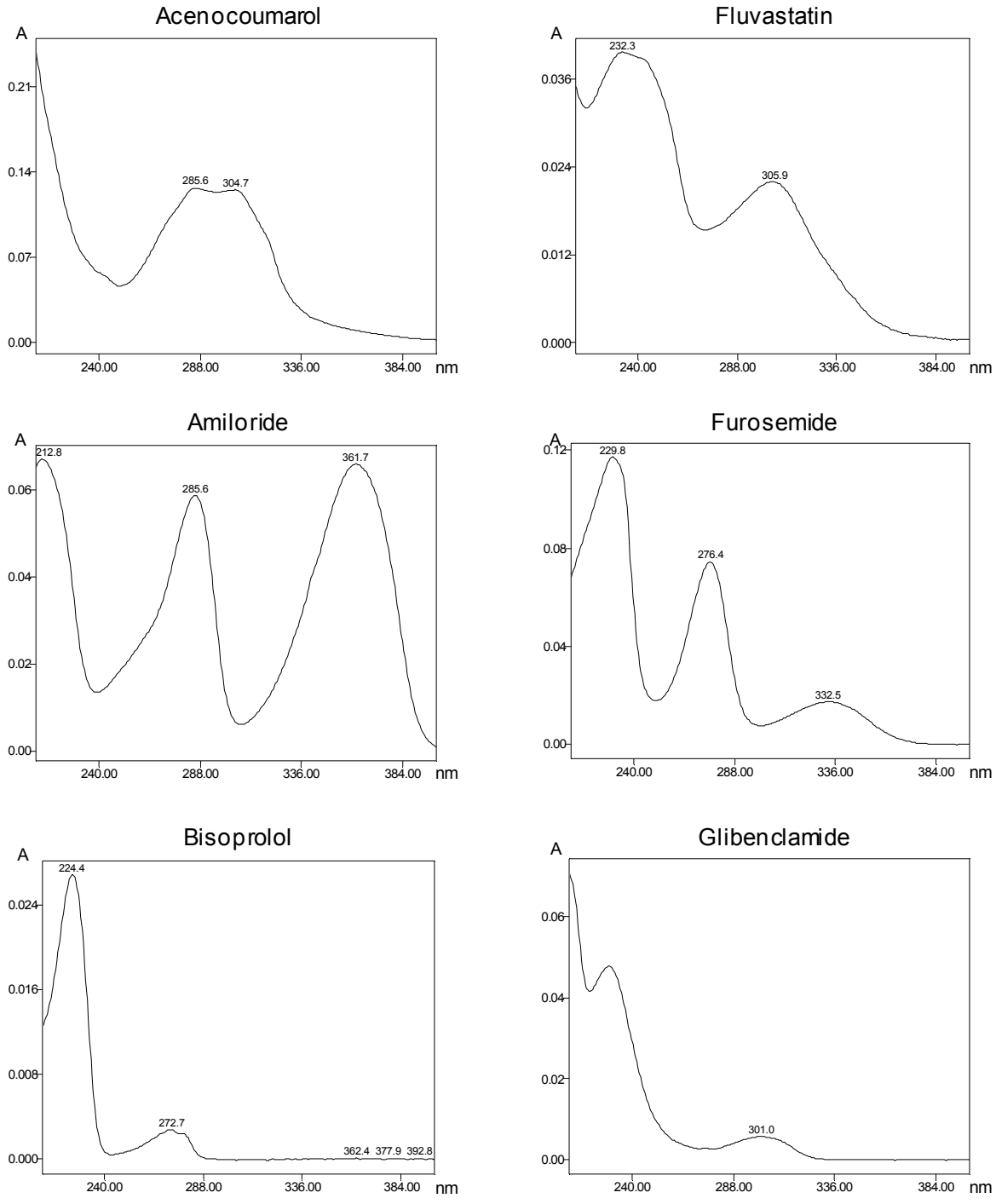

Figure 4.1. Absorption spectra obtained by PDA detector for acenocoumarol, amiloride, bisoprolol, fluvastatin, furosemide and glibenclamide.

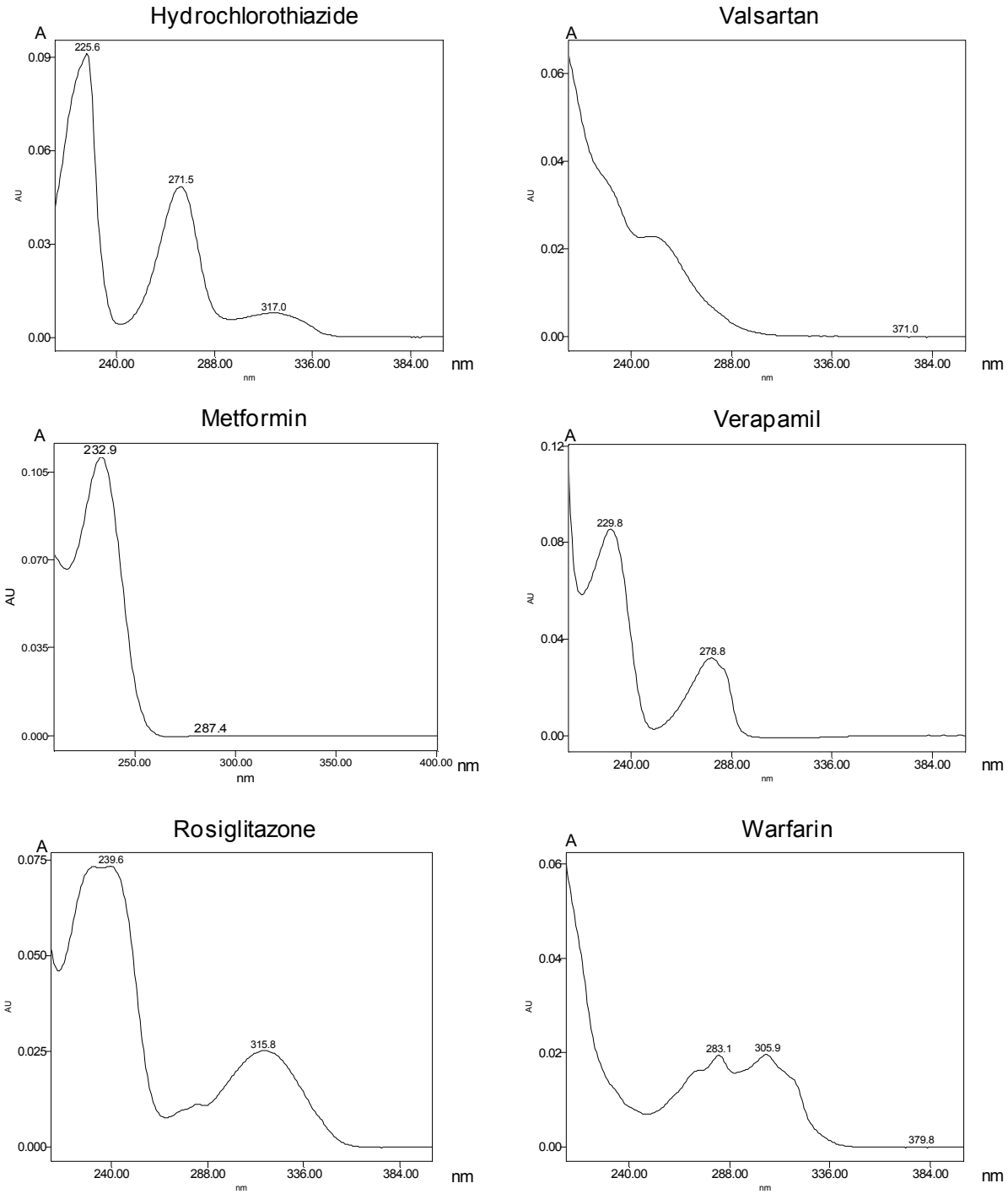

Figure 4.2. Absorption spectra obtained by PDA detector for hydrochlorothiazide, metformin, rosiiglitazone, valsartan, verapamil and warfarin.

4.3.2. Chromatographic optimization

4.3.2.1. Choice of mobile phase pH, organic solvent and stationary phase

The chromatograms obtained with the 12 possible combinations are gathered in the following figures. Figures 4.3 and 4.4 show the separations obtained using methanol as organic modifier at different pH values with BEH C18 and PFP columns respectively. The separations obtained with acetonitrile can be observed in Figures 4.5 and 4.6.

Using PFP column verapamil only elutes with acetonitrile at pH 5.5 and it does extremely late. Working with this column would result in a higher solvent and time consuming method. Bisoprolol shows similar retention problems but it can be eluted with acetonitrile at every pH. Nevertheless, its chromatographic peak shape is broad and asymmetric. On the other hand, using BEH C18 column metformin coelutes with the elution peak due to its high polarity. Taking all these facts into consideration, it is not possible to achieve the separation of the 12 analytes simultaneously with any of the two columns. Taking into account that with BEH C18 column the analysis of a greater number of analytes can be performed satisfactorily it was chosen as chromatographic column and the determination of metformin was sacrificed. This compound is highly polar and its quantitation by reversed phase chromatography is complicated. Moreover, its chemical structure is completely different to the other analytes, thus, it would make a further selective sample treatment unachievable.

Observing all the separations obtained using BEH C18, those performed with methanol as organic modifier show better resolution than the ones obtained using acetonitrile. Anyway, both at pH 3.5 and 5.5 analytes coelute. Thus, the best chromatographic separation obtained, in terms of resolution, is the one corresponding to methanol as organic modifier, C18 as stationary phase and pH 4.5 buffer (acetic acid/acetate) solution as aqueous mobile phase (Figure 4.3).

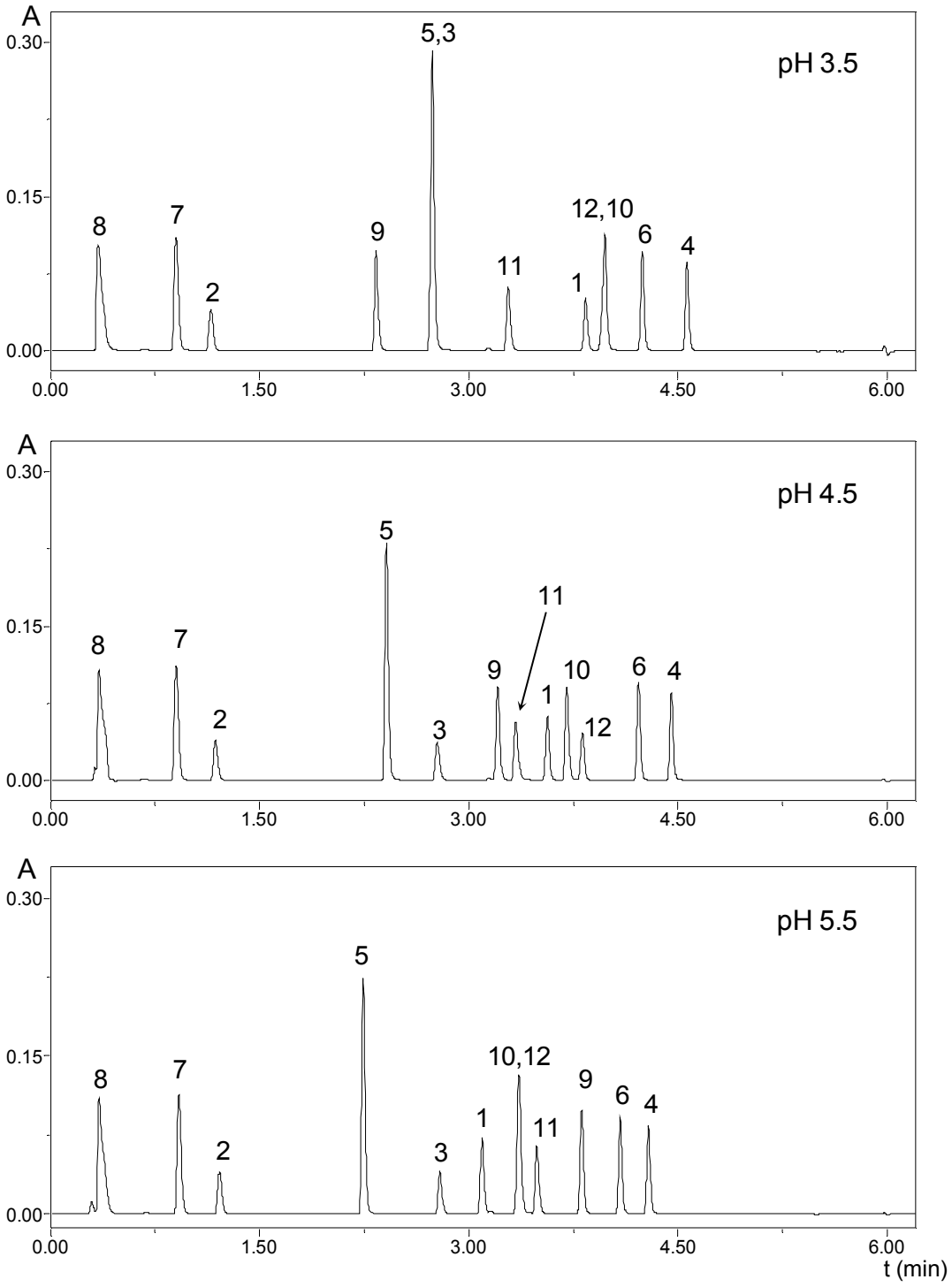

Figure 4.3. Chromatographic separations obtained with BEH C18 column at different pH using methanol as organic modifier for a 5 µg/mL standard solution. Gradient described in section 4.5.2.1. Acenocoumarol (1), amiloride (2), bisoprolol (3), fluvastatin (4), furosemide (5), glibenclamide (6), hydrochlorothiazide (7), metformin(8), rosiglitazone (9), valsartan (10), verapamil (11) and warfarin (12). Working wavelength 230 nm.

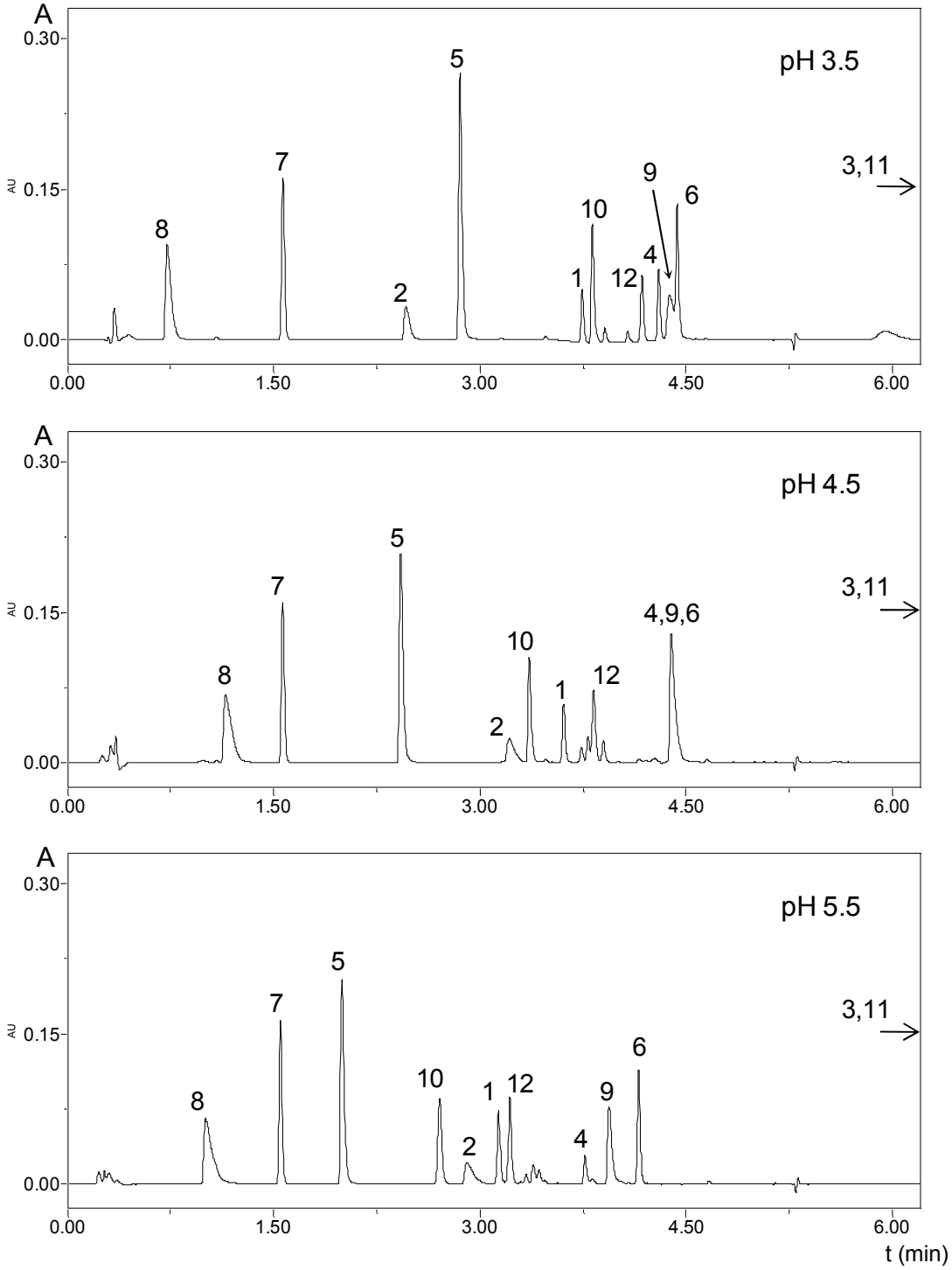

Figure 4.4. Chromatographic separations obtained with PFP column at different pH using methanol as organic modifier for a 5 µg/mL standard solution. Gradient described in section 4.5.2.1. Acenocoumarol (1), amiloride (2), bisoprolol (3), fluvastatin (4), furosemide (5), glibenclamide (6), hydrochlorothiazide (7), metformin (8), rosiglitazone (9),valsartan (10), verapamil (11) and warfarin (12). Working wavelength 230 nm.

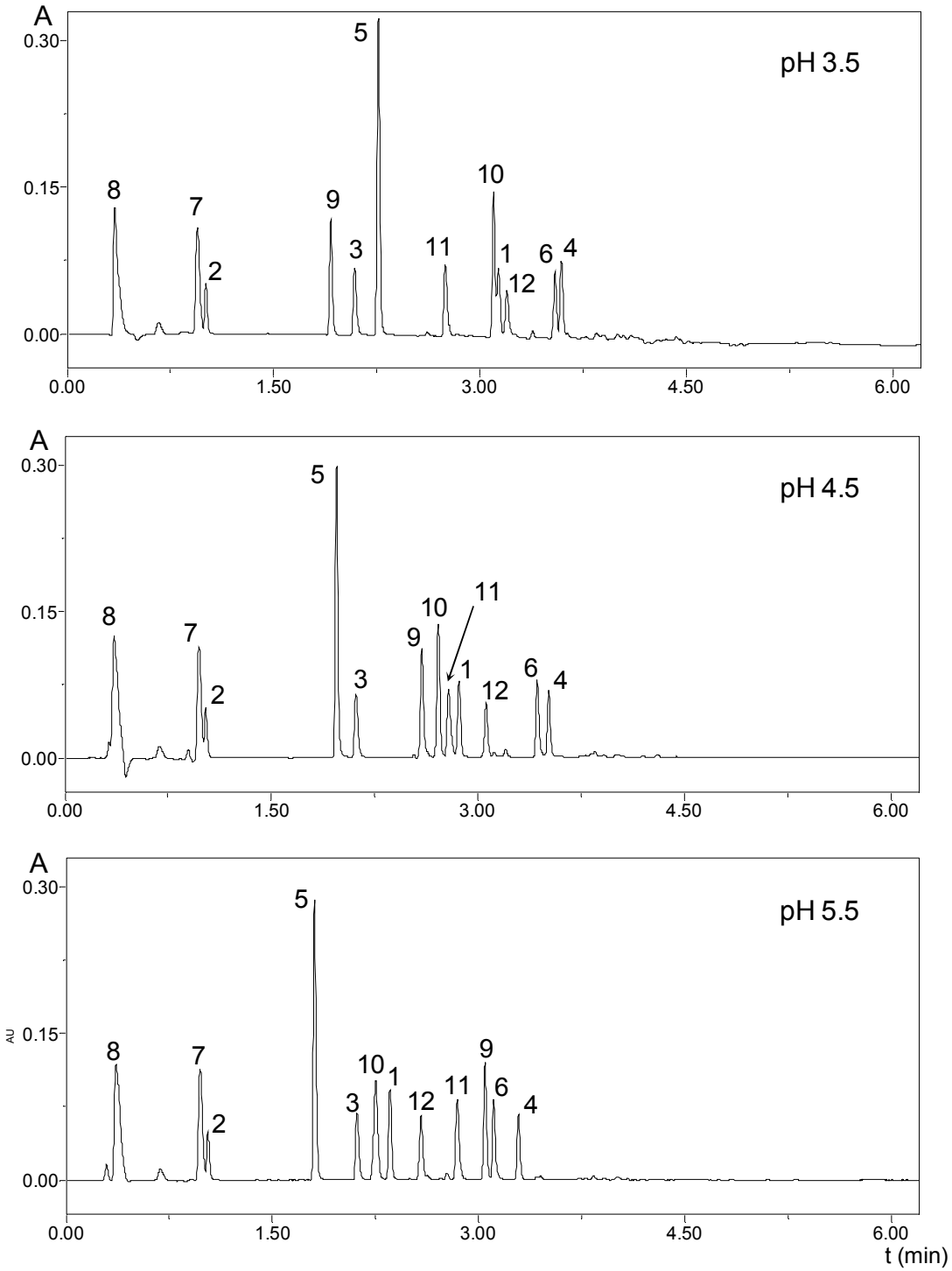

Figure 4.5. Chromatographic separations obtained with BEH C18 column at different pH using acetonitrile as organic modifier for a 5 µg/mL standard solution. Gradient described in section 4.5.2.1. Acenocoumarol (1), amiloride (2), bisoprolol (3), fluvastatin (4), furosemide (5), glibenclamide (6), hydrochlorothiazide (7), metformin (8), rosiglitazone (9),valsartan (10), verapamil (11) and warfarin (12). Working wavelength 230 nm.

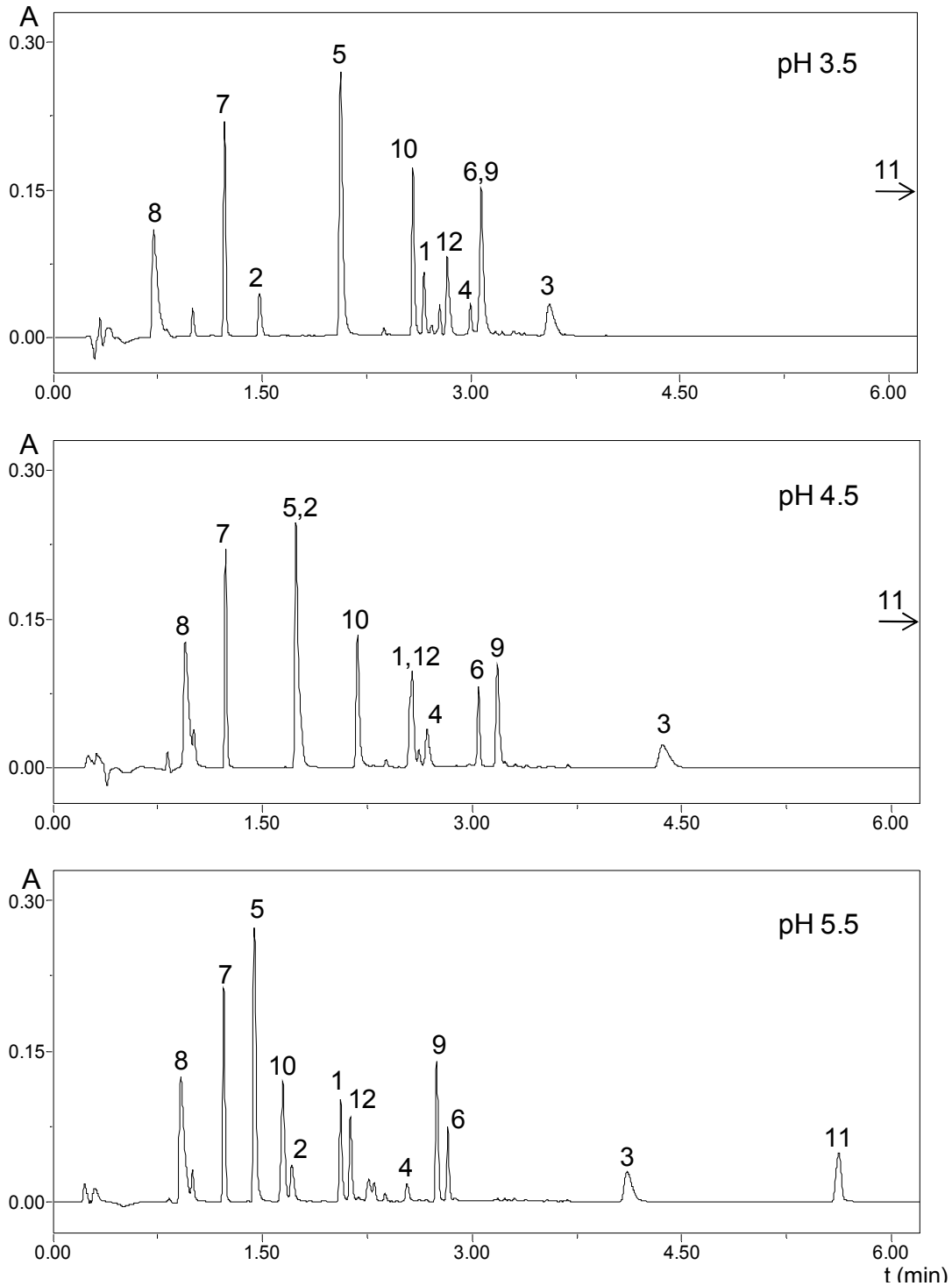

Figure 4.6. Chromatographic separations obtained with PFP column at different pH using acetonitrile as organic modifier for a 5 µg/mL standard solution. Gradient described in section 4.5.2.1. Acenocoumarol (1), amiloride (2), bisoprolol (3), fluvastatin (4), furosemide (5), glibenclamide (6), hydrochlorothiazide (7), metformin (8), rosiglitazone (9),valsartan (10), verapamil (11) and warfarin (12). Working wavelength 230 nm.

4.3.2.2. Fine-tune of the method

Once the values for the most important variables were fixed and after testing several gradient slopes, flow rates and column temperatures the best chromatographic separation was achieved with the gradient showed in Table 4.4, working at 30 ºC and 0.4 mL/min. In this way, the 11 analytes can be separated in less than 8.5 minutes and warfarin and valsartan can be separated to baseline, being this the most critical separation (Figure 4.8).

Table 4.4. Optimum chromatographic gradient (%B: Methanol percentage)

Time (min)	% B
0	0
2	38
4	60
6	95
7	95
7.5	0
8.5	0

During the optimization of the reconstitution solution it was observed that at lowest methanol proportions, the signal of the most apolar analytes (glibenclamide and fluvastatin) was weaker. This is probably due to problems dissolving these compounds in a highly polar environment. On the other hand, when the methanol concentration was too high the chromatographic peaks of hydrochlorothiazide and amiloride (early elution analytes) became broader and lost the symmetry. This problem is due to the difference between the eluotropic strength of the sample solution and the beginning of the gradient (Figure 4.7). The injection volume also affected the shape of these peaks. The longer the injection volume, the higher the

methanol amount injected and consequently the more eluotropic problems. Finally, the best chromatographic separation, where neither the first problem nor the second one happened, was achieved with a 45:55 (methanol: water) reconstitution solution and a 2.5 µL injection volume.

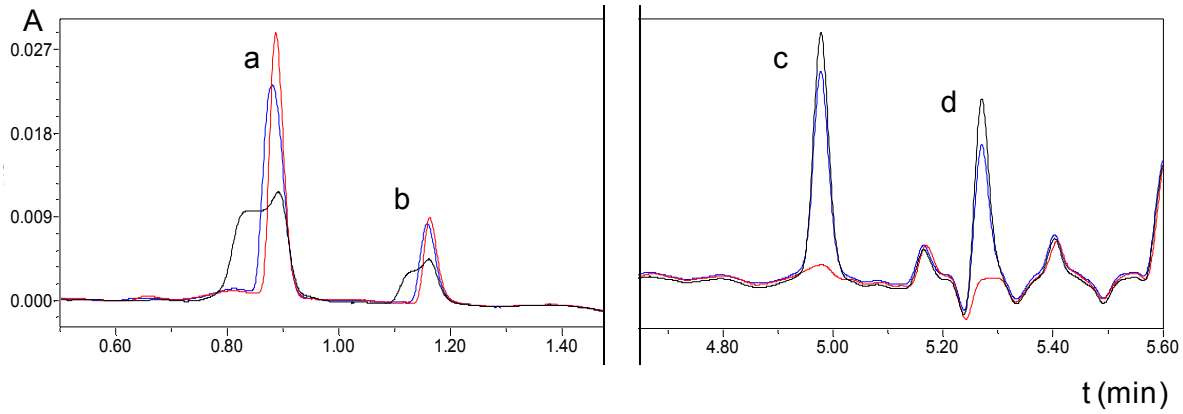

Figure 4.7. Effect of MeOH percentage of reconstitution solution on the chromatographic separation: 20% (red), 45% (blue) and 80% (black). The chromatographic signal of glibenclamide (c) and fluvastatin (d) increases with MeOH amount whereas hydrochlorothiazide (a) and amiloride (b) show peak shape problems. Chromatogram obtained at 230 nm using the optimized chromatographic method.

4.3.2.2. Choice of IS for further analysis

The 13 compounds tested as IS were injected once the method was fully developed and among them, propanolol and metoprolol proved to fit better in the chromatographic separation. Therefore, both of them can be considered possible IS for a future application of this chromatographic separation to an analytical method for the quantitation of the studied drugs in human matrices, always paying attention to a possible administration of these drugs in combined cardiovascular therapy.

In Figure 4.8 the chromatograms obtained with the optimized method for the 11 analytes and the two IS candidates, can be observed.

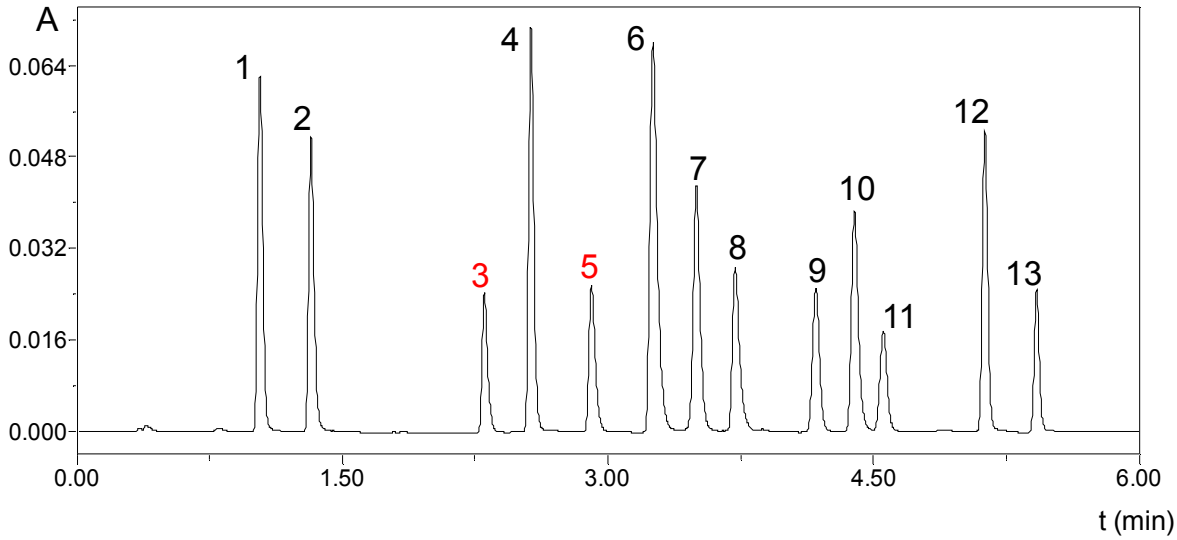

Figure 4.8. PDA chromatogram obtained for a 5 µg/mL standard solution under optimized conditions. Hydrochlorothiazide(1), amiloride(2), metoprolol (3), furosemide(4), propranolol(5), bisoprolol(6), rosiglitazone(7), verapamil(8), acenocoumarol(9), valsartan(10), warfarin(11), glibenclamide(12) and fluvastatin(13).

Once the chromatographic separation was optimized the fluorimetric detection method was developed. The fluorescence detector allows the tunable acquisition of an excitation/emission wavelength pair at an acquisition rate of 80 points per second and it is able to monitor up to 4 wavelengths pairs simultaneously, but only at 1 point per second velocity. In the studied case, it is not possible to work only at one wavelength pair mode due to the closeness of the chromatographic signals. Even if the detector changes from one detection wavelength to another extremely fast a small change in the retention times of the analytes would suppose a failure of the detection method. On the other hand, working with four detection wavelengths pairs simultaneously would be more

comfortable but the acquisition rate is not fast enough to obtain a suitable peak shape. Therefore, the best option is to work with two wavelengths pairs simultaneously, what can be done at 2 points per second acquisition rate. For this aim, two channels fluorescence detection method was developed. Channel A monitors amiloride, furosemide, bisoprolol, verapamil, valsartan and fluvastatin detection wavelengths and channel B acquires metoprolol, propranolol, rosiglitazone, warfarin and glibenclamide wavelengths. A fluorescence chromatogram obtained in this way is shown in Figure 4.9.

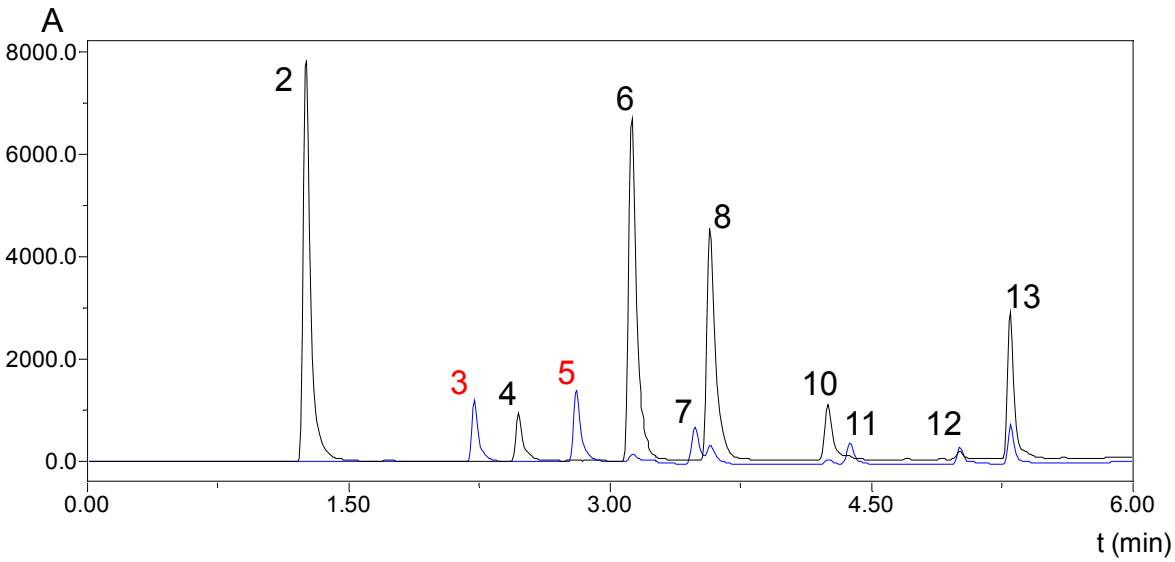

Figure 4.9. Fluorescence chromatogram obtained for a 5 µg/mL standard solution under optimized conditions. Channel A (black): amiloride(2), furosemide(4), bisoprolol(6), verapamil(8), valsartan(10) and fluvastatin(13). Channel B (blue): metoprolol (3), propranolol(5), rosiglitazone(7), warfarin(11) and glibenclamide(12).

4.3.3. Robustness of the method

After performing all the experiments of the fractional factorial design the analysis of variance was carried out in order to study the effect of the variables. In

Table 4.5 the analysis of effects obtained with The Unscrambler for a confidence level of 95% is shown. It can be observed that some responses are affected by the studied variables. The most remarkable is the influence of some factors on the resolution of rosiglitazone with propanolol and with verapamil. In the first case there is a positive effect of pH (which means that the resolution increases with the pH), whereas in the second case it is negative. This is surely due to a change in the polarity of the rosiglitazone: when the pH increases the polarity of the molecule decreases, so it gets closer to the verapamil and consequently the resolution diminishes. In the same way, it moves away from propranolol and the resolution increases. Therefore, the value of the pH of the aqueous phase has a great effect in the quality of the chromatographic separation and must be carefully controlled to guarantee a reproducible method.

Moreover, the composition of the organic phase has a significant positive effect on the area of glibenclamide and fluvastatin. The higher the methanol proportion, the bigger the area of these analytes. This can be attached to the fact that these compounds do not completely dissolve with a 45% percentage of methanol, and when increasing the organic solvent proportion the dissolution improves. Nevertheless, during reconstitution solution optimization it was observed that there was not a big change on the response of glibenclamide and fluvastatin at higher methanol percentages and taking into account that an increase in it would affect the shape of hydrochlorothiazide and amiloride peak shapes, it was kept at 45%.

Table 4.5. Analysis of effects for the fractional factorial design. Hydro: hydrochlorothiazide, Amilo: amiloride, Gliben: glibenclamide, Fluvas: fluvastatin, Ros: rosiglitazone, Pro: propanolol, Val: valsartan, Ace: acenocoumarol, Warf: warfarin. A:pH; B:column temperature C:flow rate, D:MeOH in reconstitution solution, E:MeOH in aqueous phase. NS: Non-significant +: significant positive effect -: significan negtaive effect.

Variable	Hydro Width (s)	Amilo Width (s)	Gliben Area	Fluvas Area	Ros/Pro R	Ver/Ros R	Val/Ace R	Warf/Val R
A=BD=CE	NS	NS	NS	NS	+	- -	NS	NS
B=AD	NS	NS	NS	NS	NS	-	+	NS
C=AE	NS	NS	NS	NS	NS	+	NS	NS
D=AB	NS	NS	+	+	NS	NS	NS	NS
E=AC	NS	NS	NS	NS	NS	NS	NS	NS
BC=DE	NS	NS	NS	NS	NS	NS	NS	NS
BE=CD	NS	NS	NS	NS	NS	NS	NS	NS

4.3.4 Application to plasma samples

The PDA and fluorimetric chromatograms obtained for a blank plasma sample and a spiked plasma sample after the SPE procedure can be seen in Figures 4.10 and 4.11. Even if several endogenous compounds present in the plasma can be seen in the PDA chromatogram, the chromatographic method allows the separation of most of them from the studied analytes. Nevertheless, some interference can be observed in the chromatogram at the retention time of the hydrochlorothiazide. In order to avoid this interference 317 nm wavelength could be used for the determination of this compound, but the sensitivity would significantly decrease. A more selective sample treatment, as cationic exchange SPE could be, as well, assessed. In fact, the SPE used was a standard procedure and optimizing it the interference could be removed.

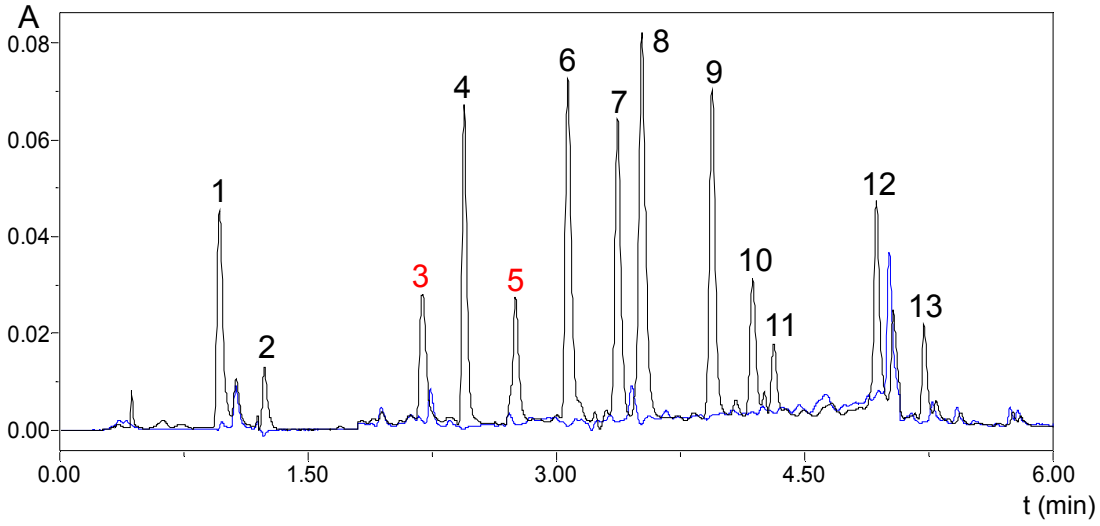

Figure 4.10. PDA chromatogram obtained for a blank plasma sample (blue) and a 2 µg/mL spiked plasma sample (black) under optimized conditions and treated as explained in point 4.2.6. Hydrochlorothiazide (1), amiloride (2), metoprolol (3), furosemide (4), propranolol (5), bisoprolol (6), rosiglitazone (7), verapamil (8), acenocoumarol (9), valsartan (10), warfarin (11), glibenclamide (12) and fluvastatin (13).

Fluorimetric detection offers better selectivity and indeed very few interferences can be observed in the chromatograms. Therefore, this detection seem to be the most adequate for the determination of the fluorescent analytes (all of them except hydrochlorothiazide and acenocoumarol), even if the sensitivity for glibenclamide may not be enough due to its poor fluorimetric intensity.

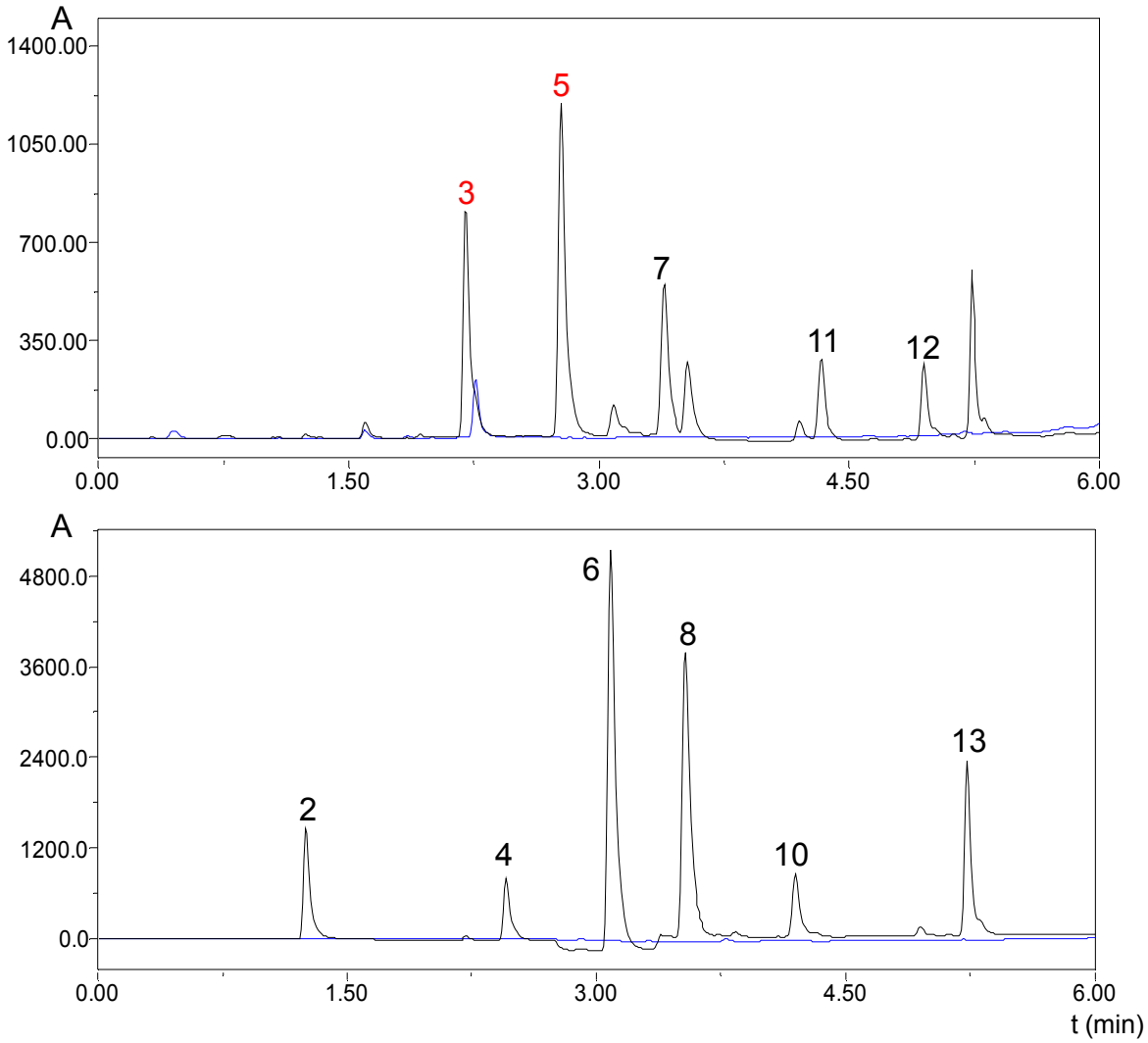

Figure 4.11. Fluorescence chromatograms obtained for a blank plasma sample (blue) and a 2 µg/mL spiked plasma sample (black) under optimized conditions and treated as explained in point 4.2.6. Channel A (above): amiloride (2), furosemide (4), bisoprolol (6), verapamil (8), valsartan (10) and fluvastatin (13). Channel B (below): metoprolol (3), propranolol (5), rosiglitazone (7), warfarin (11) and glibenclamide (12).

4.4. Conclusions

The proposed systematic approach proved to be an ideal tool for the optimization of chromatographic separations which offers a fast method development. The 12 initial experiments last around an hour, even if including the start up of the instrument (change of column and mobile phases, purge, conditioning…) it can take a whole day. Nowadays, especial instruments for method development shorten this time by changing from one column or mobile phase to another automatically. The time spent on the fine-tune of the method is very variable depending on the separation requirements. We strongly recommend fixing a clear objective before starting the optimization procedure. Since a chromatographic separation can slightly vary with a small change in the studied parameters (as it was observed when robustness was studied), there is always a chance of improving the separation, becoming the optimization procedure endless.

This optimization approach can be applied to different drugs and chromatographic techniques. Anyway, this procedure to work the studied parameters must be carefully selected for each case. For example, if none of the variables combination assessed offer a suitable separation, different columns or mobile phases should be taken into consideration. In the reported optimization 12 experiments (3x2x2) were carried out, but this value depends on the researcher's own experience, discernment and judgment: the more experiments assessed the more information is obtained, but, as well, the longer the optimization lasts.

Summing up, this procedure is a halfway between the traditional optimization and the experimental design [9]. It may not offer the total simplicity of the first one or the immense information of the second one but is sufficient for a suitable and fast optimization of chromatographic separations, both in research and routine laboratories.

Furthermore, the method is likely to be applied to the quantitation of these drugs in human plasma. On a first approach to a complete analysis method including a SPE procedure for the sample treatment, the obtained results confirm the possibility of applying of the procedure to the analysis of the studied drugs in human plasma. Nevertheless, the sample treatment should be carefully optimized in order to obtain the highest recovery for the drugs and to eliminate as much plasma's endogenous compounds as possible. Finally, the method must be properly validated (linearity, selectivity accuracy, precision…) to prove its suitability for the analysis of these drugs in human plasma [10].

Acknowledgements

The authors thank the Basque Country Government, Ministry of Science and Technology, Ministry of Science and Innovation for financial support (GIU07/02, CTQ2006-11285, CTQ2009-11690) and the pharmaceutical companies for the kind supply of studied drugs. Oskar Gonzalez thanks the University of Basque Country for his predoctoral grant.

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CHAPTER V

DEGRADATION OF FLUVASTATIN IN ACIDIC MEDIA

Abstract

In the current investigation the degradation of fluvastatin under acidic conditions was studied. Fluvastatin stability was initially tested by UV-Vis spectrometry following the change of its absorption spectrum with time. The drug was subjected to different acidic conditions and the degradation products were adequately separated using two different HPLC elution gradients. Photodiode array detector was employed to acquire the spectra of the formed products and fluorescence detector to detect the fluorescent analytes. Different degradation compounds were found depending on the acidic conditions used. Under weak acidic conditions (phosphate buffer pH 2) some intermediate compounds were found that tend to disappear with time giving rise to the final compounds. These final compounds are immediately obtained when the parent drug is subjected to a stronger acidic condition (HCl 1M). In order to identify each degradation product ultra high performance liquid chromatography combined with quadrupole/time-of-flight mass spectrometry (UPLC-Q-TOF) methods were developed. The degradation products formed under different acidic conditions were separated using a BEH C18 column with the gradient elution methods transferred from the HPLC analysis. Scans of degradation products in MS and MS/MS modes and accurate mass measurements were performed. The chemical structures of degradation products were proposed by comparing the accurate molecular masses and fragment ions with those of the parent drug. Fluvastatin degradation in acidic media proved to be complex and pH and organic modifier depending.

5.1. Introduction

Fluvastatin (*R*,S*-(E)]-(±)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1H-indol-2-yl]-3,5-dihydroxy-6-heptenoic acid)* is a well known lipid lowering drug frequently prescribed in combined cardiovascular therapy. Due to its wide use, many analytical methods have been developed for its determination both in pharmaceutical tablets and in biological matrices [1-9]. Usually, when one of these methods is developed, the main concern focuses on achieving the simplest, fastest, most sensitive, selective, accurate and precise method. Nevertheless, the optimization of an analytical method without taking into account the stability of the studied compounds can lead us to misleading results. This was indeed the problem we faced during the development of an analytical method for the quantitation of fluvastatin together with other cardiovascular drugs in human plasma [10]. As many other drugs, fluvastatin is bonded to plasma proteins, thus, previous to the quantitative analysis a protein precipitation step must be carried out. One of the earliest and most common procedures to achieve this aim is the addition of a strong acid to the plasma sample such as phosphoric acid [11,12]. In the fluvastatin case, when this acid was used as protein precipitation agent following the procedure optimized in a previous method [13], a degradation of the statin was observed.

The importance of the stability of the analytes during the sample treatment leads us to study the degradation of fluvastatin in acidic media. Furthermore, the information obtained could be useful to understand the behavior of fluvastatin in human body, taking into consideration that it is orally administered and may react

under the low pH of the stomach. Degradation of fluvastatin was initially studied based on the difference of the degradation compounds spectra and following the absorption spectrum shift with time. Afterwards, in order to understand the reaction mechanism and kinetics, liquid chromatography was used to separate the different degradation compounds. Taking into account the absorption and fluorescent properties of fluvastatin, photodiode array detector (PDA) and fluorescence detector were used. Finally, the degradation compounds were identified by a High Resolution Mass Spectrometer (HRMS) coupled to an Ultra High Performance Liquid Chromatography (UPLC) instrument.

5.1.1. Fluvastatin

Fluvastatin (Figure 5.1), the first totally synthetic statin, reduces the level of LDL (low density lipoprotein) by inhibiting HMG-CoA reductase, which is an essential enzyme for the synthesis of cholesterol. Due to this effect, it decreases the risk of cardiovascular diseases as several studies have corroborated [14-16]. Its metabolism is mainly hepatic and it is fast and complete, with a peak plasma concentration at 0.5-1.5 h and a half-life of 1.5-2.5 h [17,18]. The original molecule is almost fully converted into five metabolites [6] that are excreted predominantly in faeces.

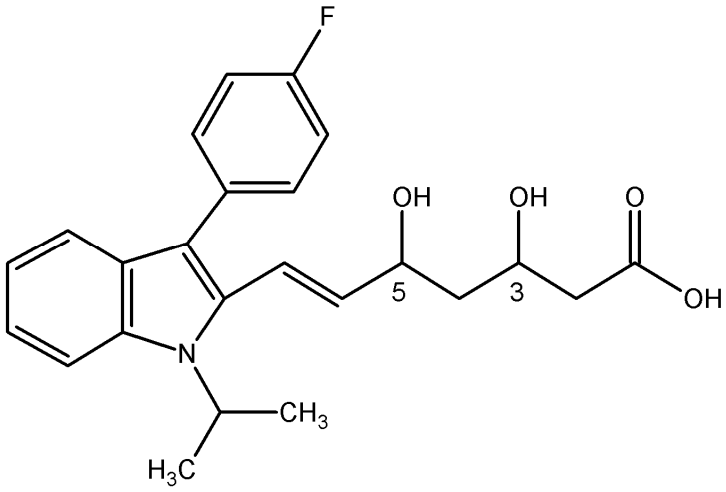

Figure 5.1. Chemical structure of fluvastatin

Fluvastatin is a weak acid (pK_a=5.5) due to its β-hydroxyacidic chain bound to an indolic ring that makes the molecule fluorescent [19,20]. Moreover, fluvastatin is extremely sensitive to light exposure, suffering from a photodegradation that transforms the original molecule in different degradation products [21,22], which is a fact to take into consideration when an analysis method is developed. Fluvastatin has two carbon estereocenters in the mevalonic chain, thus, it can be found as two pairs of enantiomers. The commercialized product is a racemic mixture of the sodium salt of (3R, 5S) and (3S, 5R) enantiomers, being the first one 30 times more active.

5.1.2. High Resolution Mass Spectrometry

Since its appearance in the 1950`s mass spectrometry has become a powerful tool for the structural determination of chemical compounds [23], complementary to another elucidation techniques such as NMR or IR. The chance

of coupling a mass spectrometer to a liquid chromatography instrument enhances the analysis possibilities, combining both separation and elucidation.

Traditionally, the main drawback of the widely used low resolution mass spectrometers such as quadrupoles or ion traps has been the poor mass accuracy that can be achieved. This is a problem when the aim is to determine the molecular formula of an unknown analyte due to the fact that the number of possible elemental compositions increases when the accuracy decreases [24]. Obviously, the number of possible molecular formulas increases with the mass, becoming especially challenging the elucidation of large molecules. Fortunately, the high resolution mass spectrometers solve this difficulty and offer a mass accuracy below 5 ppm, which minimizes the number of possible elemental compositions. The importance of the mass accuracy can be easily understood by means of an example. During metabolic processes, methylation and hydroxylation followed by oxidation at a double bond are usual. If the nominal resolution is limited to the unit both processes look like +14 Da respect to the precursor molecule and cannot be distinguished (CH_2 addition produces an increase of +14.0157 and the hydroxylation and oxidation +13.9792 Da). With a higher mass accuracy the reaction that takes place in the metabolic process can be easily identified.

Another improvement of the latest mass spectrometers is the enhanced resolution, it is not only important to detect the accurate mass but to differentiate it from any other mass/charge ratio. Even if the *m/z* measurements of ions are

usually represented as discrete lines in MS spectra, they have, in fact, a Gaussian distribution with its corresponding width (like chromatographic peaks). Therefore, it is possible to speak about resolution where the *m/z* replaces the time in the x axis. The most widely accepted definition for resolution consists on measuring the width of the peak at half height (Full Width at Half Maximum, FWHM) and compare it with the mass to charge ratio. For instance, if the width of a 1800 m/z ion is 0.6 amu, the resolution is 3000 FWHM. Low resolution spectrometers' resolution ranges around this value, whereas the best ion cyclotron spectrometers in the market can achieve 1500000 FWHM (even if the usual values for HRMS varies around 10000-40000 FWHM) [25].

Among the different HRMS instruments, the most popular may be the quadrupole time-of-flight (Q-TOF) mass spectrometer. This combination offers a great versatility and is useful for accurate mass measurement, fragmentations experiments and high quality quantitation. One of the latest Q-TOF in the market is the *Synapt G2*, this instrument offers a resolution over 40000 FWHM, 1 ppm mass accuracy, linear dynamic range of up to 10^5 and it can acquire 20 spectra per second [26].

In Figure 5.2 a diagram of the Synapt G2 instrument is shown. It consists of a quadrupole, an ion mobility section (Triwave) and a dual stage reflectron TOF (QuanTof). Ions are transported through the quadrupole analyzer to the mobility separation section where the fragmentation takes place [27,28]. There, they can

be separated according to their mobility before reaching the orthogonal acceleration time-of-flight detector.

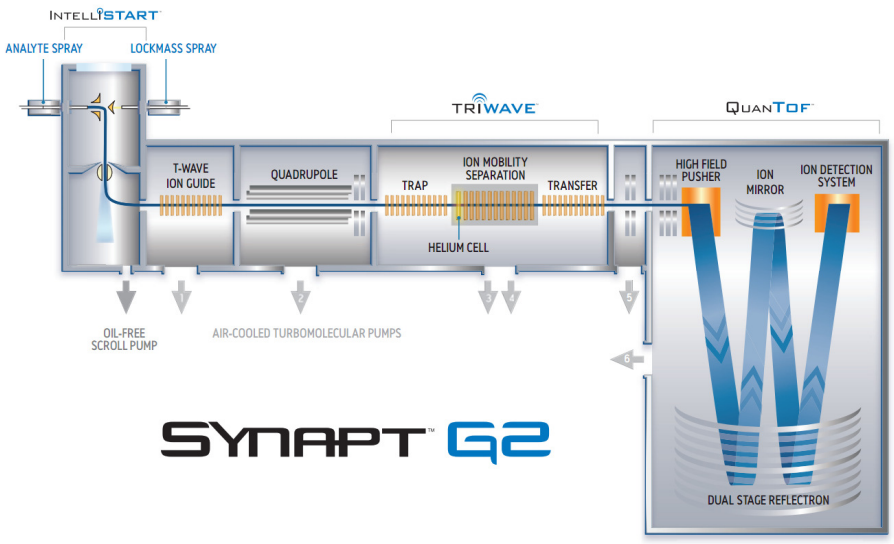

Figure 5.2. Synapt G2 instrument diagram

The QuanTof technology provides a high resolution, exact mass and quantitative performance. Thanks to the dual stage reflectron the instrument can operate in three different modes: sensitivity mode, resolution mode and high resolution mode. The first and the second work in single-pass mode, which means that ions travel from the pusher to the reflector and then to the detector. In the high resolution or double-pass mode, the ions are reflected into the ion mirror, which makes them travelling again to the dual stage reflector before arriving to the detector. All these modes provide a high acquisition rate compatible with UHPLC systems allowing faster analysis than with traditional liquid chromatography [29,30].

5.2. Experimental

5.2.1. Instrumentation

UV-Vis spectra were recorded with a Cary 5000 UV-Vis-NIR spectrophotometer (Varian, Palo Alto, USA) in 1 cm quartz cells from 190 to 500 nm wavelengths. The instrument was controlled by Cary WinUV software.

The chromatographic system used for the separation of the degradation compounds consisted of a Waters Alliance 2695 separation module connected to a Waters 996 PDA and to a Water 747 Fluorescence detector (Milford, MA, USA). A Phenomenex Luna C18(2), 150×4.6 mm I.D., 3 µm, 100 Å, column was used to perform the separation with a Security Guard cartridge of the same material (4×3 mm) placed prior to the analytical column in order to prevent its degradation. The software used to control the instrument and for the data treatment was *Empower 5.0.*

High Resolution Mass Spectrometry analysis were performed on an Acquity UPLC system coupled to a PDA detector and to a Synapt G2 HDMS Q-TOF mass spectrometer (Waters, Milford, USA). The chromatographic column used for the separation was a BEH C18, 1.7 µm, 50×2.1 mm (Waters, Milford, USA) System control, data collection and data processing were accomplished using *MassLynx 4.1* software.

The pH of the mobile phases and buffer solutions was measured with a Crison GPL 22 pH-meter (Barcelona, Spain) using a Crison glass-combined

electrode model 5209 with a reference system Ag/AgCl and KCl 3 M saturated in AgCl as electrolyte.

5.2.2. Chemicals and reagents

Sodium fluvastatin was kindly supplied by Novartis Pharma AG (Basel, Switzerland). A 1000 µg/mL stock solution of the drug was prepared in methanol.

Ammonium formate, 99% purity, was purchased from Alfa Aesar (Karlsruhe, Germany) and formic acid, LC–MS quality, from Fluka (Buchs, Switzerland).

Gradient quality methanol (Romil, Cambridge, USA) and acetonitrile (Baker, Deventer, Holland) were used as organic modifiers for HPLC analysis. Purified water from a Milli-Q system (Millipore, MA, USA) was used in the preparation of buffer and reagent solutions. For the HRMS analysis, acetonitrile, methanol and water of LC/MS quality from Fisher Scientific (MA, USA) were used.

5.2.3. Spectrophotometric study of the degradation of fluvastatin solutions in acidic media

In order to follow the degradation of fluvastatin in different acidic media 10 µg/mL solutions were prepared at pH 2, 3 and 4 using 50 mM buffer solutions (phosphoric acid/phosphate for pH 2 and 3, and acetic acid/acetate for pH 4). These solutions were immediately introduced in the spectrophotometer and their spectra collected every minute during one hour. Moreover, the spectrum of a 10

μg/mL fluvastatin solution in 1M HCl was registered to compare it with the previous ones.

The experiments performed at three different pH values were carried out as well, in 30% methanol and in 30% acetonitrile in order to study the influence of the solvent in the degradation.

5.2.4. Separation and study of the degradation compounds of fluvastatin solutions in acidic media by HPLC

After the spectrophotometric study, the separation of the degradation products was performed by HPLC. The PDA detector operated from 190 to 400 nm in order to collect the absorption spectra of the analytes and the fluorescence detector worked at 307 nm excitation and 390 nm emission wavelengths, which are the optimal detection wavelengths for fluvastatin.

According to the results obtained in the spectrophotometric study, the separation of two different degradation compounds groups was performed. On the one hand, a 10 μg/mL fluvastatin solution exposed to strong acidic conditions (1M HCl, 30% MeOH), and on the other hand a 10 μg/mL fluvastatin solution under weaker acidic conditions (50 mM phosphate buffer at pH 2, 30% MeOH). For the separation of all the compounds two different chromatographic methods were necessary, one for each acidic condition.

The separation developed for compounds produced in strong acidic media was carried out at 30 ºC and a flow rate of 0.8 mL/min using A (10 mM ammonium formate with 0.01 % formic acid in water) and B1 (10 mM ammonium formate with 0.01 % formic acid in acetonitrile) as mobile phases. The separation of the degradation compounds in weak acidic conditions was performed at 45 ºC using the same flow rate and aqueous mobile phase, but replacing the acetonitrile with methanol in the organic phase (B2). The gradients used in each case are collected in Table 5.1. In both methods the injection volume was 10 µL.

Table 5.1. Gradient used for the separation of the degradation compounds obtained under strong (Gradient 1) and weak (Gradient 2) acidic conditions

Gradient 1		Gradient 2	
t (min)	**% B1**	**t (min)**	**% B2**
0	40	0	70
4	52	20	70
9	90	30	80
11	90	31	80
12	40	33	70
17	40	40	70

The 1st separation method was used to find the degradation compounds of fluvastatin formed under strong acidic conditions both in 30% MeOH and in 30% ACN. The 2nd separation method was used to follow the degradation of fluvastatin in weaker acidic conditions, both in 30% MeOH and in 30% ACN. For this study, fluvastatin solutions in weak acidic conditions were repeatedly injected after 1, 2, 6, 12, 24, 48 and 72 hours. In the kinetics studies the peak area (A) obtained for the fluorimetric chromatographic peak corresponding to fluvastatin at different hours was used as a measurement of the relative degradation. The obtained area

(A), the neperian logarithm of the area (ln A) and (1/A)-(1/A_0) were plotted versus time (in hours) to see whether the kinetics was of zero, first or second order. All the solutions were injected again after two months to follow the degradation process in the long term.

5.2.5. Identification of the degradation compounds of fluvastatin solutions in different acidic media by UPLC-HRMS

5.2.5.1. Transfer from HPLC to UPLC

Once the number of degradation compounds was known and the separation methods developed their characterization was carried out by a High Resolution Mass Spectrometer. This instrument was coupled to an UPLC system, thus, previously to the MS experiments the chromatographic methods developed for HPLC had to be transferred. Moreover, ammonium formate was not added to the mobile phases following the recommendation given by the commercial house of avoiding salts in HRMS analysis. To overcome this fact the pH was adjusted to pH 4.3 with formic acid in order to maintain the same analysis pH.

UPLC instruments work at high pressure allowing the use of smaller size particles and smaller columns without losing separation quality (see Chapter IV). When a chromatographic separation method is to be transferred either from HPLC to UPLC (the most common case) or from UPLC to HPLC, the column internal diameter (i.d.) and volume ($V = \pi \cdot r^2 \cdot l$) difference must be taken into account. The first step in method transfer is to adjust the flow rate to the square diameter of

the column, which is known as geometrical scaling. In this case, the HPLC column i.d. and flow rate were 4.6 mm and 0.8 mL/min, respectively, and the UPLC column i.d. was 2.1 mm. Therefore, the adequate flow rate for the UPLC separation was 0.167 mL/min. The next step is to adjust the gradient in order to maintain constant the column volumes of solvent that go through the column in each segment, considering the difference in flow rates and in column volumes. The method transfer applied to the 1st separation and 2nd separation is summarized in Table 5.2.

Table 5.2. Method transfer from HPLC to UPLC for 1st and 2nd separation methods

1st Separation method

HPLC				UPLC		
t (min)	**% B1**	**Volume (mL)**	**Column Volumes**	**Volume (mL)**	**t (min)***	**%B1**
0	40				0	40
		3.2	1.28	0.22		
4	52				1.33	52
		4.0	1.60	0.28		
9	90				3.00	90
		1.6	0.64	0.11		
11	90				3.67	90
		0.8	0.32	0.06		
12	40				4.00	40
		4.0	1.60	0.28		
17	40				5.67	40

2nd Separation method

HPLC				UPLC		
t (min)	**% B1**	**Volume (mL)**	**Column Volumes**	**Volume (mL)**	**t (min)***	**%B1**
0	70				0	70
		16.0	6.42	1.11		
20	70				6.67	70
		8.0	3.21	0.56		
30	80				10.00	80
		0.8	0.32	0.06		
31	80				10.33	80
		1.6	0.64	0.11		
33	70				11.00	70
		5.6	2.25	0.39		
40	70				13.33	70

*$V_{HPLC\ COL}$= 2.493 mL, $V_{UPLC\ COL}$= 0.173 mL, $Flow_{HPLC}$= 0.8 mL/min, $Flow_{UPLC}$= 0.167 mL/min

5.2.5.2. Identification of the degradation compounds

In order to identify the degradation compounds, mass scan and product ion scan analysis were performed using the Q-TOF in ESI+ mode. First, 5 µL of the solutions prepared to study fluvastatin degradation (in strong and weak acidic media, and in 30% methanol and acetonitrile) were injected, each one using its corresponding chromatographic separation. Mass scans were performed from 50 to 1200 m/z at 5, 15, 30 and 45 V cone voltages and a scan time of 0.1 s. In this way, the molecular ion for each compound was found and its exact molecular mass was obtained. Using the tools provided by *MassLynx* software the most likely elemental compositions corresponding to the obtained masses were calculated. Besides the high accuracy of the mass spectrometer, the scrutiny of the isotope pattern [31] narrowed the number of possibilities.

Once the molecular ions were determined Product Ion scans were carried out to obtain the mass fragmentation spectra of each degradation compound. Cone voltage was kept at 30 V whereas the Collision Energy was set as a ramp from 10 to 30 V.

Additionally, the mass and product ion scans were performed for a 10 µg/mL fluvastatin solution in methanol:water (30:70) to get its mass fragmentation spectra for comparing it with the ones obtained for the degradation compounds. With all the gathered information chemical structures of the different degradation compounds were proposed.

During all the MS experiments the capillary voltage was 3.2 kV, the source temperature 120 ºC, the desolvation temperature 350 ºC and the desolvation gas flow 1000 L/h.

5.2.6. Degradation of fluvastatin in plasma samples during protein precipitation procedure

Once the first degradation studies were carried out and all the degradation compounds were found a 10 µg/mL fluvastatin spiked plasma sample was treated as reported by *Iriarte et al* [13]. This method includes a protein precipitation step with H_3PO_4 (0.5 M) prior to a SPE procedure, where fluvastatin is expected to degrade. In order to find the formed degradation compounds the eluate obtained after the SPE procedure was reconstituted in a 200 µL methanol:water (30:70) solution and analyzed by UPLC-HRMS using the geometrically scaled 2nd separation (Table 5.2) and working in mass scan mode with a cone voltage of 30 V (as described in the previous section).

5.3. Results

5.3.1. Spectrophotometric study of the degradation of fluvastatin solutions in acidic media

Absorption spectra obtained for fluvastatin solutions at different pH values during one hour are shown in Figure 5.3 together with the spectra corresponding to a 10 µg/mL fluvastatin in 1M HCl. It can be observed that the lower the pH is, the faster the spectral transformation happens. In fact, fluvastatin seems to be

stable at pH 4 (the sudden absorption decay at 225 nm is due to the absorption of the buffer solution). The spectral change observed at pH 2 suggests a complex reaction involving at least two degradation compounds. Initially, the absorption spectrum of fluvastatin exhibits two maxima around 235 and 305 nm. Exposure of fluvastatin to acidic conditions produces changes in the spectrum that involves a decrease in these bands and the appearance of a new absorption band around 230 nm. Immediately, this band decreases while the absorption band around 255 nm maintains its absorbance. Regarding to the spectral shape obtained for the solution in HCl media it bears certain resemblance to the one obtained at pH 2 after one hour. In fact, if a spectrum of the pH 2 solution is recorded after a given time, both spectra are very similar. Furthermore, at this point, the spectrum does not change anymore; thus, it seems to be the spectrum of the final degradation product. This compound is almost instantly obtained when 1M HCl is used whereas with higher pH the reaction is slower and the formation of an intermediate compound can be assumed.

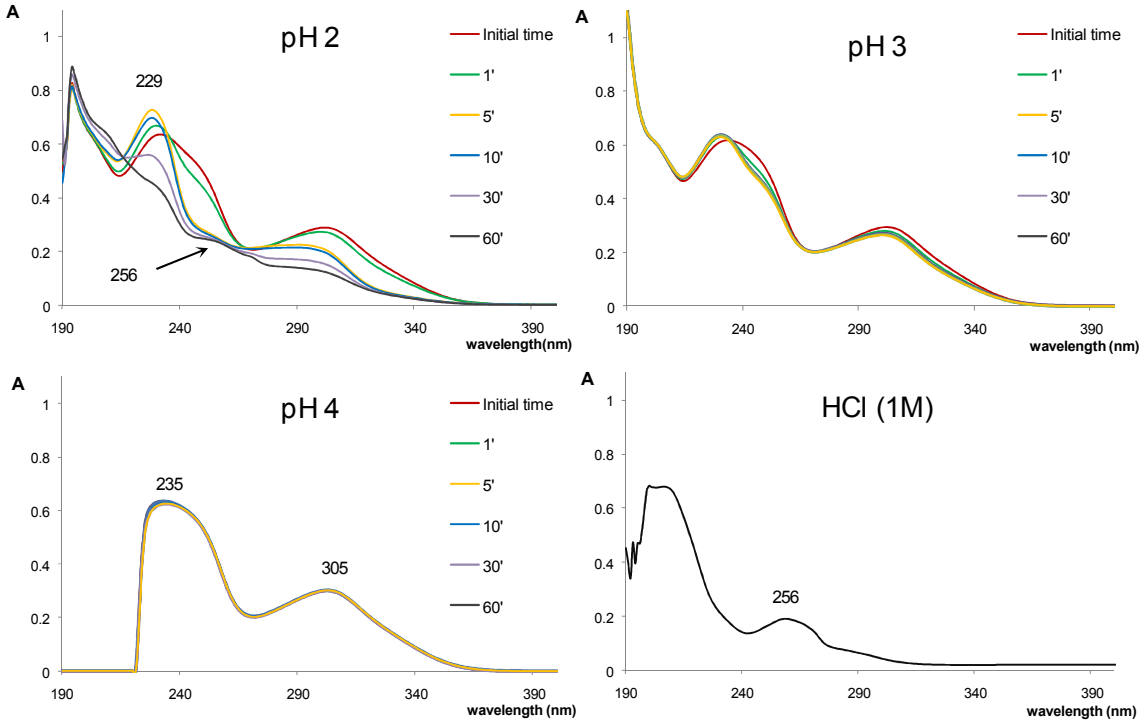

Figure 5.3. Absorption spectra monitoring for 10 µg/mL fluvastatin solutions at pH 2, 3, 4 and 1M HCl

When the experiments were performed including a 30% of methanol or acetonitrile in the solution, the degradation process was slower. Moreover, there is a considerable effect of organic solvent in the degradation process, being this much faster in methanol than in acetonitrile. In Figure 5.4, the spectral changes for a 10 µg/mL fluvastatin solution at pH 2 both in 30% methanol and in 30% acetonitrile are shown.

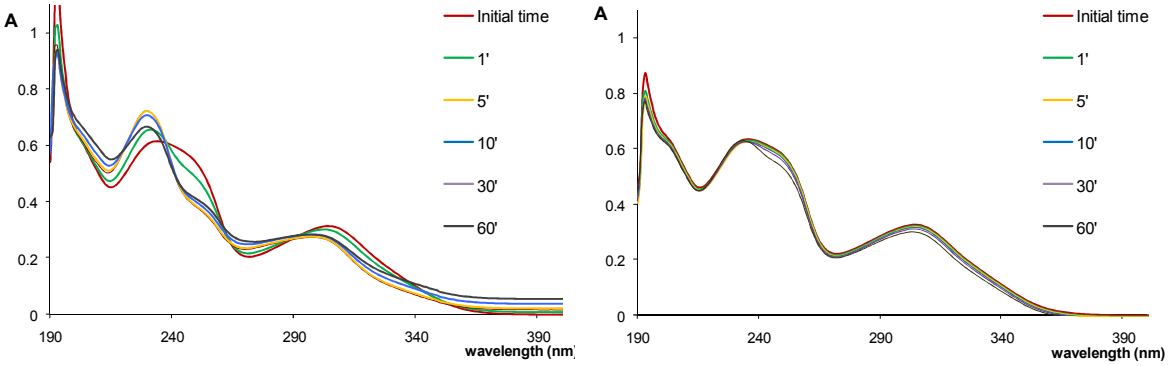

Figure 5.4. Absorption spectra monitoring for 10 µg/mL fluvastatin solutions in pH 2 buffer:methanol (70:30) (left) and in pH 2 buffer:acetonitrile (70:30) (right).

5.3.2. Separation and study of the degradation compounds of fluvastatin solutions in acidic media by HPLC

The chromatogram obtained for a 10 µg/mL fluvastatin solution in 1M HCl:MeOH (70:30) can be seen in Figure 5.4. Five chromatographic peaks presumably belonging to different degradation compounds can be found (**A**, **B**, **C**, **D** and **E**), of which two (**C** and **D**) do not appear when acetonitrile is used as organic solvent. This may indicate that compounds **C** and **D** are formed due to the presence of methanol.

When the samples were injected after two months no significant changes in the chromatographic signals were observed, thus, all these compounds can be considered as the "final" products of the degradation process. The absorption spectra (Figure 5.7) are similar for the five analytes and they match with the spectrum obtained in the spectrophotometer for a fluvastatin solution in 1M HCl

(Figure 5.3). None of these compounds was fluorescent, which indicates that the indolic group of fluvastatin is transformed or lost during the process.

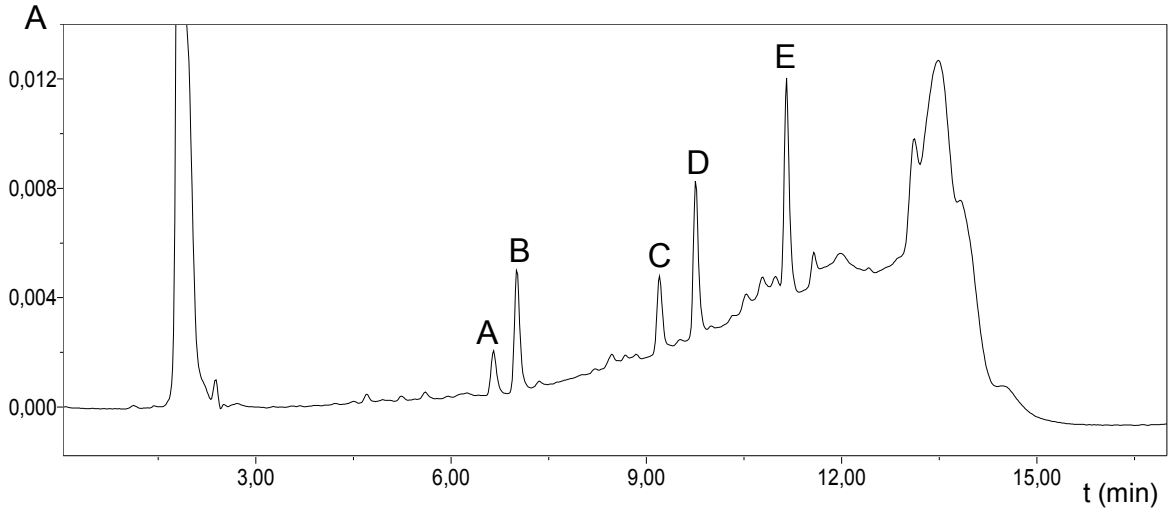

Figure 5.5. Chromatograms obtained at 256 nm for 10 µg/mL fluvastatin solutions in 1M HCl:MeOH (70:30) using separation 1.

In Figure 5.6 the chromatograms obtained for a 10 µg/mL fluvastatin solution in phosphate buffer (pH=2, 50 mM):MeOH (70:30) and in 50 mM phosphate buffer (pH=2, 50 mM):ACN (70:30) after 72 hours can be observed.

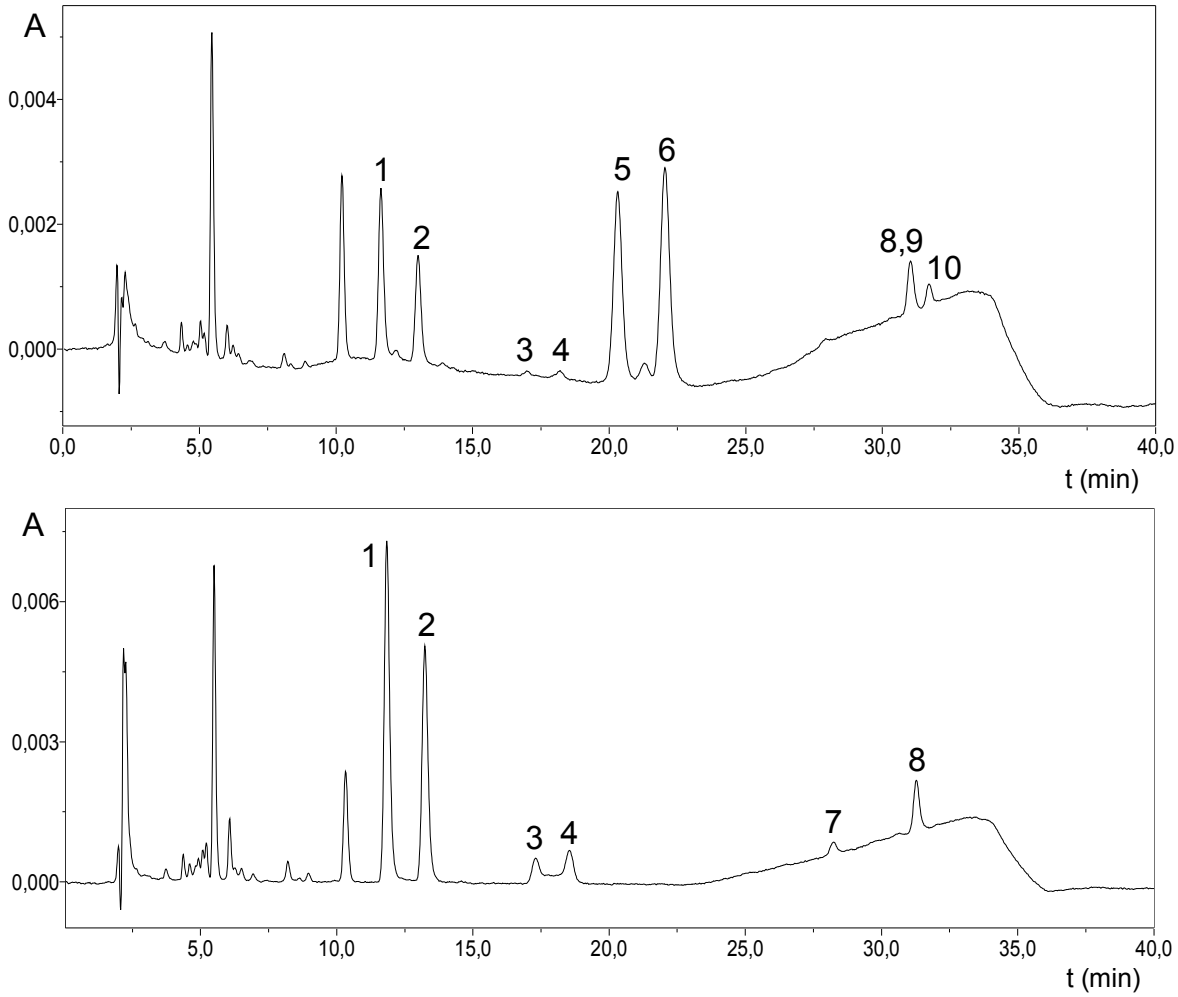

Figure 5.6. Chromatograms obtained at 256 nm for 10 µg/mL fluvastatin solutions in 50 mM phosphate buffer (pH=2):MeOH (70:30) (above) and in 50 mM phosphate buffer (pH=2):ACN (70:30) (below) after 72 hours using 2nd separation.

The chromatographic peaks eluting earlier than fluvastatin (**1**) corresponds to the "final" degradation compounds (**A** and **B** coelute), while all the other ones are intermediate products (**2** to **10**) that disappear with time. Some of these compounds (**5**, **6**, **9** and **10**) are only present in the methanolic solution, suggesting the formation of methylated derivatives of fluvastatin.

From the point of view of the absorption properties, all these compounds show an absorption spectrum similar to fluvastatin's one, except compounds **7** and **8**. Their absorption spectra (Figure 5.7) show a narrow band at 230 nm and a broad band around 275 nm that agree with the results obtained during spectrophotometric study. In fact, the spectral shape observed for an acidic solution of fluvastatin in a certain point of the degradation (Figure 5.3), can be interpreted as a combination of the spectra of the different degradation compounds. All the absorption spectra can be observed in Figure 5.7.

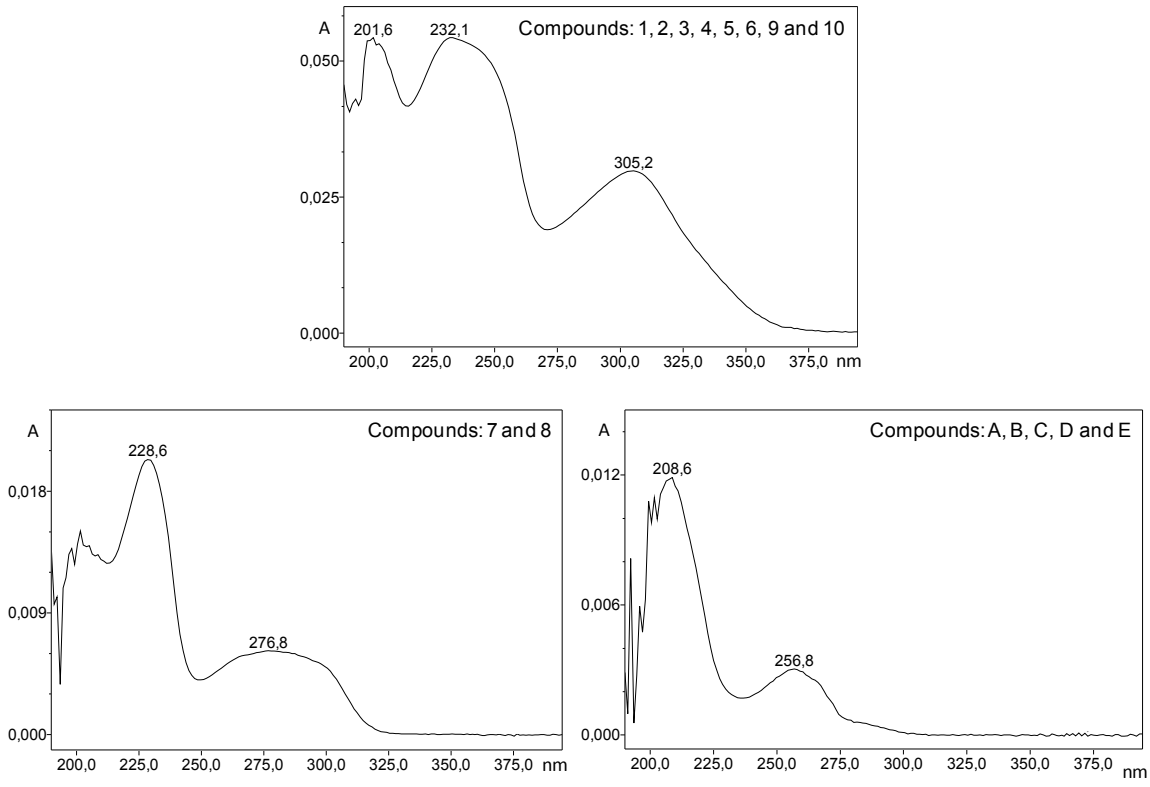

Figure 5.7. Absorption spectra obtained by PDA for the degradation compounds. Above, the corresponding to compounds 1 to 6, 9 and 10; below the corresponding to compounds 7 and 8 (left) and to A, B, C, D and E (right).

According to the fluorescence detection, compounds **1** to **10** are fluorescent, thus, the indolic group may remain intact during the degradation process.

With regard to the kinetics study, the area decrease with time of the chromatographic peak corresponding to fluvastatin at different hours for the stability experiment carried out at pH 2 in methanol, responds to a first order kinetics, Figure 5.8.

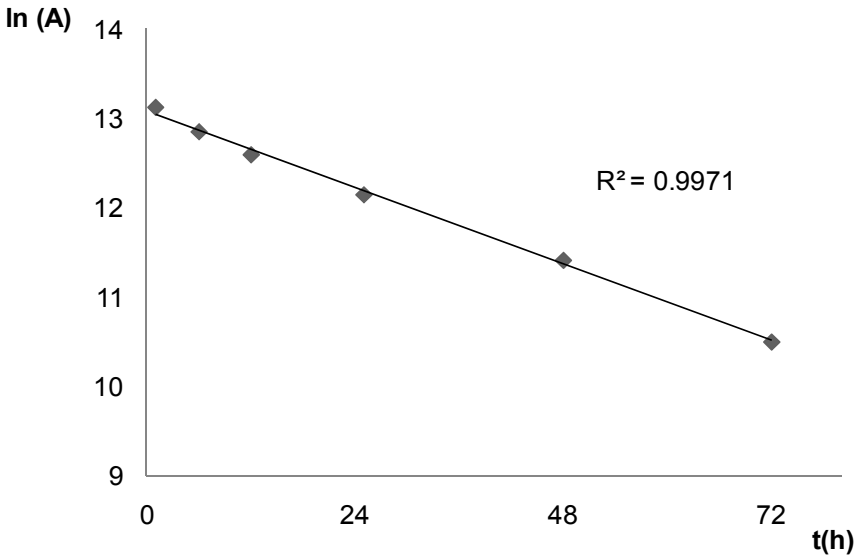

Figure 5.8. Variation of lnA with time for a 10 µg/mL fluvastatin solution in 50 mM phosphate buffer (pH=2):MeOH (70:30).

When the 10 µg/mL fluvastatin solution at pH 2 in methanol was injected after two months, only the degradation products observed in strong acid media were observed (**A** to **E**). Thus, the reaction process is completed and only the "final" degradation compounds were present in the solution.

5.3.3. Identification of the degradation compounds of fluvastatin solutions in different acidic media by UPLC-HRMS

5.3.3.1. Transfer from HPLC to UPLC

Chromatographic methods were satisfactorily transferred from HPLC to UPLC as it can be observed in the chromatograms obtained for a 10 µg/mL fluvastatin solution in 1M HCl:MeOH (70:30) (Figure 5.9) and for a 10 µg/mL fluvastatin solution in 50 mM phosphate buffer (pH=2):MeOH (70:30), injected after one week in order to observe all the degradation products (Figure 5.10). Even if the separation resolution was worse, it was suitable for the HRMS experiments. Moreover, the analysis time was reduced 3 times and solvent consumption almost 15 times.

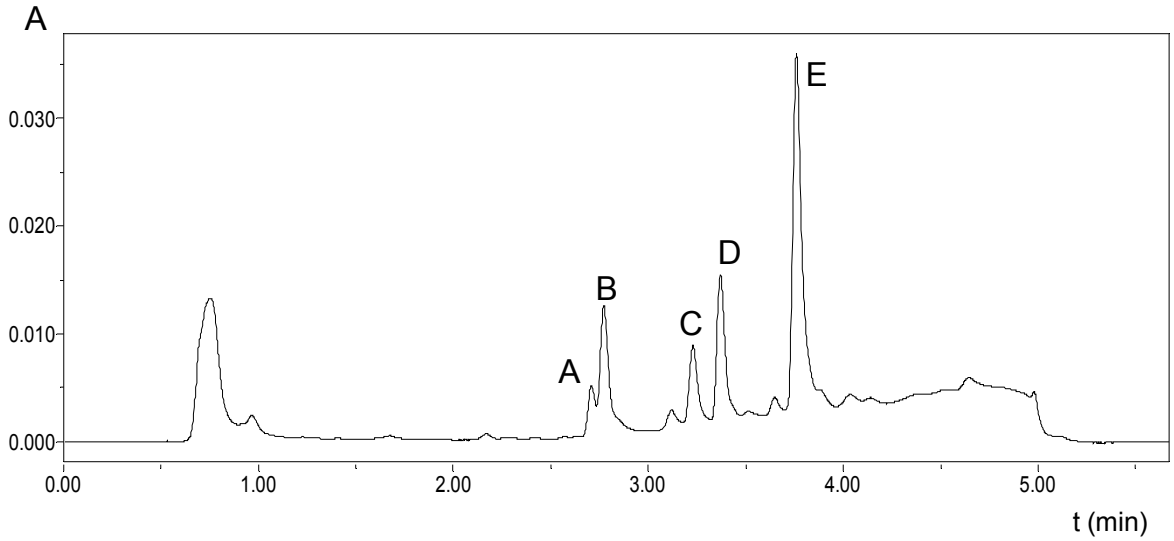

Figure 5.9. Chromatogram obtained at 256 nm for 10 µg/mL fluvastatin solutions in 1M HCl:MeOH (70:30) using 1st separation geometrically scaled to UPLC.

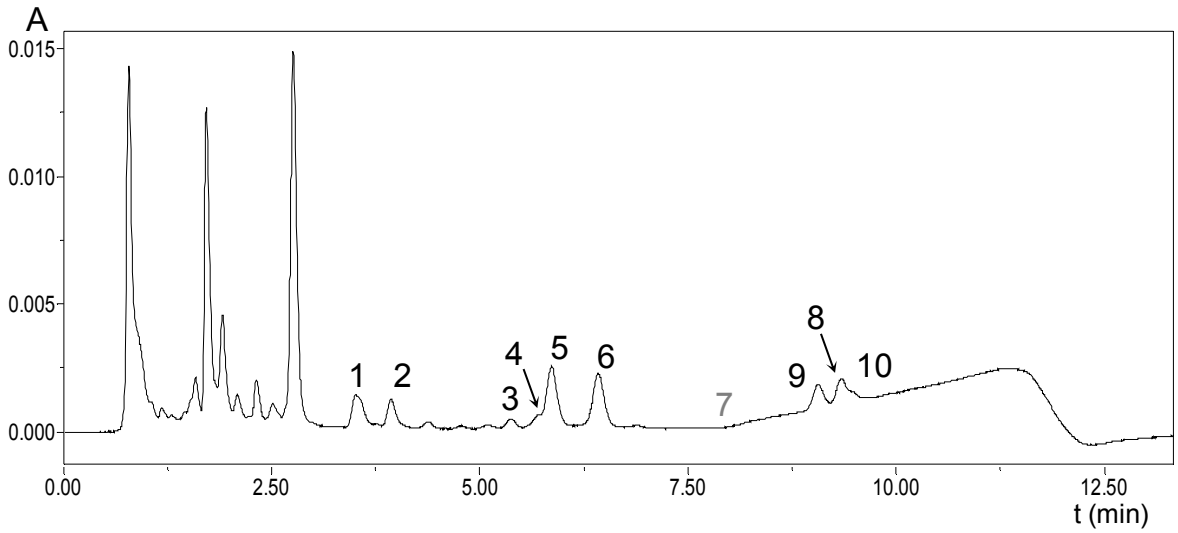

Figure 5.10. Chromatogram obtained at 256 nm for 10 µg/mL fluvastatin solutions in 50 mM phosphate buffer (pH=2):MeOH (70:30) after one week using 2nd separation geometrically scaled to UPLC

5.3.3.2. Identification of the degradation compounds

The molecular mass found for the molecular ion of the degradation compounds and the corresponding molecular formulas are shown in Table 5.3. They are gathered in two groups. The first group is composed of the final compounds formed under strong acidic conditions while the second group contains the intermediate compounds that can be found during the degradation process. Among the final compounds one that had not been detected during PDA analysis was found (compound **F**). This compound had not been photometrically observed because it coelutes with compound **E** and has the same spectrum. Actually, compound **E** only appears in presence of methanol while compound **F** appears both in presence of methanol and acetonitrile.

Table 5.3. Exact mass and molecular formulas of the degradation compounds

Intermediate compounds			Final compounds		
ID	**$[M+H]^+$ (m/z)**	**Mol. formula**	**ID**	**$[M+H]^+$ (m/z)**	**Mol. formula**
1 FV	412.1924	$C_{24}H_{26}NO_4F$	A	444.1822	$C_{24}H_{26}NO_6F$
2	412.1924	$C_{24}H_{26}NO_4F$	B	444.1822	$C_{24}H_{26}NO_6F$
3	394.1818	$C_{24}H_{24}NO_3F$	C	458.1979	$C_{25}H_{28}NO_6F$
4	394.1818	$C_{24}H_{24}NO_3F$	D	458.1979	$C_{25}H_{28}NO_6F$
5	426.2080	$C_{25}H_{28}NO_4F$	E	472.2135	$C_{26}H_{30}NO_6F$
6	426.2080	$C_{25}H_{28}NO_4F$	F	270.1294	$C_{17}H_{17}NOF$
7	394.1818	$C_{24}H_{24}NO_3F$			
8	394.1818	$C_{24}H_{24}NO_3F$			
9	440.2236	$C_{26}H_{30}NO_4F$			
10	440.2236	$C_{26}H_{30}NO_4F$			

The chromatographic peaks appearing close to each other (**1** and **2**; **5** and **6**; **9** and **10**; **A** and **B** and **C** and **D**) are isomers with a presumably similar chemical structure as can be assumed from the almost identical mass fragmentation spectra (product ion scans can be observed in Appendix III at the end of this chapter). This suggests the appearance of diasteromeric mixtures as a result of the degradation process. It could be explained as well, by a cis-trans isomerism in the double bond of the aliphatic chain as reported by Mielcarek et al. [22].

It can be observed that analytes that only appear in the methanolic solutions in weak acidic conditions (compounds **5**, **6**, **9** and **10**) differ from the parent drug in the addition of methyl groups, which confirms the hypothesis of a methylation. Fluvastatin is likely to esterificate to form compounds **5** and **6**, by the

addition of a methoxy group in the acid functional group with the subsequent water loss. Compounds **9** and **10** may be ethers coming from this ester by etherification of the allyl alcohol (in C-5) (Figure 5.12). The fragmentation spectra of these compounds reinforce this hypothesis, since the characteristic ions belonging to the indolic structure appear in all the product ion scans, suggesting a modification of the β-hydroxyacidic chain.

In the same way, there is a parallelism with the degradation products obtained in strong acidic conditions. **C** and **D** isomers pair and compound **E** differ from compounds **A** and **B** in the addition of one and two methyl groups, respectively. Thus, compounds **C** and **D** can be esters coming from compound **A** and **B**, as well as compound **E** could be the ether produced from the ester.

Carefully examining the molecular formula of compounds **A** and **B**, it can be noticed that they have two more oxygen atoms than fluvastatin. This indicates an oxidation of fluvastatin or a reorganization of the molecule including two oxygen atoms. The latter hypothesis suits better taking into account the lack of fluorescence of the “final” compounds, probably due to the rupture of the indolic group. Predicting the reaction taking place turns difficult due to the complexity of the molecule and to the fact that the elucidation of these compounds is unachievable only by mass spectrometry (NMR techniques would be helpful). *Cermola et al.* [21] proposed some photodegradation products that fit the results obtained and could be produced by a bishydroxylation of the indolic ring. These molecules can be observed in Figure 5.12. Compound **F** seems to be a part of the

skeleton of the final degradation compounds since its molecular ion fit in with a fragment ion of all these compounds ($[M+H]^+$: 270.1294) and follows the same fragmentation pathway.

Finally, four isomers with a molecular weight of 394.1818 ($C_{24}H_{24}NO_3F$) were found when fluvastatin was exposed to weak acidic conditions: compounds **3**, **4**, **7** and **8**. Their formation is favoured in the absence of methanol because parallel reactions are avoided. These four compounds are unlikely to be stereoisomers taking into account the wide difference in the retention times (and consequently in polarity). Nevertheless, the fragmentation mass spectra obtained for each compound are almost identical as can be observed in Appendix III. The mass difference of these compounds respect to fluvastatin is a water loss that could be justified by the formation of the fluvastatin lactone. In fact HMGCo-A reductase inhibitors have an equilibrium involving the lactone and the hydroxyacid forms [32-34]. In the case of fluvastatin, lactone form is favoured at low pH being the reaction reversible [34]. Therefore, one of these “intermediate” compounds is probably the lactone form of fluvastatin. According to the spectrophotometric study, compounds **7** and **8** have absorption spectra significantly different to fluvastatin. Absorption spectrum is not supposed to change due to the cyclation of the aliphatic chain, thus, the proposed lactone should be compound **3** or compound **4** (or both of them taking into account the possibility of diastereoisomerism). In order to justify both the mass spectra similarities and the absorption spectra differences among these four compounds a pH favoured dehydration reaction is proposed. The dehydration of the alcohol in C-5 would

suppose a new double bound in the aliphatic chain in resonance with the aromatic moiety that could change the absorption properties of the molecule. Nevertheless, this hypothesis does not agree with the fragmentation spectra of compounds **7** and **8**, which should be similar to the parent drug's one. Furthermore, the proposed change in absorption should move the absorption bands to higher wavelengths. Thus, once again NMR techniques turn necessary for the elucidation of the degradation compounds.

Even if characterization of the degradation products was not fully successful the complex degradation pathway of fluvastatin can be globally described as in Figure 5.11. The chemical structures proposed for the compounds taking place in these reactions are shown in Figure 5.12.

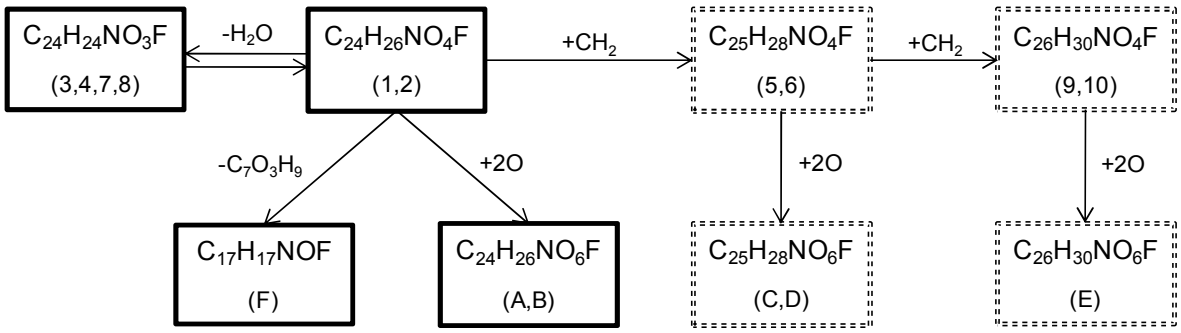

Figure 5.11. Proposed reaction scheme for fluvastatin degradation. Degradation compounds in dashed boxes were only found in methanolic solutions

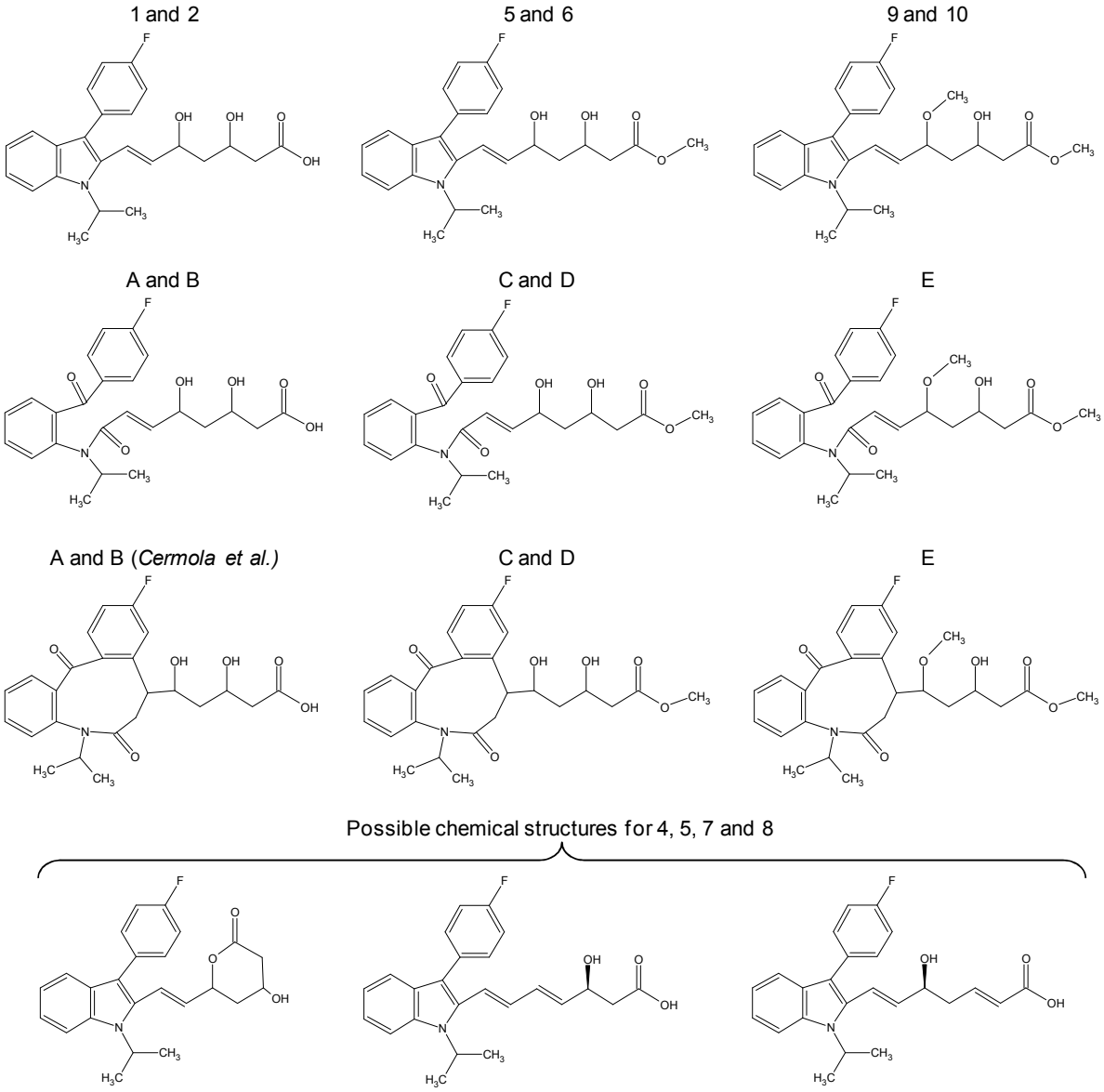

Figure 5.12. Chemical structures proposed for the degradation compounds of fluvastatin in acidic media

5.3.4. Degradation of fluvastatin in plasma samples during protein precipitation procedure

After analyzing the fluvastatin plasma sample exposed to acidic protein precipitation during sample treatment, the degradation compounds identified in the previous steps were searched. In Figure 5.13 the extracted ion chromatograms for the *m/z* ratio detected are shown.

All the intermediate compounds were found whereas only one of the final compounds (**F**) was detected. Fluvastatin was no more than 30 minutes exposed to the acidic conditions, thus, longer exposure times would suppose a stronger degradation. Compounds **5**, **6**, **9** and **10** that only were found in the degradation studies in the presence of methanol can be observed at low intensities. This means that a small amount of the methylated compounds were formed during sample treatment. In fact, SPE procedure involves using methanol both in cleaning and elution steps, which can lead to the formation of the mentioned degradation compounds.

The ion chromatogram extracted for *m/z* 394.1818 showed more chromatographic signals than the corresponding to compounds **3**, **4**, **7** and **8** because it is as well a fragment ion of fluvastatin (**1**) and other degradation products (**5** and **6**).

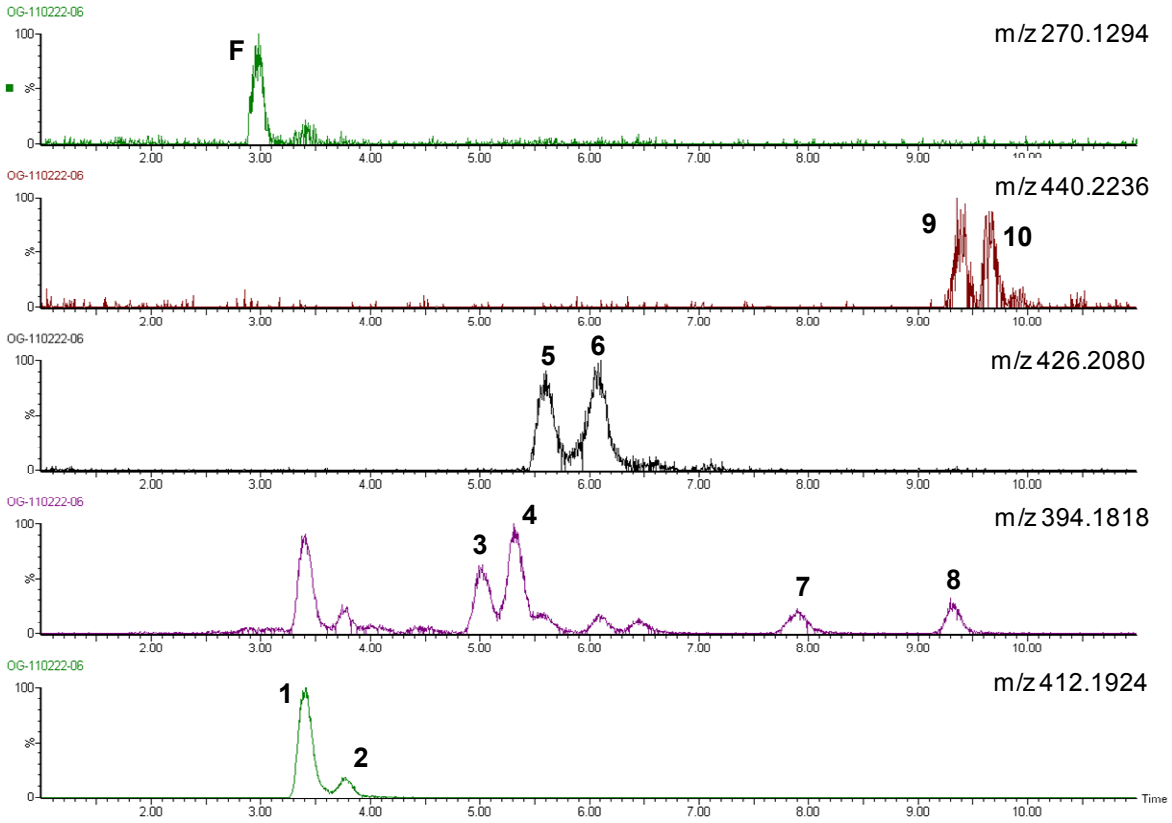

Figure 5.13. Extracted ion chromatograms for the degradation compounds present in a plasma sample spiked with 10 µg/mL fluvastatin and treated with 0.5 M H_3PO_4 solution for protein precipitation

5.4. Conclusions

This work has demonstrated the instability of fluvastatin in acidic media, an important point to take into account when an analysis method for this drug is developed. According to the obtained results, acids must be avoided as protein precipitant agents during plasma sample treatment or fluvastatin would be converted into several degradation compounds. Therefore, other precipitant agents such as acetonitrile or methanol should be used.

This degradation can affect fluvastatin determination in other points of the analysis such as the chromatographic separation. If the mobile phases used in HPLC are acid, fluvastatin can be degraded during the separation process. This degradation would be affected by the pH of the mobile phase, the length of the chromatographic separation and the nature of the organic modifier. According to the results obtained by spectrophotometric analysis, aqueous mobile phases below pH 3 should be avoided unless acetonitrile is used as organic modifier. Nevertheless, the effect of the mobile phase should be carefully checked to prevent fluvastatin degradation in each particular case.

From the point of view of the pharmacokinetics of the drug, the instability of fluvastatin under acidic conditions can affect its bioavailability. In fact, some literature reports indicate that concomitant administration of fluvastatin with an acidic carbonated beverage results in decreased bioavailability [17,35]. According to these researches the maximum concentration, the time of maximum concentration and the area under the curve of fluvastatin were affected by the acidic beverage intake surely due to the degradation of the active ingredient in acidic media. In the same way, the simple fact of being at stomach low pH conditions may decrease fluvastatin bioavailability.

Among the identified degradation compounds the formation of fluvastatin lactone should not affect the bioavailability of fluvastatin since after absorption into the bloodstream (pH = 7.4) the pH dependent equilibrium should be shifted towards the active hydroxy acid form [34].

On another note, the Q-TOF proved to be a powerful and useful instrument for the determination of the molecular formula of the degradation compounds. Anyway, structural elucidation is a more complex task and even if some structural conformations have been proposed they should be confirmed by NMR or other characterization techniques. In this respect, preparative chromatography would be necessary to isolate each degradation product.

In order to confirm the fragmentation pathway of fluvastatin molecule, further analysis using ion mobility will be carried out to provide a better knowledge of the possible structural conformation of the degradation compounds.

5.5 References

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APPENDIX III

PRODUCT ION SCANS OF FLUVASTATIN DEGRADATION COMPOUNDS

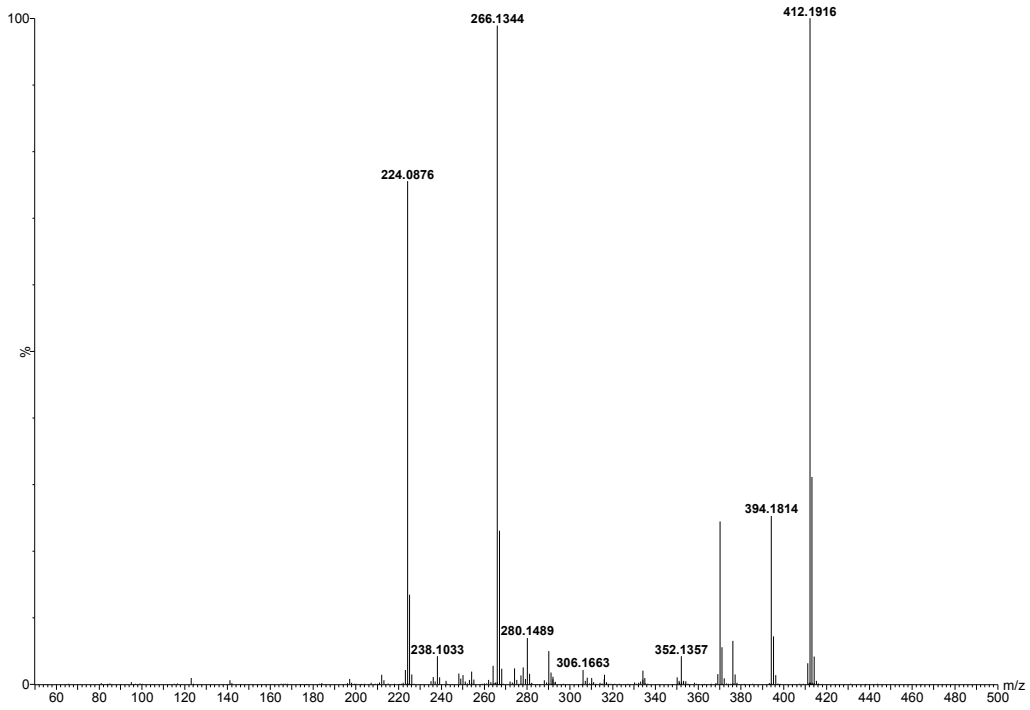

Figure 5.14. Product ion scan obtained for Compound 1 ($[M+H]^+$: 412.1924) as described in section 5.2.5.2.

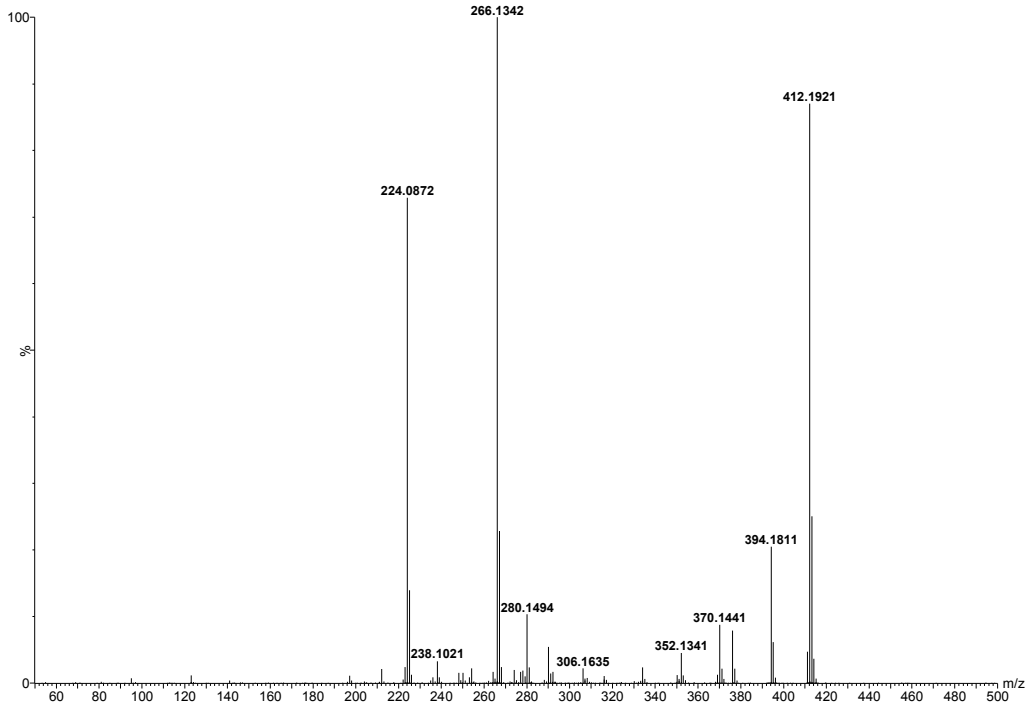

Figure 5.15. Product ion scan obtained for Compound 2 ($[M+H]^+$: 412.1924) as described in section 5.2.5.2.

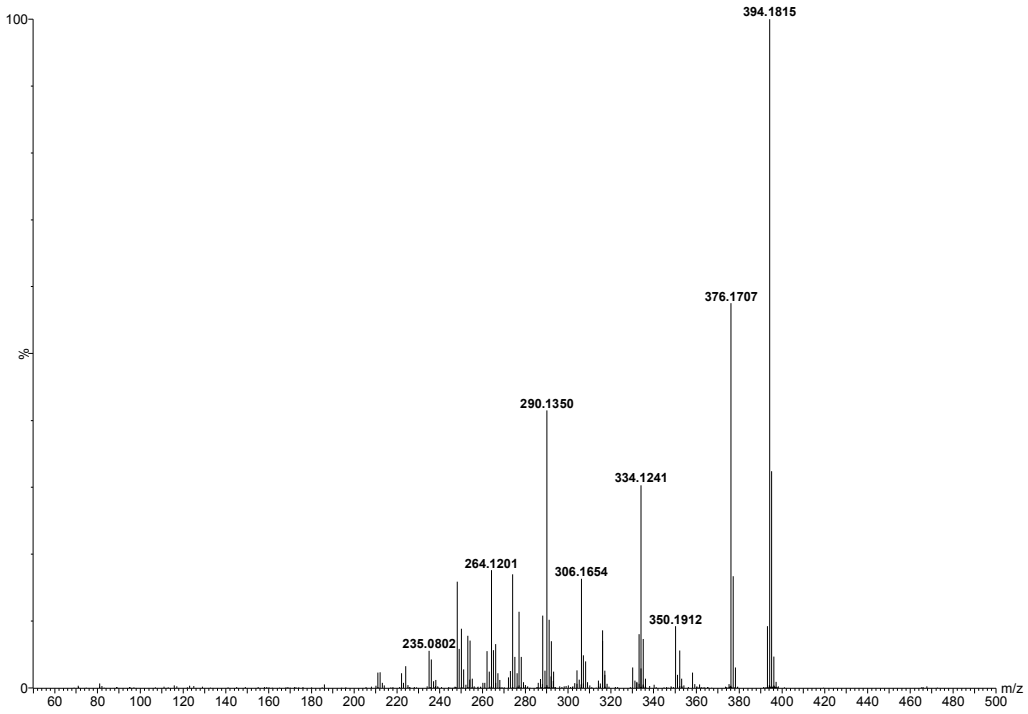

Figure 5.16. Product ion scan obtained for Compound 3 ($[M+H]^+$: 394.1818) as described in section 5.2.5.2.

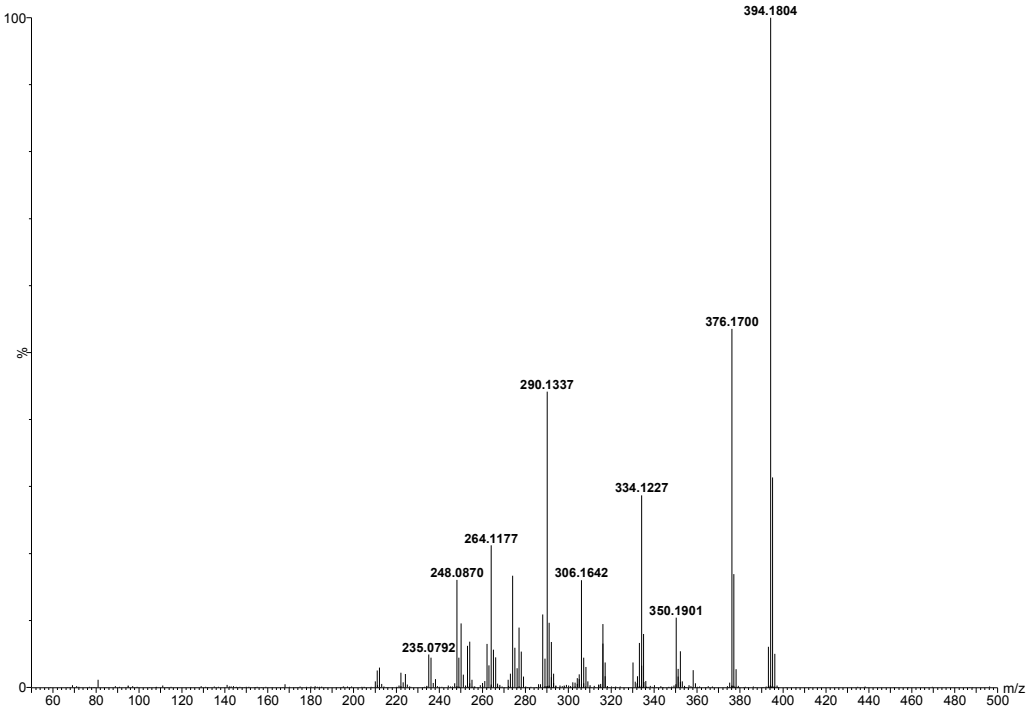

Figure 5.17. Product ion scan obtained for Compound 4 ($[M+H]^+$: 394.1818) as described in section 5.2.5.2.

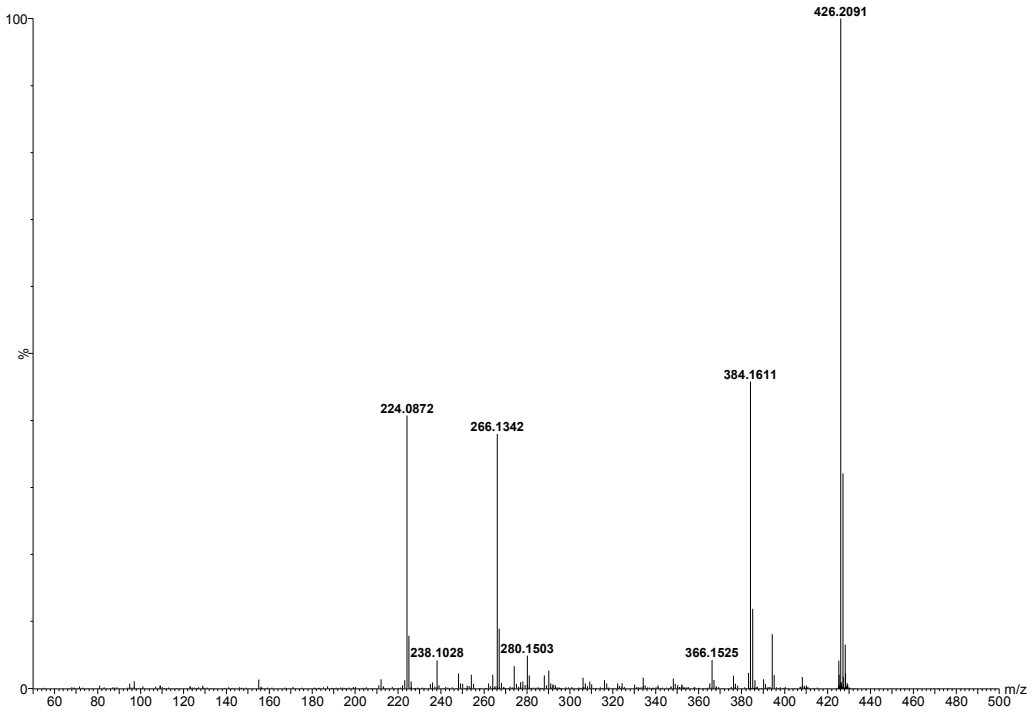

Figure 5.18. Product ion scan obtained for Compound 5 ($[M+H]^+$: 426.2080) as described in section 5.2.5.2.

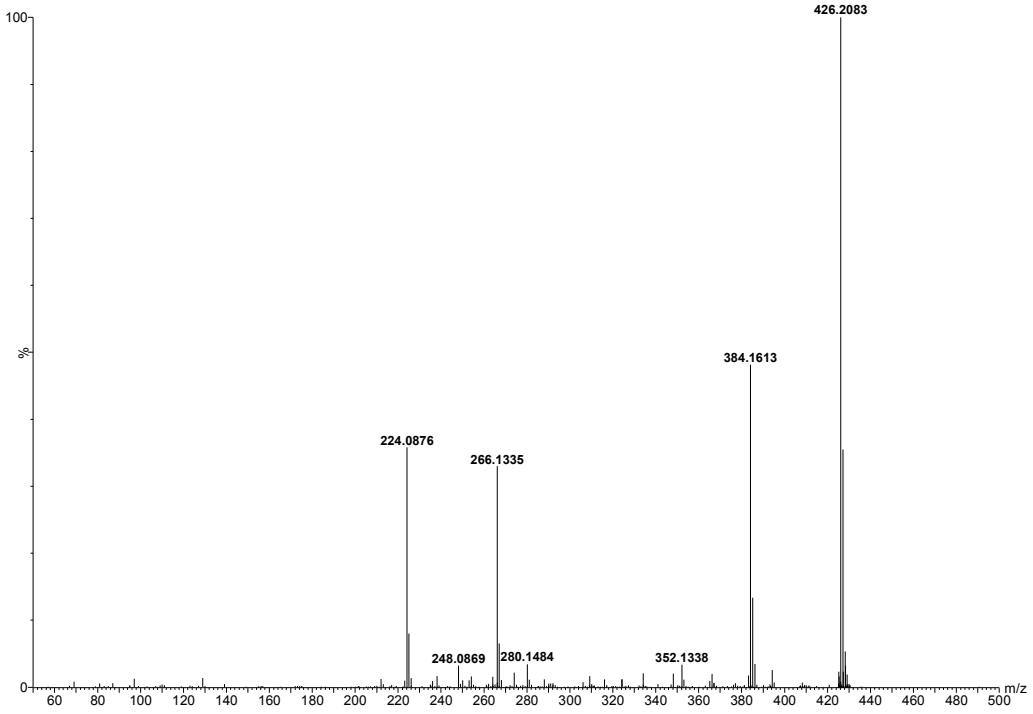

Figure 5.19. Product ion scan obtained for Compound 6 ($[M+H]^+$: 426.2080) as described in section 5.2.5.2.

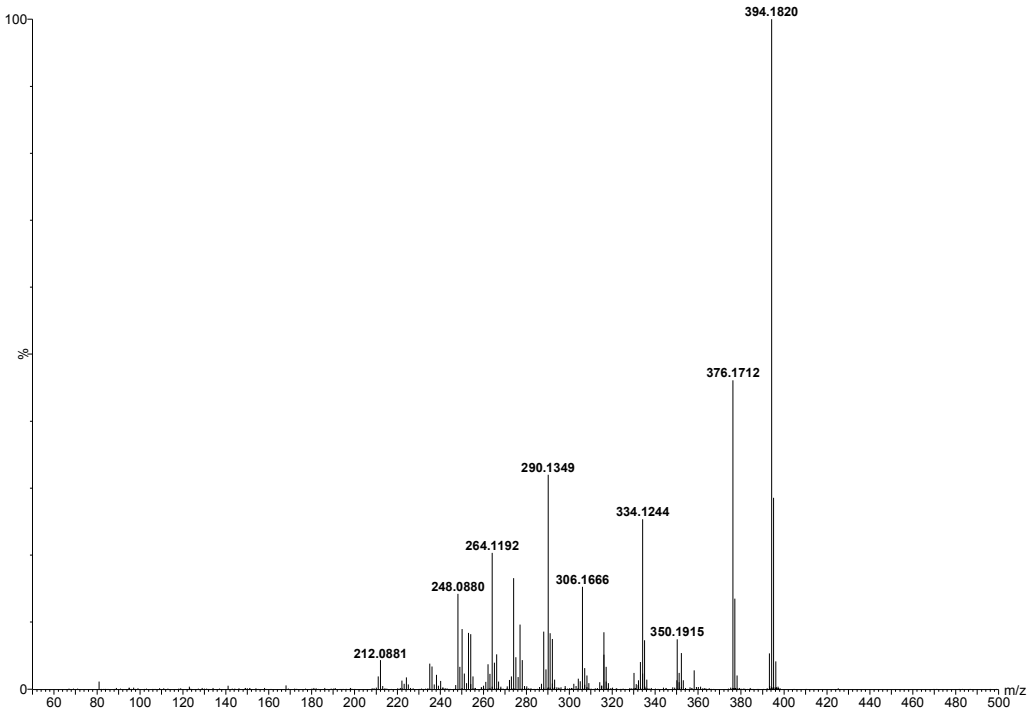

Figure 5.20. Product ion scan obtained for Compound 7 ($[M+H]^+$: 394.1818) as described in section 5.2.5.2.

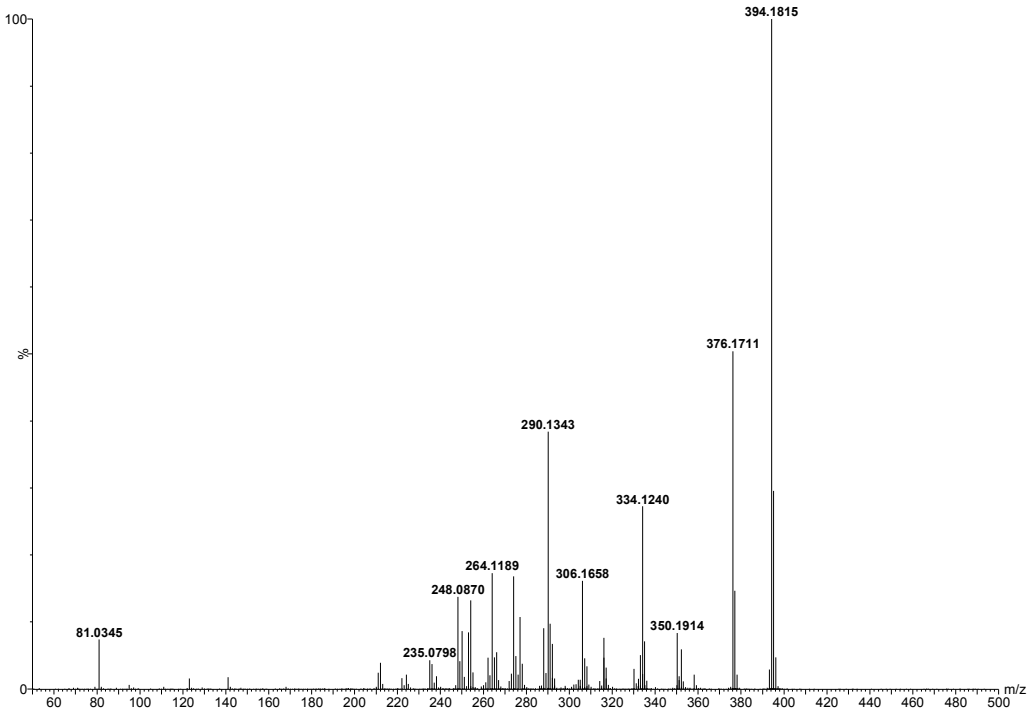

Figure 5.21. Product ion scan obtained for Compound 8 ($[M+H]^+$: 394.1818) as described in section 5.2.5.2.

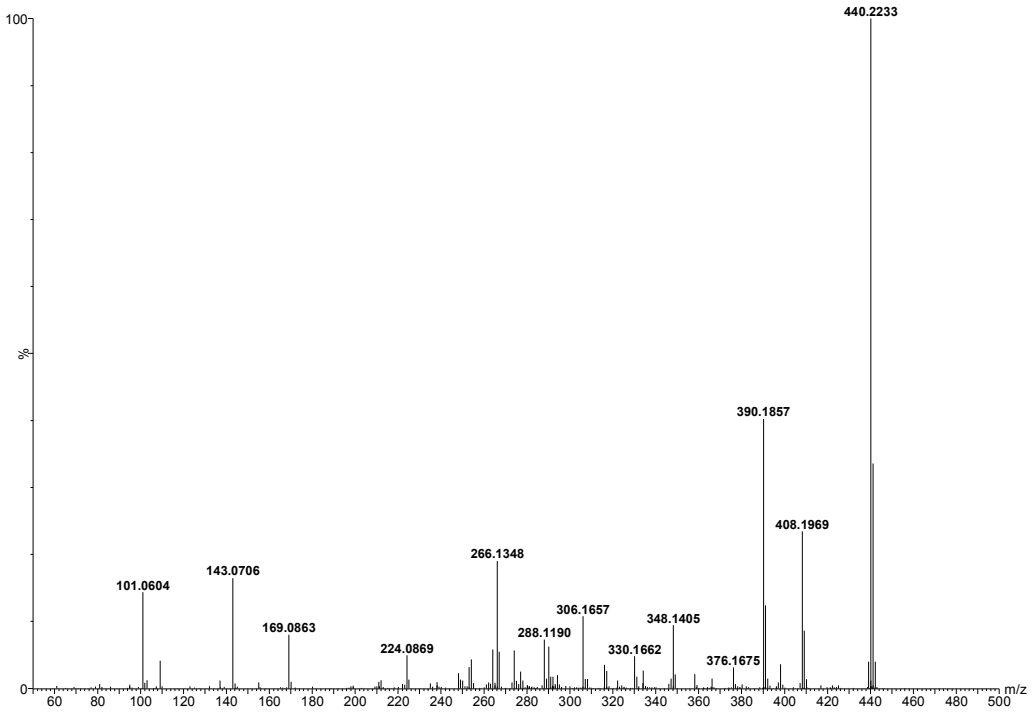

Figure 5.22. Product ion scan obtained for Compound 9 ($[M+H]^+$: 440.2236) as described in section 5.2.5.2.

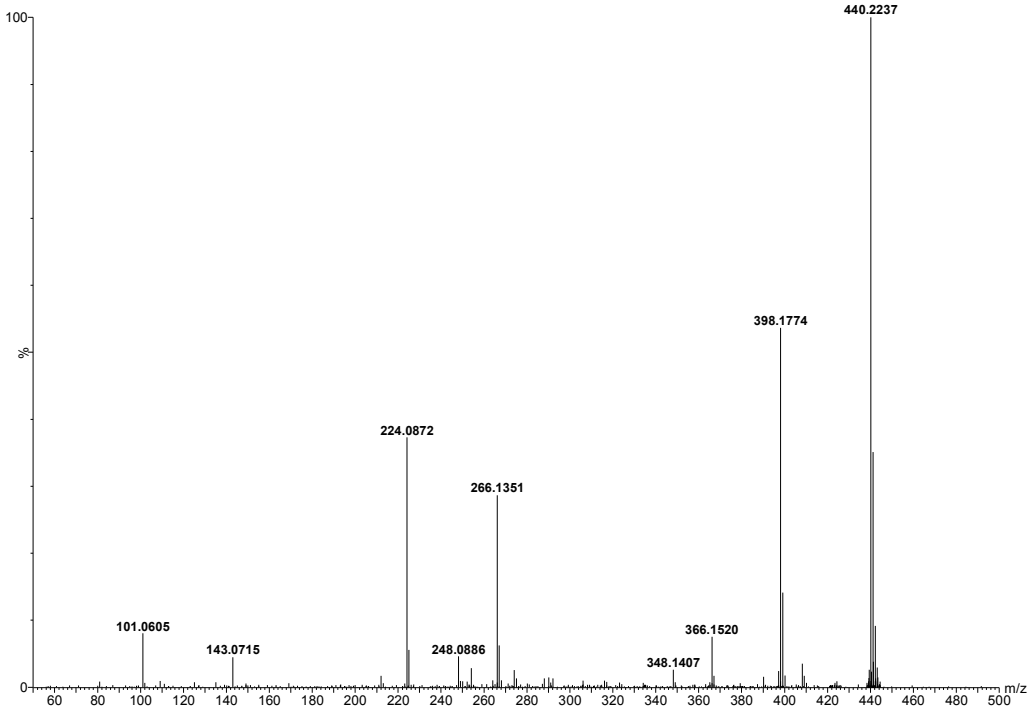

Figure 5.23. Product ion scan obtained for Compound 10 ($[M+H]^+$: 440.2236) as described in section 5.2.5.2.

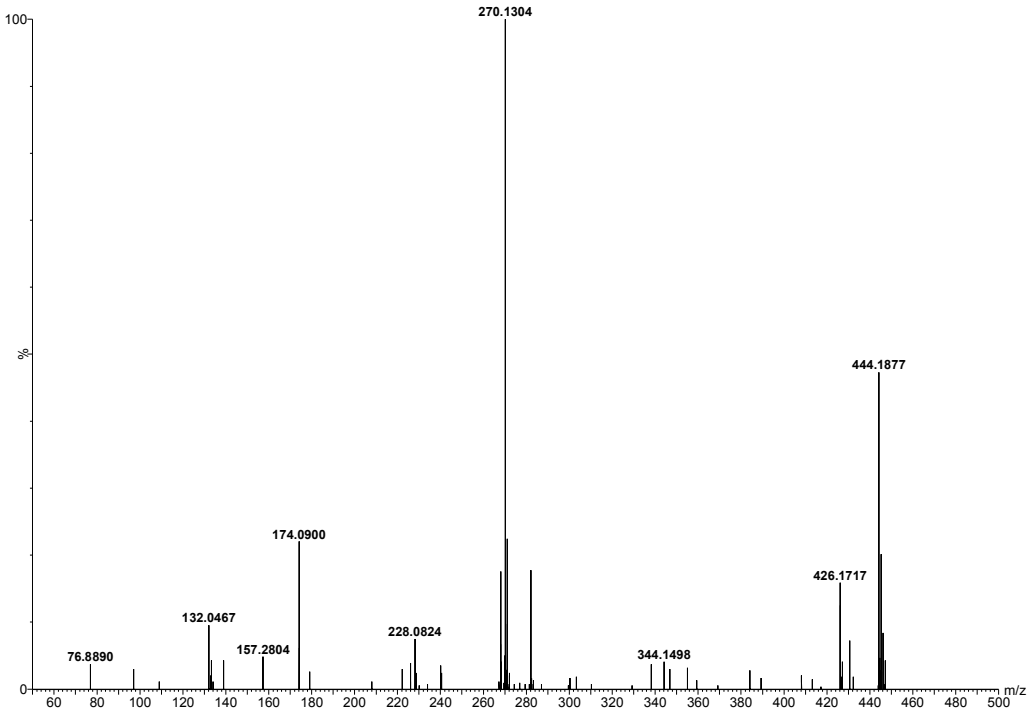

Figure 5.24. Product ion scan obtained for Compound A ($[M+H]^+$: 444.1822) as described in section 5.2.5.2.

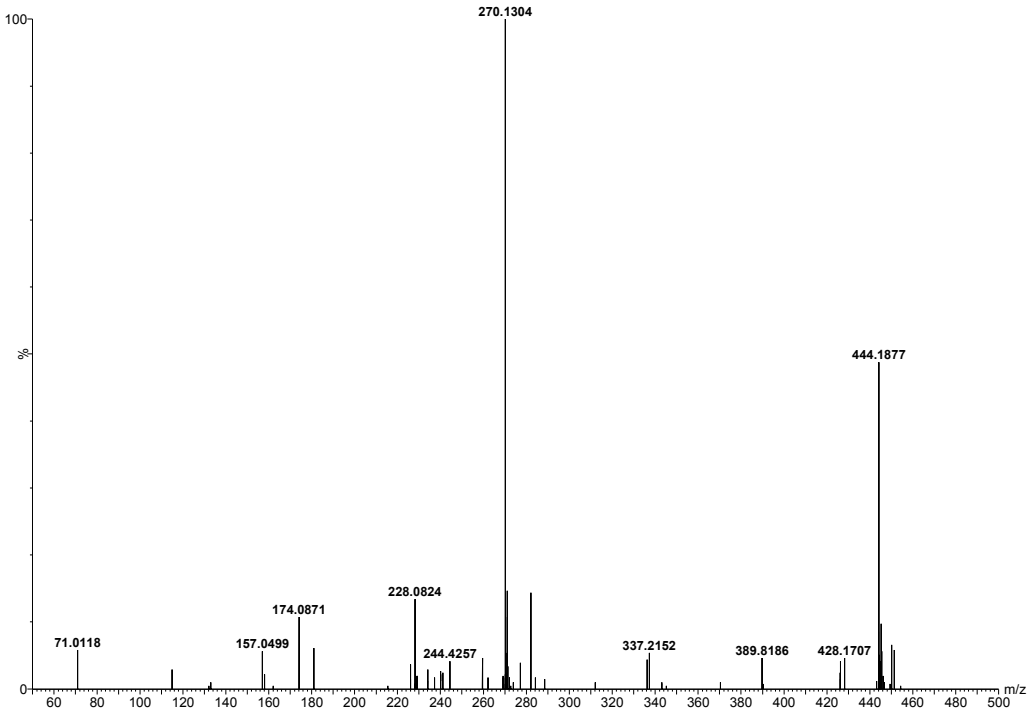

Figure 5.25. Product ion scan obtained for Compound B ($[M+H]^+$: 444.1822) as described in section 5.2.5.2.

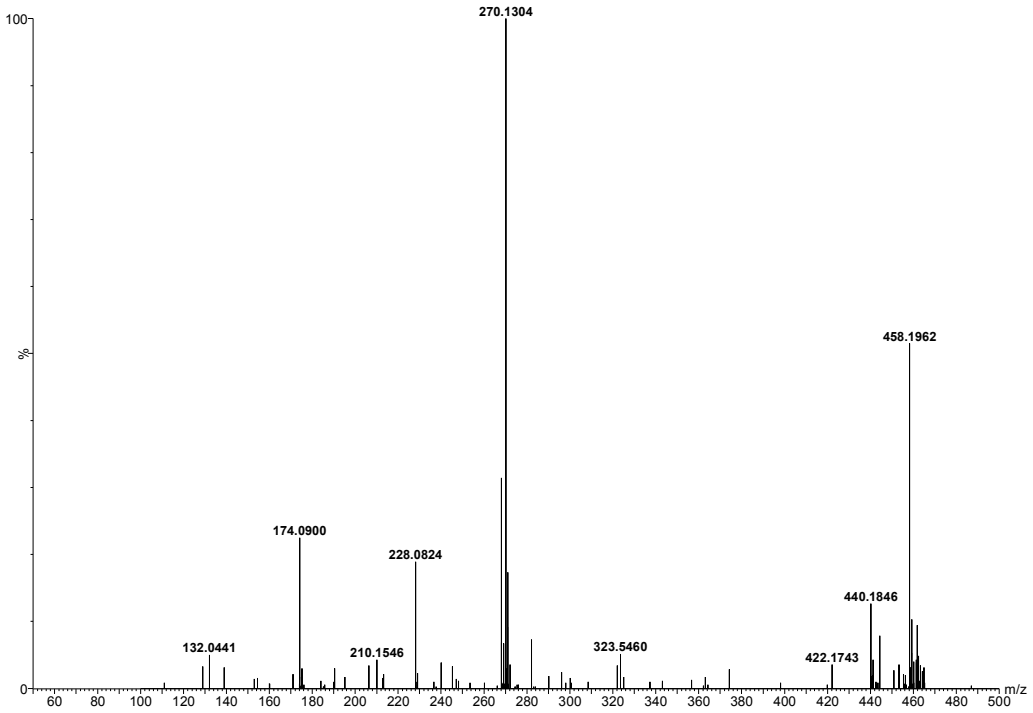

Figure 5.26. Product ion scan obtained for Compound C ($[M+H]^+$: 458.1979) as described in section 5.2.5.2.

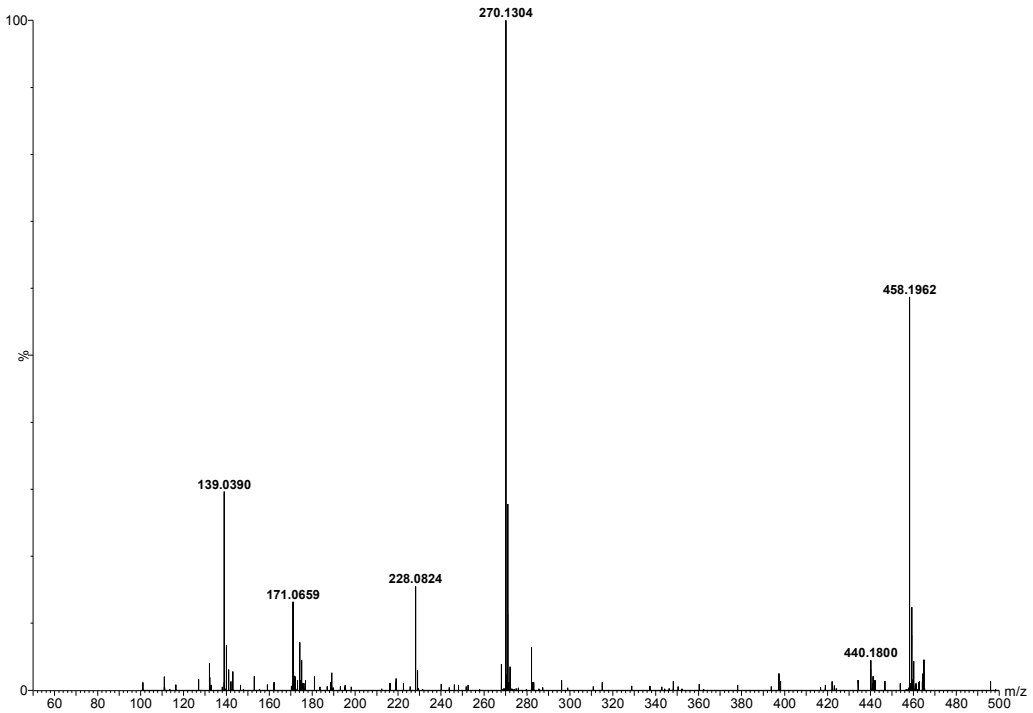

Figure 5.27. Product ion scan obtained for Compound D ($[M+H]^+$: 458.1979) as described in section 5.2.5.2.

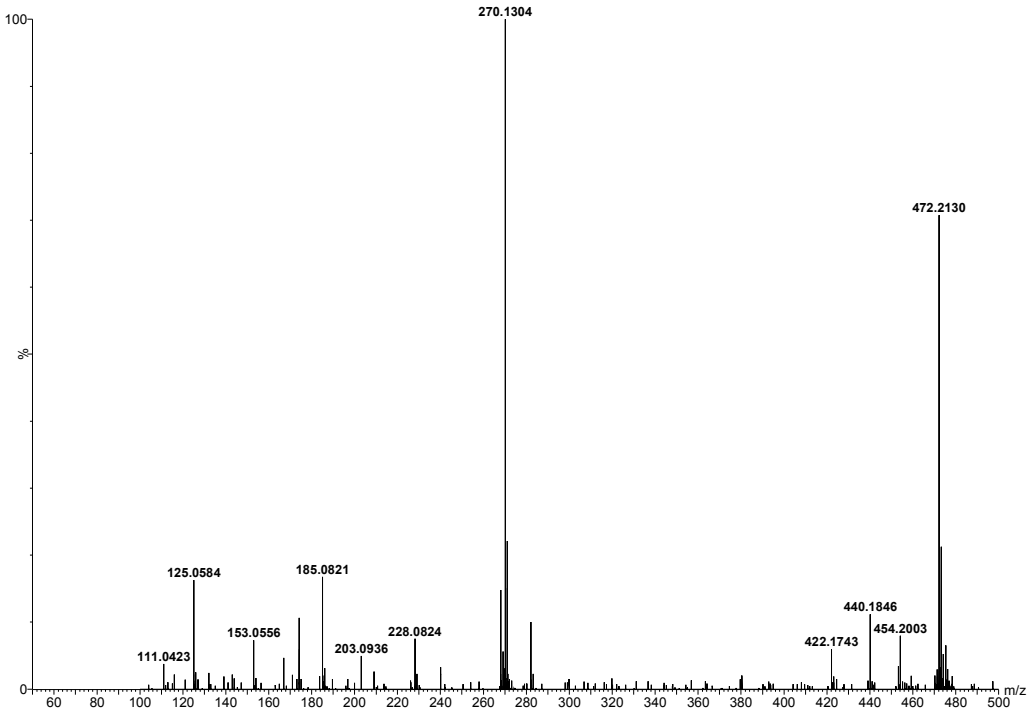

Figure 5.28. Product ion scan obtained for Compound E ($[M+H]^+$: 472.2135) as described in section 5.2.5.2.

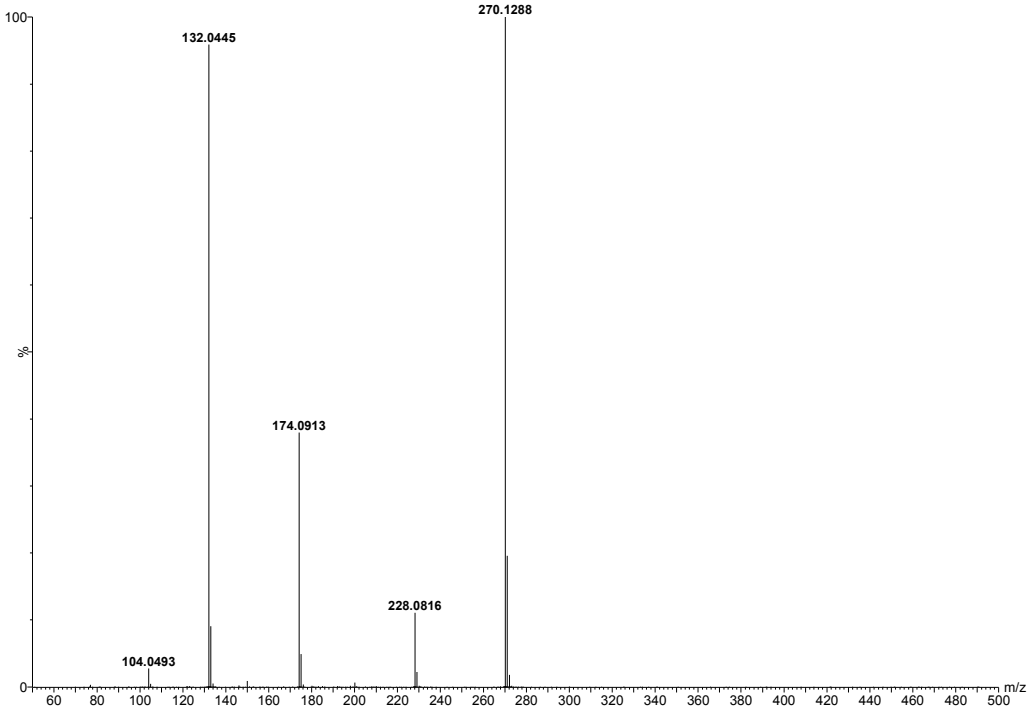

Figure 5.29. Product ion scan obtained for Compound F ($[M+H]^+$: 270.1294) as described in section

APPENDIX IV

PREVIOUS WORK:

Optimization and validation of a SPE-HPLC-PDA-fluorescence method for the simultaneous determination of drugs used in combined cardiovascular therapy in human plasma

Journal of Pharmaceutical and Biomedical Analysis

Volume 50, Issue 4, 1 November 2009, Pages 630-639

Validation of a fast liquid chromatography–UV method for the analysis of drugs used in combined cardiovascular therapy inhuman plasma

Journal of Chromatography B

Volume 877, Issue 27, 1 October 2009, Pages 3045-3053

APPENDIX V

FULL DATA OF THE VALIDATION PROCESS CARRIED OUT IN CHAPTER III.

Table 1. Intra- and inter-RSD values obtained for all the analytes at low, medium und high concentrations (5 replicas for each analyte)

	Low concentration		Middle concentration		High concentration	
Analyte	%RSD intra	%RSD inter	%RSD intra	%RSD inter	%RSD intra	%RSD inter
Acebutolol	7.13	3.29	6.95	9.30	6.11	4.16
Acenocoumarol	7.79	5.13	3.10	2.08	7.80	5.17
Alprenolol	6.13	4.19	3.04	2.92	5.19	3.76
Amiloride	11.52	5.56	6.96	7.20	8.87	3.99
Amlodipine	9.20	11.81	7.53	11.07	7.59	0.88
Atenolol	5.69	2.85	5.58	2.70	6.66	11.02
Befunolol	8.14	3.49	3.30	4.27	5.72	6.39
Betaxolol	6.59	3.07	3.65	5.54	4.77	6.41
Bopindolol	7.50	8.57	6.37	6.86	5.48	4.02
Bunitrolol	6.36	3.38	3.32	2.66	5.39	2.93
Bupranolol	5.53	2.28	2.73	2.28	5.39	3.53
Candesartan	10.61	11.99	9.90	14.23	7.38	8.96
Carazolol	7.16	2.95	2.91	2.61	4.99	3.79
Carteolol	4.84	1.30	3.77	3.96	5.53	3.20
Carvedilol	8.69	7.81	6.76	6.16	5.24	7.57
Celiprolol	5.52	5.36	5.83	7.81	5.97	7.33
Cilazapril	7.64	3.42	5.27	7.45	5.85	5.70
Clopidogrel	5.79	11.82	2.91	7.26	6.66	3.64
Enalapril	10.80	10.82	7.81	7.33	6.48	3.38
Eprosartan	10.14	1.70	5.19	2.50	8.70	12.40
Esmolol	7.58	7.74	3.26	6.63	5.22	5.53
Felodipine	10.96	7.85	5.55	7.75	9.60	8.75
Fendiline	8.02	8.02	6.11	2.41	9.55	6.67
Gallopamil	7.90	5.69	6.11	6.25	4.88	7.46
Glibenclamide	10.42	6.05	4.25	3.96	8.34	5.33
Glibornuride	7.01	7.07	4.60	13.48	8.57	10.92
Gliclazide	8.79	3.27	5.11	2.97	8.42	2.59
Glipizide	6.05	11.96	2.55	4.52	9.64	5.92
Gliquidone	7.55	1.82	6.64	12.72	6.41	3.73
Irbesartan	7.17	6.22	4.22	3.87	5.33	0.99
Lercanidipine	7.20	3.48	3.73	3.54	4.49	6.25
Levobunolol	6.46	1.08	2.90	2.69	4.85	1.70
Mepindolol	10.01	11.22	8.42	6.10	6.67	10.38
Metformin	10.79	6.07	7.00	2.52	6.01	6.81
Nadolol	6.62	2.02	3.34	6.05	6.39	6.89
Nicardipine	4.87	4.79	2.80	5.15	4.72	3.52
Penbutolol	7.35	10.48	3.33	8.98	5.94	10.38
Perindopril	9.50	6.38	2.91	6.01	5.43	8.72
Pindolol	5.90	2.78	4.44	3.45	5.90	2.74

Table 1. Continuation

	Low concentration		Middle concentration		High concentration	
Analyte	%RSD intra	%RSD inter	%RSD intra	%RSD inter	%RSD intra	%RSD inter
Practolol	11.99	7.01	10.72	6.33	7.85	0.85
Propranolol	4.44	1.30	3.03	1.29	4.85	4.51
Ramipril	7.32	0.92	3.79	6.09	5.60	2.78
Repaglinide	6.19	2.50	5.94	5.76	6.44	4.01
Rosiglitazone	6.80	1.36	4.42	3.96	4.40	3.83
Sotalol	5.93	4.47	5.93	5.86	6.89	5.79
Talinolol	7.86	14.09	3.71	7.47	3.91	4.26
Telmisartan	4.99	11.53	2.95	7.92	5.97	5.75
Tertatolol	6.77	1.88	2.91	2.11	5.38	5.72
Ticlopidine	4.98	2.66	5.88	1.49	5.04	11.04
Tolbutamide	7.38	4.46	4.54	6.78	7.35	12.78
Toliprolol	5.38	3.74	3.53	4.72	4.99	2.47
Torasemide	8.18	12.82	6.65	6.93	5.49	2.41
Valsartan	9.98	12.05	5.17	9.00	6.64	3.55
Verapamil	6.62	7.75	3.25	0.44	5.40	9.98
Warfarin	7.44	3.17	4.75	0.95	9.66	0.58
Bisoprolol*	5.96	0.52	3.13	5.62	-	-
Carbutamide*	7.87	2.51	3.51	3.97	-	-
Labetalol*	7.50	3.26	2.21	1.67	-	-
Metoprolol*	7.86	3.06	3.20	4.13	-	-
Oxprenolol*	4.64	1.86	3.15	1.03	-	-
Timolol*	6.68	2.46	4.00	5.97	-	-
Tolazamide*	9.62	1.97	6.33	4.11	-	-
Atorvastatin	8.33	8.17	1.98	8.23	4.37	10.67
Cand Cilex	11.26	14.81	4.01	14.68	5.97	6.79
Cerivastatin	13.07	11.12	4.22	9.28	6.60	11.41
Enalaprilat	4.27	13.44	2.30	8.52	6.03	6.92
Fluvastatin	4.81	5.72	3.72	7.69	6.44	6.97
Glimepiride	9.76	9.22	6.16	6.25	2.27	8.47
Imidapril	5.24	13.45	2.37	14.41	5.16	2.55
Lisinopril	6.28	6.60	3.58	10.42	5.28	5.61
Lovastatin	9.12	7.74	10.50	2.02	4.19	6.18
Quinaprilat	4.23	13.10	3.86	13.99	3.77	5.85
Simvastatin	6.90	8.84	4.11	10.60	4.24	11.99

Table 2. Intra- and inter-RE values obtained for all the analytes at low, medium und high concentrations (5 replicas for each analyte)

	Low concentration		Middle concentration		High concentration	
Analyte	%RE intra	%RE inter	%RE intra	%RE inter	%RE intra	%RE inter
Acebutolol	3.54	3.30	6.19	3.46	7.35	8.53
Acenocoumarol	5.87	6.51	2.11	1.51	3.54	0.16
Alprenolol	12.31	12.99	5.79	6.44	3.10	1.94
Amiloride	5.19	3.31	5.15	1.20	10.09	11.31
Amlodipine	7.83	0.23	7.38	6.92	6.82	7.68
Atenolol	8.92	9.59	2.45	1.29	8.01	3.10
Befunolol	3.86	3.70	4.76	5.41	5.92	5.75
Betaxolol	7.78	8.43	8.96	9.63	6.03	7.22
Bopindolol	5.83	3.02	4.81	0.53	2.36	2.74
Bunitrolol	3.20	3.85	6.56	7.22	9.26	10.44
Bupranolol	2.34	0.01	4.18	4.82	10.08	11.29
Candesartan	9.34	1.85	9.78	0.32	6.81	1.85
Carazolol	10.61	11.30	10.24	10.93	4.14	3.11
Carteolol	13.98	14.68	8.36	9.03	1.87	0.73
Carvedilol	9.47	8.92	7.60	7.93	7.07	7.18
Celiprolol	4.18	0.99	5.73	1.57	8.30	9.53
Cilazapril	3.08	2.59	4.68	3.04	8.92	8.06
Clopidogrel	11.03	11.67	11.29	10.72	3.78	4.94
Enalapril	7.68	2.11	6.25	5.32	12.23	13.45
Eprosartan	10.51	11.19	4.31	4.94	11.01	4.50
Esmolol	6.20	1.25	5.96	4.19	3.40	2.74
Felodipine	6.43	4.11	5.00	4.70	6.96	3.54
Fendiline	8.64	6.54	2.66	2.74	9.47	10.71
Gallopamil	10.80	11.48	6.91	7.59	6.98	6.24
Glibenclamide	4.58	0.04	3.88	4.16	7.90	9.06
Glibornuride	5.03	4.54	9.55	0.17	8.80	1.24
Gliclazide	7.35	8.03	9.39	10.06	10.49	11.68
Glipizide	7.98	0.09	3.56	1.39	5.59	6.06
Gliquidone	1.29	1.14	9.48	4.61	7.82	5.85
Irbesartan	4.95	4.66	7.42	8.09	11.46	12.68
Lercanidipine	2.26	0.85	4.11	3.91	4.92	6.10
Levobunolol	12.52	13.22	11.69	12.39	1.82	0.73
Mepindolol	7.73	0.02	3.91	0.89	9.52	6.67
Metformin	4.79	0.66	2.37	0.32	6.42	3.32
Nadolol	10.85	11.52	4.00	4.51	7.70	6.65
Nicardipine	9.24	9.92	8.36	9.03	2.86	3.99
Penbutolol	8.53	1.39	6.61	1.42	7.03	2.50
Perindopril	5.72	3.32	4.82	4.24	8.12	5.52
Pindolol	5.65	6.30	4.09	4.05	3.76	4.91
Practolol	4.43	3.14	4.42	1.47	1.14	2.05
Propranolol	11.32	12.01	7.77	8.44	3.34	1.88
Ramipril	4.61	4.02	4.20	2.78	12.58	13.83

Table 2. Continuation

	Low concentration		Middle concentration		High concentration	
Analyte	%RE intra	%RE inter	%RE intra	%RE inter	%RE intra	%RE inter
Repaglinide	1.68	0.57	4.20	2.97	5.30	6.43
Rosiglitazone	12.70	13.39	9.80	10.48	3.36	1.29
Sotalol	3.72	4.34	5.22	2.08	3.75	4.80
Talinolol	12.54	4.08	4.98	1.76	2.85	1.73
Telmisartan	8.77	3.23	6.07	0.05	7.57	8.71
Tertatolol	8.89	9.56	3.76	4.41	4.24	0.05
Ticlopidine	10.27	10.27	7.53	7.53	7.41	5.07
Tolbutamide	3.62	3.62	4.92	0.25	11.76	4.78
Toliprolol	13.58	14.27	7.72	8.37	4.19	3.13
Torasemide	8.24	1.26	6.20	4.89	9.16	10.34
Valsartan	7.75	1.20	6.30	0.04	8.98	10.18
Verapamil	10.58	11.06	3.51	3.99	6.30	1.79
Warfarin	11.73	12.26	8.92	9.43	4.32	3.53
Bisoprolol*	12.92	13.62	9.83	9.26	-	-
Carbutamide*	10.70	11.23	3.93	1.38	-	-
Labetalol*	13.97	14.69	14.43	13.91	-	-
Metoprolol*	12.01	12.71	3.79	3.10	-	-
Oxprenolol*	13.86	14.57	5.15	5.80	-	-
Timolol*	10.40	11.07	4.17	0.87	-	-
Tolazamide*	11.84	12.54	3.54	1.84	-	-
Atorvastatin	7.54	4.86	6.53	2.98	9.59	6.57
Cand Cilex	11.58	2.72	10.71	1.63	5.03	3.65
Cerivastatin	9.37	4.28	5.95	0.14	9.08	3.94
Enalaprilat	10.40	3.02	5.49	4.48	4.32	3.08
Fluvastatin	4.06	1.00	5.74	1.06	6.08	2.79
Glimepiride	7.55	5.74	6.92	6.33	7.61	4.74
Imidapril	10.65	3.62	10.05	0.57	1.49	0.96
Lisinopril	7.35	7.78	9.36	7.14	3.54	2.46
Lovastatin	6.77	4.45	8.47	7.91	5.91	4.71
Quinaprilat	10.80	3.48	10.69	0.79	5.24	6.42
Simvastatin	6.76	5.60	6.70	1.49	8.29	0.74

eman ta zabal zazu

Universidad del País Vasco

Euskal Herriko Unibertsitatea

Gaur egun, eritasun kardiobaskularrak lehenengo heriotza-arrazoia dira mundu mailan hildako guztien % 30 eraginez. Gaixotasun kardiobaskularrak pairatzeko aukerak hainbat arrisku faktoreren menpekoak dira: hipertentsioa, kolesterola, diabetesa, loditasuna… Arrisku faktore hauen konbinazioa **sindrome metabolikoa** bezala ezagutzen da eta mundu osoan hedatuta dagoen pandemia da. Sindromea osatzen duten faktoreak banaka tratatu behar dira eta ondorioz farmako ezberdinez osaturiko **terapia kardiobaskular konbinatua** beharrezkoa da. Ikerkuntza lan honen helburua terapia mota honetan erabiltzen diren farmako ezberdinen aldibereko kuantifikazioa ahalbidetzen duten metodo kromatografikoen garapena da.

Cardiovascular diseases are nowadays the first cause of mortality worldwide, causing around the 30% of global deaths each year. The risk of suffering from cardiovascular illnesses is strongly related to some factors such as hypertension, high cholesterol levels, diabetes, obesity… The combination of these different risk factors is known as **metabolic syndrome** and it is considered a pandemic due to the high prevalence worldwide. The pathology of the disorders implies a **combined cardiovascular therapy** with drugs which have different targets and mechanisms of action, to regulate each factor separately. The aim of this work is to develop analytical chromatographic methods capable of quantifying the different drugs prescribed in combined cardiovascular therapy simultaneously in human plasma.

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