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DIPLOMA THESIS

Development of LC/MS/MS method for determination of ambrisentan, midazolam and its metabolite in human plasma

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I hereby declare that this thesis is my own original work. All literature and other sources I used are stated in the list of references and are cited properly. This thesis was not used for obtaining of any other degree.

Hradec Králové 10th May 2011   _____

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ABSTRACT

Development of LC/MS/MS method for determination of ambrisentan, midazolam and its metabolite in human plasma

Diploma thesis

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Ambrisentan (ABT) is a new potent drug for treatment of pulmonary arterial hypertension, which is metabolized by CYP3A4. Pharmacokinetic profile of midazolam (MDL) and its main metabolite 1-hydroxymidazolam (OH-MDL) can be used as a marker of CYP3A4 activity. Simultaneous determination of ambrisentan, midazolam and 1-hydroxymidazolam is advantageous for studying of potential influence of CYP3A4 induction or inhibition on ambrisentan pharmacokinetics. HPLC/MS/MS method for their analysis in human plasma was developed and validated. After solid phase extraction on a Strata-X (Phenomenex) SPE column, the analytes were separated on a Synergi MAX-RP (150×4.6 mm, 4 μm) (Phenomenex) analytical column using isocratic elution with acetonitrile/5mM ammonium acetate 41:59 (v/v) as a mobile phase. Detection was achieved using tandem mass spectrometry on a triple quadrupole mass spectrometer. Ambrisentan-d_3, midazolam-d_5 and $^{13}C_3$-1-hydroxymidazolam were used as internal standards. The calibration curves were linear between 2.03 and 150 ng/ml for MDL ($r^2 = 0.9993$) and OH-MDL ($r^2 = 0.9987$) and between 5.40 and 400 ng/ml for ABT ($r^2 = 0.9974$). Accuracy and precision variabilities of all analytes were within 15 %. Limit of quantification was 2.02 ng/ml for MDL and OH-MDL and 5.40 ng/ml for ABT using 0.1 ml plasma samples. The recoveries of all analytes were above 80 %. The method was applied to a midazolam pharmacokinetic study following oral dose of 4 mg and intravenous dose of 2 mg of MDL.

KEYWORDS: ambrisentan, midazolam, 1-hydroxymidazolam, HPLC/MS/MS, SPE

ABSTRAKT

Vývoj LC/MS/MS metody pro stanovení hladin ambrisentanu, midazolamu a jeho metabolitu v lidské plazmě

Diplomová práce

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Ambrisentan (ABT) je nová účinná látka pro léčbu pulmonální arteriální hypertenze, která je metabolizována cytochromem CYP3A4. Farmakokinetický profil midazolamu (MDL) a jeho hlavního metabolitu 1-hydroxymidazolamu (OH-MDL) se používá jako marker aktivity CYPu 3A4. Simultánní stanovení ambrisentanu, midazolamu a 1-hydroxymidazolamu je výhodné pro studium vlivu indukce či inhibice CYPu 3A4 na farmakokinetiku ambrisentanu. HPLC/MS/MS metoda pro jejich analýzu v lidské plazmě byla vyvinuta a validována. Po extrakci na pevné fázi na SPE koloně Strata-X (Phenomenex) byly analyty separovány na analytické koloně Synergi MAX-RP (150×4.6 mm, 4μm) (Phenomenex) s použitím izokratické eluce a acetonitrilu/5mM ammoniumacetátu 41:59 (v/v) jako mobilní fáze. Pro detekci byla využita tandemová hmotnostní spektrometrie na trojitém kvadrupólu. Ambrisentan-d_3, midazolam-d_5 a $^{13}C_3$-1-hydroxymidazolam byly použity jako vnitřní standardy. Kalibrační křivky byly lineární mezi 2,03 a 150 ng/ml pro MDL ($r^2 = 0,9993$) a OH-MDL ($r^2 = 0,9987$) a mezi 5,40 a 400 ng/ml pro ABT ($r^2 = 0,9974$). Variabilita přesnosti a opakovatelnosti všech analytů byla nižší než 15 %. Limit kvantifikace byl 2,02 ng/ml pro MDL a OH-MDL a 5,40 ng/ml pro ABT při použití 0,1 ml plasmy. Výtěžnost všech analytů byla přes 80 %. Metoda byla aplikována na studii farmakokinetiky midazolamu po orální dávce 4 mg a intravenózní dávce 2 mg.

KLÍČOVÁ SLOVA: ambrisentan, midazolam, 1-hydroxymidazolam, HPLC/MS/MS, SPE

ABBREVIATIONS

$^{13}C_3$-OH-MDL	1-hydroxymidazolam with ^{13}C carbons – internal standard
ABT	Ambrisentan
ABT-d$_3$	Deuterated ambrisentan – internal standard
ACN	Acetonitrile
CAL	Calibration (sample)
CID	Collision-induced dissociation
CYP3A4	Cytochrome P450 3A4
ESI	Electrospray ionization
ET$_A$	Endothelin-1 receptor A
ET$_B$	Endothelin-1 receptor B
FDA	Food and Drug Administration
HPLC	High performance liquid chromatography
HPLC/MS/MS	High performance liquid chromatography with tandem mass spectrometry
IS	Internal standard
LC	Liquid chromatography
LOQ	Lower limit of quantification
MDL	Midazolam
MDL-d$_5$	Deuterated midazolam – internal standard
MS	Mass spectrometry
NH$_4$Ac	Ammonium acetate
NYHA	New York Heart Association
OH-MDL	1-hydroxymidazolam
PAH	Pulmonary arterial hypertension
QC	Quality control
SJW	St. John's wort
SPE	Solid phase extraction

CONTENTS

1 INTRODUCTION

Ambrisentan is a new potent member of endothelin receptor antagonist family designated for treatment of pulmonary arterial hypertension. As CYP3A4 substrate, ambrisentan pharmacokinetics is predisposed to be influenced by cytochrome P450 inducers and inhibitors. It is necessary to determinate whether the effect of various potent CYP3A4 inducers or inhibitors on ambrisentan plasma concentrations is clinically important.

Midazolam is a benzodiazepine usually used for sedation. It is also a CYP3A4 substrate. Measuring of midazolam and its metabolite plasma concentrations can be used for determination of CYP3A4 activity or its inhibition or induction.

Clinical study called "Influence of CYP3A4-induction by St. John's wort (SJW) on the steady state pharmacokinetics of ambrisentan" is held at University Hospital of Heidelberg. Its main goal is evaluating the impact of St. John's wort, as known CYP3A4 inductor, on the ambrisentan pharmacokinetics using midazolam as CYP3A4 activity marker. For this purpose, a validated analytical method for simultaneous determination of ambrisentan, midazolam, and 1-hydroxymidazolam in human plasma is needed.

High performance liquid chromatography with tandem mass spectrometry was chosen as the most accurate and sensitive available method.

This thesis was worked out at Department of Clinical Pharmacology and Pharmacoepidemiology of University Hospital of Heidelberg, Germany.

2 THEORETICAL PART

2.1 Pulmonary arterial hypertension

2.1.1 Definition and classification

Pulmonary arterial hypertension (PAH) is a subgroup of pulmonary hypertension, which is defined as elevation of blood pressure in pulmonary arteries over 25 mmHg at rest or 30 mmHg with exercise. According to mechanism of development the pulmonary hypertension can be divided into precapillary, postcapillary and hyperkinetic. In order of seriousness pulmonary hypertension can be divided into mild (medium pressure in pulmonary artery 26-35 mmHg), moderate (36-45 mmHg) and severe (over 45 mmHg) form (1).

The incidence of primary PAH is estimated at 2-3 per million per year. Females suffer from it 2.3 times more frequently than males. From this number about 6 percent can be classified as familiar and the rest is idiopathic. But with combination with other disorders the incidence is rapidly rising: in patient with scleroderma it is 6-60 %, with systemic lupus erythematosus 4-14 %, with rheumatoid arthritis 20 % and about 2 % in case of portal hypertension. Among patient infected by HIV approximately 0.5 % suffers from pulmonary hypertension as well. Severe forms of chronic obstructive lung disease lead to PAH in more than 50 % of cases (1, 2, 3).

Since 1970´ there have been four World Symposiums on pulmonary hypertension endorsed by the World Health Organization, where international groups of experts were discussing the best classification of various kinds of pulmonary hypertension according to current scientific knowledge: Geneva 1973, Evian 1998, Venice 2003 and the last one so far took place in 2008 in Dana Point, California. The Venice classification was there slightly modified and 5 categories have been designated: Pulmonary arterial hypertension, Pulmonary hypertension owing to left heart disease, Pulmonary hypertension owing to lung diseases and/or hypoxia, Chronic thromboembolic pulmonary hypertension and Pulmonary hypertension with unclear multifactorial mechanisms (4).

Symptoms of PAH are often classified according to New York Heart Association (NYHA) Functional Classification (table 1).

Table 1. NYHA functional classification (5)

Class	Symptoms
I	No symptoms during ordinary physical activity.
II	Ordinary physical activity results in fatigue, dyspnea, or anginal pain.
III	Less than ordinary activity causes fatigue, dyspnea, or anginal pain.
IV	Symptoms of heart failure or the anginal syndrome are present even at rest.

2.1.2 Clinical symptoms

Symptoms of pulmonary hypertension are not specific for particular types of disease and they usually appear when the blood pressure of the patient exceeds twice the normal value.

The most frequent symptoms of PAH are dyspnea and fatigue, which occur in about 60 percent of patients. Their seriousness usually correlates well with seriousness of the disease. Almost 50 percent of patients also suffer from angina pectoris. In some cases syncope or presyncope may appear as consequence of lower cardiac output. Peripheral oedema as a result of right ventricular failure is often present in advanced pulmonary hypertension (1, 6).

2.1.3 Pathogenesis and etiology

The primary initiating factors of the rise in pressure in pulmonary arteries are heterogeneous and in most forms of pulmonary arterial hypertension they have not been fully clarified yet. However, it has been found out on the basis of lung biopsy and autopsy records that the main pathological mechanisms in the lung vessels are vasoconstriction and proliferation of endothelial and smooth muscle cells. It has been observed that inflammation and thrombosis play a role in the pathological process as well. Probably the most important element in development of these phenomena is imbalance in secretion of vascular effectors, vasoconstrictors and vasodilators, cell division promoters and inhibitors or prothrombotic and antithrombotic factors. Usually the blood pressure is controlled by regulated releasing of thromboxane A2, endothelin-1

and serotonin as vasoconstrictive and mitogenic factors on the one hand and nitric oxide, prostacyclin and vasoactive intestinal peptide as vasodilators and growth inhibitors on the other. In patients with PAH, increased levels of thromboxane A2, endothelin-1 and serotonin and decreased levels of prostacyclin, vasoactive intestinal peptide and lower occurrence of nitric oxide synthase against general population were observed. This imbalance leads to reduction of internal diameter of arteries, vascular remodelation and thrombocytes activation with increased risk of thrombosis (7, 8).

Besides idiopathic PAH, where the particular causes of development of increased blood pressure in pulmonary circulation are unknown, Dana Point classification states heritable, drug- and toxin-induced and form connected with other diseases (8, 9, 10).

2.1.4 Role of endothelin-1

Endothelin-1 is one of the substances, which are produced by vascular endothelial cells to control blood pressure. Smaller amounts can be also released from the heart, kidney, CNS and posterior pituitary. The secretion of endothelin-1 is stimulated by vasoactive hormones, growth factors, hypoxia, shear stress, lipoproteins, free radicals, endotoxin and cyclosporine and inhibited by nitric oxide, nitrovasodilatators, natriuretic peptides, heparin and prostaglandins.

The particular effect of endothelin-1 release depends on the bound to a receptor. There are two main endothelin receptors, ET_A and ET_B. Both of them belong to G-protein-connected family of receptors. ET_B-receptors are located in vascular smooth muscle cells and on endothelial cells, whereas ET_A-receptors are localized just in smooth muscle cells. Activation of both receptors on smooth muscle cells mediates vasoconstriction and proliferation of these cells. These effects of endothelin-1 release are prevailing, although activated ET_B-receptors on endothelial cells stimulate secretion of nitric oxide, which has vasodilating and antiproliferative effects. It follows that one of the ways in treating PAH is using of endothelin-1 antagonists, non-selective (like bosentan) or better ET_A-selective (ambrisentan), to reduce unwanted effects of endothelin-1.

Besides vascular effects endothelin-1 also influences the hearth (positive chronotropic and inotropic), kidneys (decrease renal plasma flow and glomerular filtration) and stimulates angiotenzin-converting enzyme and aldosterone release (11).

2.1.5 Treatment

➢ Calcium channel blockers

Calcium channel blockers (nifedipine, diltiazem and amlodipin) are suitable only for minority of patients with PAH (about 13 %), who have preserved acute vasoreactivity, defined as a reduction in mean pulmonary artery pressure by at least 10 mmHg to at most 40 mmHg, with normal cardiac output during exposition to an acute, short-acting vasodilator (12, 13).

➢ Prostanoids

Epoprostenol, synthetic prostacyclin, is a short-acting vasodilator and antiproliferative agent. It was the first drug approved by FDA for the treatment of PAH and for a long time it was the first-choice drug for those patients, who did not respond positive in the acute vasoreactivity test. Epoprostenol is very unstable and therefore it has to be administrated by continuous intravenous infusion, which brings a lot of risks. Treprostinil is a synthetic prostacyclin analogue with increased stability in comparison with epoprostenol, which enables subcutaneous administration using a minipump. Iloprost is a stable synthetic analogue of prostacyclin, which is administrated by inhalation. Beraprost is an orally active prostacyclin analogue used in Japan. It showed short-term improving of PAH symptoms, but no long-term benefits of its use have been proved (12, 13).

➢ Endothelin receptor antagonists

Bosentan is a dual antagonist of endothelin receptors (both ET_A and ET_B). It has vasodilating and antiproliferative effects, which brings improvement of the health state of patients with PAH. Bosentan is administrated orally. It is indicated for NYHA class III and IV (in Europe only class III) patients, who are not able to use calcium channel blockers. The most significant adverse effect of bosentan is the elevation of liver enzymes. At least three times higher levels of aminotransferase occur in 5-12 % of

patients treated with bosentan. It can be usually managed by reduction of dose or temporary interruption of treatment.

Sitaxentan was an orally active, ET_A selective antagonist. Theoretically, selective antagonists should be more effective in treating PAH than dual ET_A and ET_B antagonists. However several clinical trials have not confirmed this assumption. The incidence of liver enzymes elevation did not seem to be lower than that with bosentan. Sitaxentan was removed from the marked in 2010 (12, 13).

The latest selective endothelin-1 antagonist, ambrisentan, will be discussed further.

➢ Phosphodiesterase-5 inhibitors

Phosphodiesterase-5 is an enzyme that inactivates cyclic guanosine monophosphate (cGMP), which plays important role in mediation of vasodilatation induced by nitric oxide. Increased levels of phosphodiesterase-5 can be found in lung tissue of PAH patients, therefore use of its inhibitors can be successful way of treatment. Sildenafil was approved by FDA for the treatment of PAH in 2005 and tadalafil, with longer effect than sildenafil, in 2009. Both of them show good efficacy and are well tolerated (12, 13).

➢ Latest treatment possibilities

There are several new prospective approaches to PAH treatment. All of them are nowadays under clinical testing and more information is needed to prove their efficacy. Well-known drugs such as a member of the statin family, simvastatin, or imatinib, tyrosine kinase inhibitor used in chronic myelogenous leukaemia show certain potential in PAH treatment. Fasudil, a rho-kinase inhibitor, belongs among new promising substances (14, 15, 16).

➢ Lung or heart-lung transplantation

For those patients, who do not respond to any medication, lung or heart-lung transplantation or atrial septostomy are the last options. Lung transplantation is very complicated and risky operation. 70 – 80 % of transplanted patients survive first year, about 50 % five years and 25 % survive more than ten years (12, 13).

2.2 Ambrisentan

After first isolation of endothelin-1 from endothelial cell cultures by Yanagisawa in 1988 (17) and describing its function and role in vascular and blood-pressure-related diseases, a lot of attention was paid to research and development of possible endothelin receptors antagonists. Non-selective ET-receptor antagonists, which were the most successful in preclinical studies, contained the sulphonamide functional group, such as bosentan. On the basis of knowledge of different specific effects of ET_A and ET_B, searching for ET_A-selective antagonists began (18).

Riechers and co-workers were screening the compound library of the chemical company BASF in 1996 and they discovered that two diphenyl propionic acid derivates, which were originally developed as herbicides, have good affinity to the ET_A-receptor. These structures were then simplified and modified and the final molecules with enhanced binding potential and possible availability for oral administration were named LU 135252 and LU 208075. The first was than clinically tested as darusentan and the second as ambrisentan (18, 19).

Ambrisentan is produced and sold in the USA as Letairis by Gilead (Foster City, California) and in the EU as Volibris by Glaxo Group Ltd (Greenford, UK) in doses 5 mg and 10 mg.

2.2.1 Chemical properties

Ambrisentan is (+)-(2*S*)-2-[(4,6-dimethylpyrimidin-2-yl)oxy]-3-methoxy-3,3-diphenyl-propanoic acid (Fig. 1). Its molecular formula is $C_{22}H_{22}N_2O_4$ and molecular weight 378.42. It is a carboxylic acid with a pKa of 4.0. Ambrisentan is practically insoluble in water and good soluble in methanol (20). LogP of ambrisentan is 3.8 (21).

Fig. 1. Ambrisentan

2.2.2 Pharmacokinetics

The affinity of ambrisentan is >4000 times higher for ET_A receptor than for ET_B receptor (22). In comparison with bosentan, that is about 100 times more specific for ET_A than for ET_B, ambrisentan is much more selective (23). However, clinical studies have not shown any contribution of greater ET_A selectivity to improvement of clinical symptoms of PAH (24).

After an oral administration, ambrisentan is rapidly absorbed and its maximal concentration is reached after approximately 2 hours in healthy subjects and patients with PAH. Bioavailability is not affected by food (20). Mean C_{max} values were 539 and 1147 ng/ml for repeated 5 or 10 mg/daily doses. Trough plasma concentrations were 63 and 163 ng/ml, which is 15% of C_{max} (25). About 99 % of ambrisentan is bound to plasma proteins. The main way of elimination is via glucuronidation. As a substrate for P-glycoprotein, metabolism of ambrisentan can be affected by its strong inhibitors. The mean oral clearance of ambrisentan is 38 ml/min in healthy subjects and 19 ml/min in PAH patients. Terminal half-life after long-term dosing is about 15 hours. The main metabolite of ambrisentan occurring in plasma is 4-hydroxymethyl ambrisentan, which AUC is approx. 4 % relative to ambrisentan (20, 23).

2.2.3 Drug interactions

In vitro experiments revealed, that ambrisentan is metabolized in human probably by CYP3A4 and CYP2C19 (20). Therefore it was expected, that their strong inhibitors or strong inducers can influence plasma concentrations of ambrisentan. However, no

clinically relevant changes of ambrisentan concentrations have been observed during simultaneous administration with neither ketoconazole (CYP3A4 inhibitor) (26), omeprazole (CYP2C19 inhibitor) (27), nor rifampicin (CYP3A4 inducer) (28). No dose adjustments of ambrisentan are required in case of co-administration of these drugs.

On the other hand, cyclosporine A, as an inhibitor of CYP3A4 and P-glycoprotein, is able to significantly increase ambrisentan concentrations. In open-label, parallel treatment study, C_{max} of ambrisentan was 1.5 times and AUC twice higher in presence of cyclosporine A than in case of ambrisentan alone. Therefore a maximum ambrisentan dose of 5 mg is recommended if it is administrated together with cyclosporine A (29).

Patients with PAH are in higher risk of embolization, so they are often medicated with warfarin, which has a significant interaction potential. An open-label, cross-over study showed that no clinically relevant changes in C_{max} or AUC of ambrisentan and warfarin appear with their co-administration and any dose adjustments should not be required (30).

Combined therapy with ambrisentan and phosphodiesterase-5 inhibitors (sildenafil, tadalafil) is one of the ways of treating patients with severe PAH. Therefore their potential mutual influence was studied. No significant interactions were found between ambrisentan and sildenafil or ambrisentan and tadalafil (31, 32).

2.2.4 Clinical studies

Several studies have proved efficacy and safety of ambrisentan in the treatment of PAH.

A double blind, dose-ranging study included 64 patients with idiopathic PAH or PAH associated with collagen vascular disease, use of weight-loss agents or HIV infection. These patients received 1, 2.5, 5 or 10 mg of ambrisentan per day for 12 weeks, followed by 12 weeks administration of open-label ambrisentan. After 12 weeks of treatment, the 6-minute walk distance was significantly improved in all groups combined (+36.1 m). Six-minute walk test is very easy and repeatable and well correlates with severity of pulmonary hypertension (33). The improvements were similar regardless of dose, etiology of PAH and whether patients were in NYHA functional class II or III. Improvements were also observed in the Borg dyspnea index (−1.3), NYHA functional class (36.2% of patients improved by ≥1 functional class; 95%

CI, 25.7–46.7), subject's global assessment (+23.2%), mean pulmonary arterial pressure (–5.2 mm Hg) and cardiac index (+0.33 L/min/m^2). Reported adverse effects were mild and unrelated to dose. The most common were peripheral oedema (25.0%), nasal congestion (18.8%), upper respiratory tract infection (18.5%), headache (15.6%), flushing (12.5%), and nausea (12.5%) (34).

Similar results were provided by ARIES 1 and 2 (Ambrisentan in Pulmonary Hypertension, Randomized, Double-Blind, Placebo-Controlled, Multicenter, Efficacy Studies). There were 201 patients in ARIES 1, who received 5 or 10 mg of ambrisentan once daily or placebo. In ARIES 2, ambrisentan 2.5 or 5 mg or placebo were administrated to 192 patients. Otherwise the design of these studies was identical. After 12 weeks, the 6-minute walk distance was increased in ARIES 1 in the ambrisentan 5-mg group (+31 m; P = 0.008) and in the ambrisentan 10-mg group (+52 m; P = 0.001) and in ARIES 2 in the 2.5-mg group (+22 m; P = 0.022) and in the 5-mg group (+59 m; P ≤ 0.001). No elevations of aminotransferase higher than 3 times the normal value were observed. The mean frequency of adverse events in all ambrisentan groups compared to placebo group was 17%, vs. 11% for peripheral oedema, 6% vs. 2% for nasal congestion, 5% vs. 2% for palpitation, 4% vs. 1% for flushing, 3% vs. 1% for abdominal pain, 3% vs. 1% for nasopharyngitis, 3% vs. 0% for sinusitis and 4% vs. 2% for constipation. The efficacy and safety of ambrisentan were then confirmed in a long-term study, where the patients from ARIES 1 and 2 continued using ambrisentan for 2 years (35, 36).

2.2.5 Methods of ambrisentan determination

Ambrisentan is usually determined from plasma using liquid chromatography connected to tandem mass spectrometry (LC/MS/MS). Liquid-liquid extraction to acidified organic solvent (e.g. toluene) is used for elimination of plasma proteins (31, 32).

Spence at al. used BSF 127041 (2-(4,6-Dimethoxy-pyrimidin-2-yloxy)-3-phenoxy-3-phenyl-butyric acid) as internal standard. Ambrisentan was measured in positive ionization mode with transition masses of 379.2 and 125.2. The lower limit of quantification was 5 ng/ml (32).

2.3 Midazolam

Midazolam is a short-acting member of the benzodiazepines family. It was first synthesized in 1976 by Walser and Fryer and nowadays it is one of the most often used benzodiazepine. Intravenous application of midazolam is effective for conscious sedation during diagnostic or therapeutic procedures, induction of anaesthesia or sedation in intensive care units. In the form of tablets, midazolam is prescribed to insomniac patients. Less usual uses of midazolam are acute management of status epilepticus and other seizures, schizophrenia or sedation in the final stages of end-of-life care (37, 38).

Midazolam enhanced the effect of the main inhibitory neurotransmitter GABA on the $GABA_A$ receptors, which leads to neural inhibition. Pharmacological results of this inhibition are sedation, hypnosis, anxiolysis, anterograde amnesia and muscle relaxation (38).

2.3.1 Chemical properties

Midazolam is 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzo-diazepine (Fig. 2). It has molecular formula $C_{18}H_{13}ClFN_3$, which signifies molecular weight 325.78. Unlike other benzodiazepines, midazolam molecule contains an imidazole ring, which is responsible for its basicity and stability in aqueous solution. Because of a pKa 6.15, it is possible to prepare midazolam salts, which are soluble in water (39). LogP of midazolam is 2.5 (40).

The most important metabolite, 1-hydroxymidazolam, is 8-chloro-6-(2-fluorophenyl)-1-hydroxymethyl-4H-imidazo[1,5a][1,4]benzodiazepine (Fig. 3) with molecular formula $C_{18}H_{13}ClFN_3O$ and molecular weight 341.77.
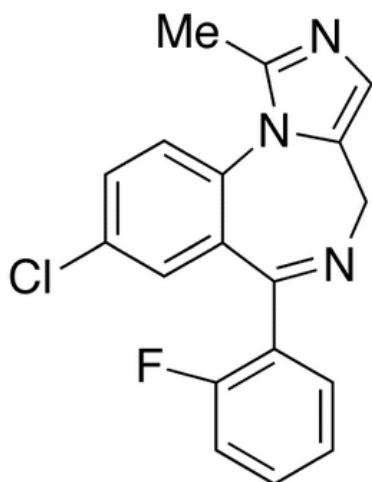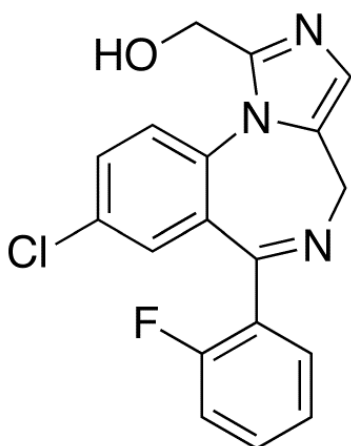
Fig. 2. Midazolam

Fig. 3. 1-hydroxymidazolam

2.3.2 Pharmacology

The absorption of midazolam after oral administration is rapid and complete, but due to high first pass effect, the bioavailability is about $30 - 50$ percent. Maximal plasma concentration is $70 - 120$ ng/ml for 15 mg dose and it is reached after $0.5 - 1.5$ hours. $96 - 98$ % of midazolam is bounded to the plasma proteins. The volume of distribution varies between 0.7 and 1.2 l/kg (41).

Midazolam is hydroxylated by CYP3A4 and CYP3A5 to 1-hydroxymidazolam and 4-hydroxymidazolam. 1-hydroxymidazolam is pharmacologically active, whereas the contribution of 4-hydroxymidazolam is unimportant. Plasma concentration of 1-

hydroxymidazolam can reach 30 – 50 % of the parent drug and it is responsible for about 34 % of pharmacological effect. Both metabolites are then conjugated by glucuronic acid and lose their effectiveness. Glucuronides are eliminated by kidneys. The plasma clearance of midazolam is 5.8 – 9.0 ml/kg per minute and the half-time of elimination ranging from 1.5 to 2.5 hours (41, 42).

2.3.3 Cytochrome P450 3A4 phenotyping

CYP3A4 has been determined as one of the most important sites of metabolism of many currently used drugs. There are many genetic mutations of CYP3A4 with different activity, which leads to clinically very important interindividual variability in drug clearence and drug – drug interactions. Therefore there are efforts to find some drugs that would be possible to use as probes to determine cytochrome activity. The most applied methods are the Erythromycin Breath Test and the Midazolam Oral Test. They are used for either CYP3A4 phenotyping, which is helpful for individual drug dosing, and evaluation of the inhibitory or inductive potential of risky drugs (43, 44).

In the midazolam test, the metabolic clearance to 1-hydroxymidazolam is usually used to assess intestinal and hepatic CYP3A4 activity. It was found that there is a good correlation between clearance of midazolam to 1-hydroxymidazolam and partial AUC of midazolam from 2 to 4 hours after administration. So there is no need to make a full pharmacokinetic and urinary excretion profile and just four plasma samples are enough for measuring of CYP3A4 activity (45).

2.3.4 Methods for midazolam determination in plasma

Most of the methods for midazolam determination in plasma samples are based on liquid – liquid extraction followed by high performance liquid chromatography with various detectors.

B. Lehmann and R. Boulieu used liquid – liquid extraction with diethyl ether and then HPLC with CN column and UV detection at 215 nm. Mean recoveries of this process were 87 % for midazolam and 85 % for 1-hydroxymidazolam. They achieved the quantification limit 2 ng/ml (for 1-ml sample) for both substances (46).

S. L. Eackhoudt et al. increased the sensitivity to LOQ 1 ng/ml for midazolam and 0.5 ng/ml for 1-hydroxymidazolam (for 1-ml sample) using C18 capillary HPLC column and UV detection at 240 and 300 nm (47).

Recently, mass spectrometry is the most frequent method of detection of midazolam and its metabolites. O. Quintela et al. published a LC/MS method, where they extracted the plasma sample to diethyl ether, separated it on a C_{18} chromatographic column and measured midazolam on a single-quadrupole mass spectrometer at m/z 326.1. Recovery of midazolam was 73 % and limit of quantification was 1 ng/ml for a 0.5-ml sample (48).

X. Xue et al. introduced a method, where they determined midazolam and 1-hydroxymidazolam by liquid chromatography connected with tandem mass spectrometry. They used tert-butyl methyl ether/dichloromethane (75:25) as an organic phase for liquid- liquid extraction, C18 column for HPLC and triple quadrupole set up on monitoring m/z 326.05 → 244.00 for midazolam and 342.02 → 168.01 for 1-hydroxymidazolam. They achieved the LOQ 2 ng/ml for both substances for a 0.1-ml sample (49).

T. Kim et al. used a mixed-mode cation exchange solid phase extraction as an extraction method, with recovery of midazolam over 80 %. With subsequent LC/MS/MS detection, they were able to work with the LOQ 0.25 ng/ml for midazolam for a 330-µl plasma sample (50).

J. Martens and P. Banditt focused to gas chromatography instead of HPLC. Using liquid-liquid extraction and mass spectrometry, they were able to quantify 0.6 ng/ml of midazolam and 0.3 ng/ml of its hydroxy metabolites in a 1-ml of human serum (51).

2.4 High performance liquid chromatography

High performance liquid chromatography (HPLC) is nowadays the most popular and the most progressive analytical method. It can be used either for separation, identification and quantification. High universality of applications is possible due to the wide range of analytical columns and detectors available.

A liquid chromatograph consists of a high pressure pump, which provides constant flow of mobile phase; injector for manual injection of samples or more usually automatic autosampler; chromatographic column, where the separation itself takes place; one or more detectors of various mechanisms of detection and computer for controlling and recording the analysis (Fig. 4) (52).
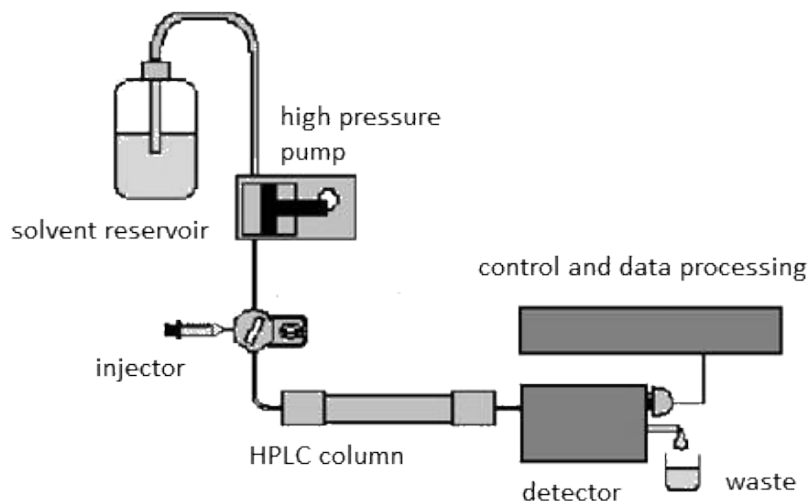

Fig. 4. Scheme of HPCL (53)

HPLC works on the principal of separation of substances on the basis of different interaction between stationary and mobile phases. There are several mechanisms of these interactions, which are typical for different columns:

- gel permeation chromatography – molecules of analytes are separated according to their size
- distribution chromatography – uses different solubility of analytes between two non-miscible liquids

- adsorption chromatography – uses different adsorption of molecules on the solid phase
- ion-exchange chromatography – uses different electrostatic interaction capability of ions (54)

2.4.1 Analytical columns

Chromatographic columns for HPLC are usually 5 – 250 mm long and their internal diameter moves from 2.1 to 4.6 mm. They are filled with a stationary phase, typically a porous matter or particles with the size of 1.7 – 5 µm. Stationary phases can be divided according to chemical structure and analytical properties into (55):

> **Phases on the basis of silica gel**

Phases on the basis of silica gel are the most often used and have wide spectrum of applications. Various functional groups with different polarity can be bound to the silica gel particles. Polar groups together with non-polar mobile phases are used in normal-phase liquid chromatography. On the other hand non-polar functional groups in connection with polar mobile phase are typical for reversed-phase chromatography (table 2). Silica gel stationary phases are stable just at temperature under 60 °C and in the pH range 2 – 7, but it is possible to increase stability by endcapping. These limitations are counterbalanced by relatively low price.

Table 2. Typical functional group for silica gel phases (56)

Polar	cyano	$(-O-Si(R_2)-(CH_2)_3-CN)$
	amino	$(-O-Si(R_2)-(CH2)_3-NH_2)$
	hydroxyl	$(-O-Si(R_2)-(CH_2)_3-O-CH_2-CH(OH)CH_2OH)$
	nitro	$(-O-Si(R_2)-(CH_2)_3-C_6H_4-NO_2)$
	polyethylene glycol	$(HO-(CH_2-CH_2-O)n-H)$
Non-polar	octadecylsilyl = C18	$(-O-Si(R_2)-(CH_2)_{17}-CH_3)$
	octylsilyl = C8	$(-O-Si(R_2)-(CH_2)_7-CH_3)$
	C6 and C4	
	phenyl	$(-O-Si(R_2)-(CH_2)_2-O-C_6H_5)$
	amide	$(-O-Si(CH_3)_2-(CH_3)_2-NHCO-C_{15}H_{31})$
	fluorated phases	$(-O-Si(R_2)-C_6F_{13})$

➢ **Phases on the basis of zirconium oxide**

The use of zirconium oxide instead of silica gel leads to enhanced stability and lifetime of the columns. They are able to work in the whole range of pH and at temperature to 200 °C, which enables faster analysis.

➢ **Polymeric phases**

Macroporous polymeric structures have large absorption capacity and high pH and temperature stability. On the other hand they suffer from lower pressure resistance and relatively lower effectiveness, which is moreover dependent on the organic component of the mobile phase.

➢ **Hybrid phases**

Hybrid columns connect the advantages of silica and polymeric phases. It means pH stability from 1 to 12, temperatures to 100 °C, better pressure resistance and separation effectiveness than pure polymeric columns.

➢ **Phases on the basis of porous graphitic carbon**

Porous graphitic carbon is often used for separation of structural isomers and chiral analytes. It is stable in wide range of pH and temperatures.

➢ **Monolithic phases**

Unlike other, monolithic columns are not filled with spherical particles but with one piece of porous material. Smaller pores (about 12 nm) enable very good separation effectiveness, whereas bigger pores (1 − 2 μm) decrease the backpressure. The main advantages of these columns are then the possibility of using high flow rates of mobile phase and even flow rate gradient, which leads to significantly shorter time of analysis comparing to common stationary phases.

2.4.2 Detectors in liquid chromatography

Ideal detectors for liquid chromatography should meet many demands. High sensitivity and selectivity are the most valuable characteristics. Besides that, good stability and reproducibility, fast response and wide linear dynamic range are also important.

Each method of detection is distinguished by various quality of these parameters (52, 56).

➤ UV–VIS detectors

UV–VIS spectrometric detectors are the most often used detectors in HPLC so far, thanks to simplicity and relatively low price. They work on the principle of interaction of the molecules with UV-VIS electromagnetic radiation, which can be measured as absorbance. Absorbance is directly proportional to the concentration of the analyte.

UV–VIS detectors have usually a changeable wavelength, often with the possibility to measure two different wavelengths at the same time. Modern UV-VIS detectors are equipped with DAD (diode array detector) / PDA (photo diode array), which enables to acquire wide absorbance spectrum for the whole time of analysis (56).

➤ Fluorescence detector

Fluorescence detector is able to detect an electromagnetic radiation, which is produced by some substances after irradiation by an external source. Only about 10 % of organic substances exhibit fluorescence themselves but much more molecules are able to fluoresce after modification with appropriate agents. The possibility of setting up an exact excitation and emission energy makes fluorescence detectors very selective and sensitive, with the low signal-noise ratio (56).

➤ Refractive index detector

This detector determines concentration of analyte by comparing refractive indexes of a solution of analyte and a pure eluent. It is very universal, because it does not depend on physical-chemical properties of determined substances. On the other hand, refractive index detectors have low sensitivity, are very dependent on the temperature and do not enable gradient elution. They are used especially for quantitative analysis of polymers (56).

➤ Detectors on the basis of aerosol

Detectors on the basis of aerosol is a common name for a group of a few detectors with similar principle. All of them are distinguished by their high universality. Unlike refractive index detectors, these detectors have better sensitivity as well as other analytical properties. In the first stage of the working process, the eluent is nebulized

by the stream of nitrogen and the solvent is evaporated by high temperature. The particles of analyte ate then detected using different methods.

Detectors on the basis of aerosol are used for determination of lipids, proteins, steroids, polymers, hydrocarbons, peptides and other substances, which do not have a chromophore in their structure (56).

➢ Electrochemical detectors

Electrochemical detectors work on the principal of measuring of various electrochemical properties of analytes, such as conductivity or current changes during oxidation and reduction (56).

➢ Mass spectrometer detectors

See next chapter.

2.5 Mass spectrometer

Mass spectrometer (MS) is the most selective and very sensitive detector, which can be connected to the HPLC. It offers wide range of applications. It is used in bio, environmental or food analytics, metabolomics, proteomics, lipidomics, drug analysis etc. A big advantage of MS lies in providing useful information about identity or structure of the analyte molecule.

Mass spectrometry consists of three main steps. After the sample enters the MS, it is ionized in the ion source, then ions are separated according to their mass-to-charge ratio (m/z) in the analyzer and finally they are detected in the ion detector (57).

2.5.1 Ionization techniques

➢ Electron ionization (EI)

Electron ionization is the oldest ionization technique. It is called hard ionization technique, because the molecule gets big surplus of energy, which leads to massive fragmentation of the molecular ion. Only this method enables comparing the mass spectra with a database and fast identification of molecules. On the other hand, the analyte must be sufficiently volatile and thermostable, therefore EI is often used in connection with gas spectrometry (57, 58).

➢ Electrospray ionization (ESI)

Electrospray ionization is the most often used method in HPLC/MS and it was also used for purposes of this thesis. It was developed originally for the analysis of biological macromolecules by John Bennett Fenn, who was awarded by the Nobel Prize in 2002. Electrospray ionization works under atmospheric pressure and it allows analysis of both small and macromolecules. Mobile phase carrying the dissolved analyte is pushed through a charged capillary and nebulized by nitrogen into a fine aerosol. The emerged drops carry a large number of charges. While the solvent is evaporating, the drops are getting smaller and the density of charges is rising. When their Rayleight limit is exceeded, so-called Coulomb fission follows and the drops divide into even smaller particles. Repeating of this process eventually leads to releasing of molecular ions. The process is shown in Fig. 5. Emerged ions are usually protonated $[M + H]^+$

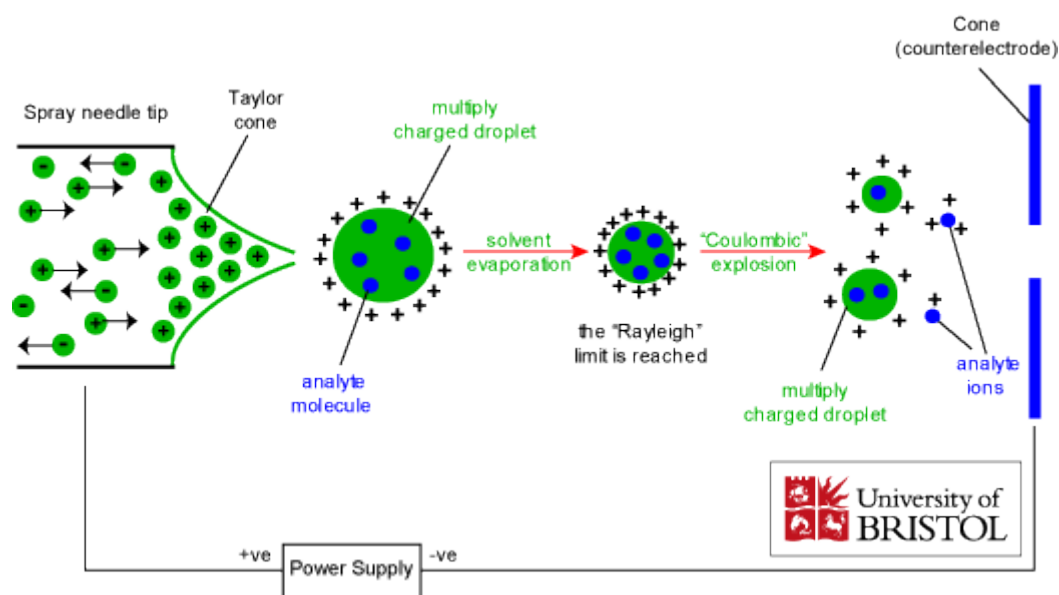or deprotonated [M + H]⁻ molecules, or they can be formed with another cation such as sodium ion (57, 58, 59).

Fig. 5. Principle of ESI (59)

➢ Atmospheric-pressure chemical ionization (APCI)

Atmospheric-pressure chemical ionization is together with electrospray ionization standard ion source used for HPLC/MS. High voltage is put on a discharge needle and a corona discharge appears. Heated mobile phase, nebulized by nitrogen, is ionized by this discharge, ionized molecules of mobile phase subsequently ionize molecules of the analyte by ion-molecular reaction and these ions are then focused to the analyzer (57, 58).

➢ Atmospheric-pressure photoionization (APPI)

Atmospheric-pressure photoionization works on a similar principle as APCI. Only the corona needle is replaced with UV lamp or other photon source. The energy of UV radiation is just able to ionize organic molecules of the analyte, but it is insufficient to ionize molecules of the mobile phase (57, 58).

➢ Matrix-assisted laser desorption/ionization (MALDI)

This technique is (together with ESI) suitable for analysis of biopolymers and synthetic polymers. The analyte is dissolved in an appropriate matrix and the sample is exposed to a laser beam. The matrix absorbs the energy and transfers it to the analyte that gets

ionized. Analyte ions are then desorbed from the matrix and focused to the analyzer. MALDI is usually connected with the time-of-flight mass analyzer (57, 58).

> ➢ **Ambient ionization techniques**

Ambient ionization techniques allow the formation of analyte ions directly from the sample without its preparation or separation. The most often used is desorption electrospray ionization (DESI), where the pure mobile phase is nebulized and charged and this stream causes the sample ionisation as well as desorption. Direct analysis in real time (DART) works similarly, only excited gas, He or N_2, is used instead of liquid mobile phase. Other methods work on a similar principal such as desorption atmospheric pressure photoionization (DAPPI) or matrix-assisted laser desorption electrospray ionization (MALDESI) (58, 60).

2.5.2 Mass analyzers

> ➢ **Sector instruments**

In a sector field mass analyzer, an electric and/or magnetic field is applied. The field forces the ions to change the direction of their movement, but due to centrifugal forces it influences heavier and less charged ions more than lighter and more charged ones. Therefore ions fly on different paths according their mass-to-charge ratio and can be detected separately (61).

> ➢ **Quadrupole (Q)**

The quadrupole consists of 4 parallel metal rods, among which is an oscillating electric field. In a certain moment, only oscillations of ions with a narrow range of m/z are stable and these ions are able to pass through to the detector. By changing the potentials on the rods it is possible to stabilize the ions with different m/z and thus the whole spectrum can be scanned continuously or in discrete steps (57, 61).

> ➢ **Triple quadrupole (QqQ)**

Three quadrupoles can be connected consecutively to increase the selectivity of analysis as shown in Fig. 6. First quadrupole (Q_1) works as a mass filter. Released ions are fragmented by collision with an inert gas (Ar, He, N_2) in the second quadrupole (q_2), so-

called collision cell. This process is called collision-induced dissociation (CID). The fragments are then analysed in the third quadrupole (Q_3) (57, 61).
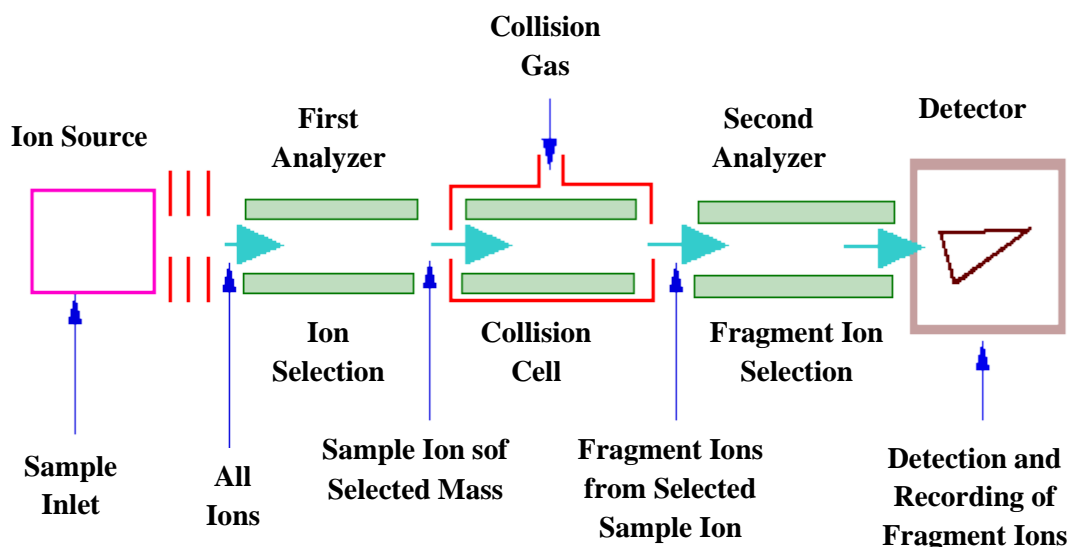

Fig. 6. Triple quadrupole (62)

Triple quadrupole can work in four main modes (63):

- **Product ion scan:** An ion of a definite mass is selected by Q1 and fragmented in q2. Q3 then scan the entire m/z range, which provides data about the size of the fragments. The structure of original ion can be deduced from this information.
- **Precursor ion scan**: Certain product ion is selected in Q3. Q1 is set to the scanning of precursor masses, detecting only ions that contain the fragment defined in Q3. This method is selective for molecules with a particular functional group.
- **Neutral loss scan**: Q1 and Q3 are scanned both, but with a constant m/z difference between them. Ions that loose a definite neutral fragment (e.g. H2O, NH3) can be selectively recognized.
- **Selected reaction monitoring (SRM)**: Both Q1 and Q3 are set to release a selected mass, so only certain product from certain precursor is detected. This mode is often used for increasing the selectivity of the analysis.

➢ Ion trap (IT)

Ion trap works on a similar principle as quadrupole. Ions are trapped among ring and two endcap electrodes and can be selectively ejected to the detector according to their m/z. In the linear ion trap (LIT), the ions are trapped in two-dimensional field, which allows more effective ions accumulation and the detector reaches higher sensitivity (57, 61).

➢ Time-of-flight analyzer (TOF)

Ions with the same kinetic energy but different m/z move possess a different speed. In the time-of-flight analyzer, ions are accelerated by an electric pulse and then it is measured the time until they reach the detector. The detectable m/z range is very wide and scanning is very fast. The resolution of TOF analyzer can be increased by using so-called reflectron that equalize the kinetic energy of ions and doubles the ion flight path (57, 61).

➢ Fourier transform ion cyclotron resonance (FT-ICR)

Strong magnetic field is applied to ions of the analyte in an electric/magnetic ion trap. Ions start to move at a cycloidal trajectory with specific cyclotron frequency according to their mass-to-charge ratio. Cyclotron frequencies are then converted to the m/z scale by Fourier transform. FT-ICR achieves very high sensitivity and resolution, but on the other hand it is very expensive (57, 61).

➢ Orbitrap

Ions are trapped in an electrostatic field, orbit around the central electrode and oscillate along the electrode's long axis. The frequency of oscillation depends on m/z of ions and can be detected and converted to mass spectra by Fourier transform. Orbitrap can achieve almost the same sensitivity and resolution as FT-ICR, but the operating costs are much lower (57, 61).

2.6 Solid phase extraction (SPE)

Solid phase extraction is one of the most often used techniques for isolation of the analyte of interest from biological or environmental matrix. The analyte is dissolved in a liquid (mobile phase) and forced through a solid stationary phase. Substances of the sample have different affinity to the stationary phase according to their chemical or physical properties. Thereby the analyte can be selectively retained on the SPE column or disc, whereas other components go through with the mobile phase. On the end, the substance of interest is eluted from the solid phase using solvent of another polarity.

Main advantages of SPE over other separation methods (like liquid-liquid extraction) are higher recoveries, increased selectivity thanks to variety of stationary phases, less solvents use and relatively easy automation.

SPE stationary phases are manufactured as 1-60ml syringe-shaped tubes, 96-well plates and 47 or 90mm discs with various amounts of the sorbent. Stationary phases contain material based on silica, alumina or others, on which different functional groups are bounded. SPE is used mainly in reversed phase, normal phase, ion exchange or adsorption modes (likewise HPLC).

Solid phase extraction process in reversed phase mode generally consists of several steps. On the beginning, the SPE sorbent must be activated by water-miscible organic solvent which is usually methanol. It wets the surface of the sorbent and penetrates bonded phases. The SPE tube or disc is then conditioned by the same liquid (usually buffer) (Fig. 7, Solvent A) that is used for dissolving of the sample and is ready for sample loading (Fig. 7, Step 2). Analysed sample is diluted and buffered if it is needed and added on the solid phase (Fig. 7, Step 3). In the next step, a solution containing less organic solvent than the final eluent (Fig. 7, Solvent B) is applied to wash off all unretained material from the sorbent (Fig. 7, Step 4). In the end, a solvent containing higher amount of organic solvent (usually methanol, acetonitrile) (Fig. 7, Solvent C) is used for washing out the retained analyte (Fig. 7, Step 5). Positive or more often negative pressure is usually used to force the liquid through the solid phase (64, 65).
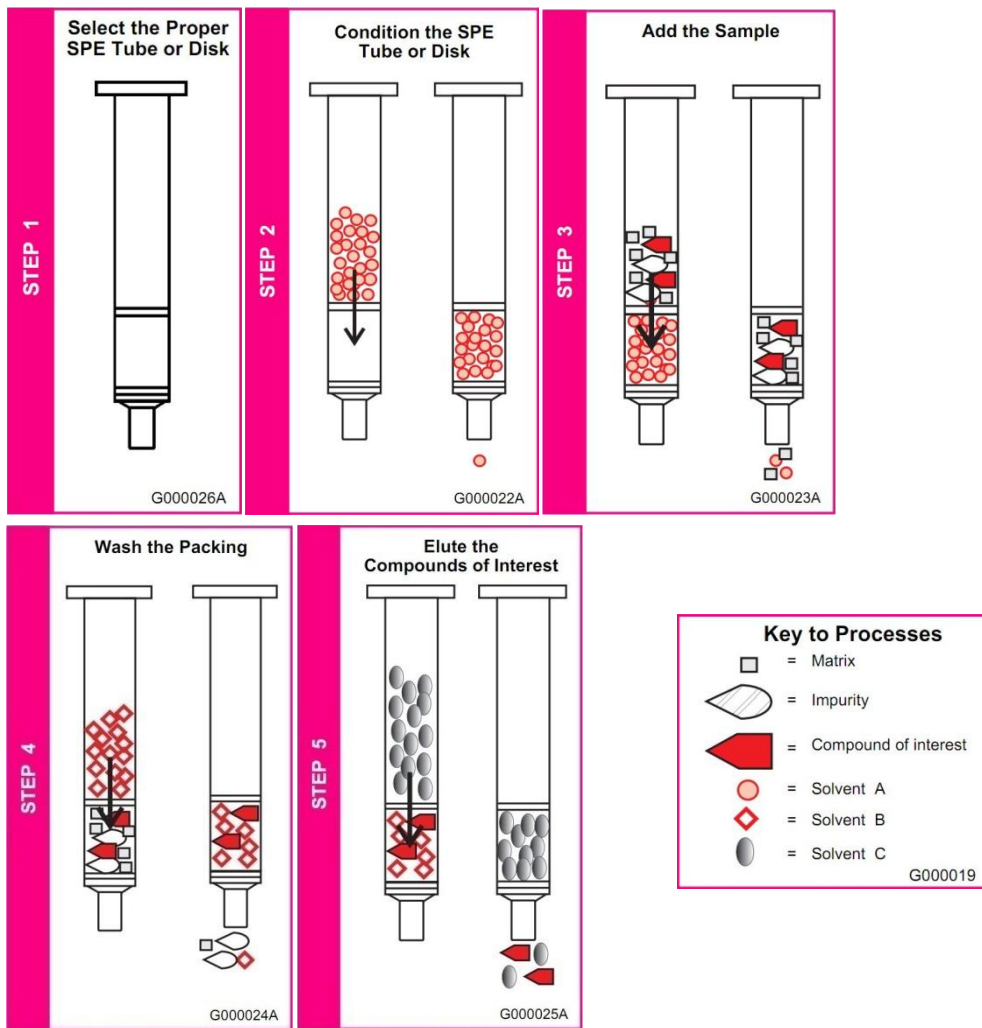
Figure 7. SPE steps (64)

3 EXPERIMENTAL PART

3.1 Chromatographic system, laboratory equipment, chemicals

> **Chromatographic system**

Liquid chromatography system Thermo Surveyor HPLC (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

Mass spectrometer Finnigan TSQ 7000 LC/MS/MS (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

HPLC column Phenomenex Synergi Max-RP 150×4.6mm 4μm (Phenomenex, Torrance, California, USA)

HPLC column Phenomenex Synergi Polar-RP 150×4.6mm 4μm (Phenomenex, Torrance, California, USA)

Precolumn SecurityGuard Column C12 4×2.0mm (Phenomenex, Torrance, California, USA)

Precolumn SecurityGuard Column Polar-RP 4×2.0mm (Phenomenex, Torrance, California, USA)

Software Xcalibur Home Page 1.3 (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

Software TSQ – Tune 1.1 (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

Software LCquan 1.3 (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

> **Other laboratory equipment**

Pipettes Eppendorf (Eppendorf, Hamburg, Germany)

Vortex Julabo paramix II (Julabo Labortechnik, Seelbach, Germany)

Evaporator Zymark TurboVap LV (Sotax, Allschwil, Switzerland)

Vacuum manifold for SPE extraction (Macherey – Nagel, Düren, Germany)

Ultrasonic bath Bandelin Sonorex RK 102 (Bandelin electronic, Berlin, Germany)

Analytical scales Mettler AT261 DeltaRange (Mettler-Toledo, Greifensee, Switzerland)

Analytical scales Sartorius BP210S (Sartorius AG, Goettingen, Germany)

Millipore TKA-LAB (0.055µS)

Laboratory glass

SPE columns Strata-X 33u Polymeric Reversed Phase 30mg/1ml (Phenomenex, Torrance, California, USA)

➢ **Chemicals**

$^{13}C_3$-1-hydroxy-midazolam (Toronto Research Chemicals, North York, Canada)

1-hydroxy-midazolam (Roche, Basel, Switzerland)

Acetonitrile LC-MS grade (Carl Roth, Karlsruhe, Germany)

Ambrisentan (Gilead Science, Foster City, California, USA)

Ambrisentan-d_3 (Toronto Research Chemicals, North York, Canada)

Hydrochloric acid 0.1 TritriPUR (Merck, Darmstadt, Germany)

Methanol for spectroscopy (Merck, Darmstadt, Germany)

Midazolam (Roche, Basel, Switzerland)

Midazolam-d_5 (Toronto Research Chemicals, North York, Canada)

3.2 Preparation of ultrapure water, stock solutions and mobile phase

➢ Ultrapure water

Ultrapure water was prepared from deionized water using Millipore system. All water mentioned below is considered to be ultrapure water.

➢ Stock solutions

Ambrisentan stock solution 500 µg/ml

5.07 mg of ambrisentan was dissolved in 5 ml of acetonitrile (ACN) and diluted to 10 ml by water.

Ambrisentan-d$_3$ stock solution 200 µg/ml

1 mg of ambrisentan-d$_3$ was dissolved in 2.5 ml of ACN and diluted to 5 ml by water.

Midazolam stock solution 500 µg/ml

5.21 mg of midazolam was dissolved in 5 ml of ACN and diluted to 10 ml by water.

Midazolam stock solution 500 µg/ml for QC

4.97 mg of midazolam was dissolved in 5 ml of ACN and diluted to 10 ml by water.

Midazolam-d$_5$ stock solution 200 µg/ml

1 mg of midazolam-d$_5$ was dissolved in 2.5 ml of ACN and diluted to 5 ml by water.

1-OH-midazolam stock solution 500 µg/ml

4.87 mg of 1-OH-midazolam was dissolved in 5 ml of ACN and diluted to 10 ml by water.

1-OH-midazolam stock solution 500 µg/ml for QC

4.78 mg of 1-OH-midazolam was dissolved in 5 ml of ACN and diluted to 10 ml by water.

^{13}C$_3$-1-OH-midazolam stock solution 200 µg/ml

1 mg of ^{13}C$_3$-1-OH-midazolam was dissolved in 2.5 ml of ACN and diluted to 5 ml by water.

➢ **Mobile phase**

Organic and inorganic component of the mobile phase were prepared separately and mixed in the HPLC pump in an appropriate ratio.

The organic component of the mobile phase was pure acetonitrile without any adjustments.

Inorganic liquid was 5mM ammonium acetate buffer with addition of 0.1 % of acetic acid and 0.1 % of acetonitrile. 19.27 g of NH_4Ac was dissolved in 50 ml of water to make 5M ammonium acetate. 1 ml of 5M ammonium acetate was then mixed with 1 ml of acetic acid and 1 ml of ACN in a 1000ml flask and the flask was filled up with water. The pH of the buffer was 4.5.

3.3 Preparation of calibration and quality control samples and internal standard solution

Calibration and quality control solutions were prepared by mixing and diluting stock solutions of analytes and internal standards. All dilutions were made by ACN/water 1:1. Calibration range was set to 0.5–200 ng/ml for MDL and OH-MDL and 1–400 ng/ml for ABT.

➢ **Calibration solutions**

Calibration solution G (CAL G) was prepared by mixing 33.2 µl of ABT 500 µg/ml, 12.8 µl of MDL 500 µg/ml and 12.3 µl of OH-MDL 500 µg/ml and diluting the solution to 10 ml. All other calibration solutions were made by dilution of CAL G as shown in table 3 together with resulting concentrations. Concentrations of calibration samples after mixing with plasma are summarised in table 4.

Table 3. Dilution of CAL G to other calibration solutions [µl] and resulting concentrations [ng/ml]

	CAL G	ACN/water 1:1	ABT	MDL	OH-MDL
CAL G	4000	0	1600.00	600.00	600.00
CAL F	2700	1300	1080.00	405.00	405.00
CAL E	1300	2700	520.00	195.00	195.00
CAL D	470	3530	188.00	72.00	72.00
CAL C	170	3830	68.00	25.52	25.52
CAL B	54	3946	21.60	8.12	8.12
CAL A	13	3987	5.20	1.96	1.96

Table 4. Concentrations of calibration samples in plasma

	ABT	MDL	OH-MDL
CAL G	400.00	150.00	150.00
CAL F	270.00	101.25	101.25
CAL E	130.00	48.75	48.75
CAL D	47.00	18.00	18.00
CAL C	17.00	6.38	6.38
CAL B	5.40	2.03	2.03
CAL A	1.30	0.49	0.49

➢ Quality control solutions

Quality control (QC) samples were made in three concentrations over whole calibration range. 30.0 µl of ABT 500 µg/ml, 12.8 µl of MDL 500 µg/ml for QC and 12.3 µl of OH-MDL 500 µg/ml for QC were diluted to 10 ml. Resulting QC stock solution had concentrations of 1446 ng/ml for ABT, 572 ng/ml for MDL and 589 ng/ml for OH-MDL, respectively. QC solutions were prepared from this stock solution. Diluting volumes, concentrations of QC solutions and resulting concentrations of QC samples in plasma are shown in table 5. Calculated concentrations were slightly adjusted on the basis of multiple results of QC samples analysis. Calibration solution CAL B was used for preparing LOQ samples.

Table 5. Quality control solutions

		LOQ	QC A	QC B	QC C
QC stock sol. [µl]			260	1300	2700
ACN/water [µl]			3740	2700	1300
Conc. of solution	**ABT**	21.60	93.96	469.88	975.92
[ng/ml]	**MDL**	8.12	37.20	186.00	386.36
	OH-MDL	8.12	38.28	191.40	397.52
Conc. in plasma	**ABT**	5.40	23.49	117.47	243.98
[ng/ml]	**MDL**	2.03	9.30	46.50	96.59
	OH-MDL	2.03	9.57	47.85	99.38
Adjusted conc.	**ABT**	5.40	23.49	100.50	222.00
in plasma	**MDL**	2.03	9.30	40.00	91.53
[ng/ml]	**OH-MDL**	2.03	9.57	39.65	92.02

➢ Internal standard solution

Ambrisentan-d_3, midazolam-d_5 and $^{13}C_3$-1-hydroxymidazolam were used as internal standards for ABT, MDL and OH-MDL, respectively. Structural formulas of their molecules are stated in Fig. 8-10. Internal standard solution was prepared by diluting 10 µl of ABT-d_3 200 µg/ml, 20 µl of MDL-d_5 200 µg/ml and 20 of $^{13}C_3$-OH-MDL 200 µg/ml in 10 ml of ACN/water 1:1. Concentration of the solution was 200 ng/ml

for ABT and 400 ng/ml for MDL and OH-MDL, concentrations of internal standards in plasma sample were then 50 ng/ml and 200 ng/ml.
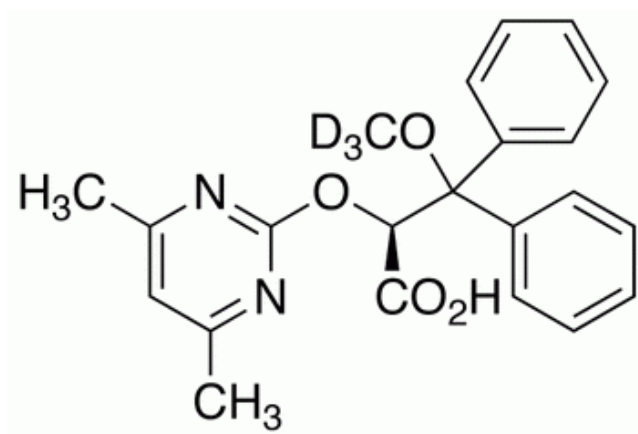
Fig. 8. Chemical structure of ABT-d$_3$
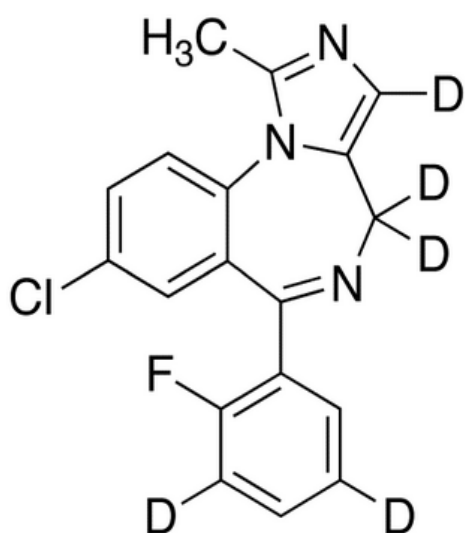
Fig. 9. Chemical structure of MDL-d$_5$
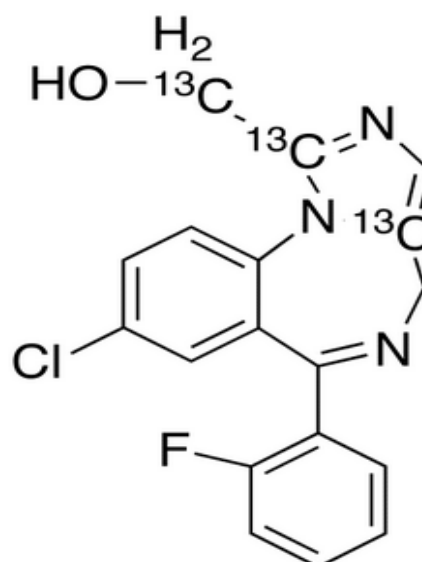
Fig. 10. Chemical structure of $^{13}C_3$-OH-MDL

> ➢ **Calibration and quality control samples**

100 µl of human plasma was transferred into a glass tube and spiked with 25 µl of calibration or QC solution and 25 µl of IS solution. 100 µl of 0.1M HCl was added and mixture was vortexed properly. Samples were processed by SPE (see further) and eluted by 1.5 ml of methanol. Methanol was evaporated under the stream of nitrogen. Samples were reconstituted by 200 µl of HPLC mobile phase (ACN/buffer 41:59) and injected into HPLC.

➢ Solid phase extraction

SPE columns Strata-X 33µm Polymeric Reversed Phase (Phenomenex, USA) with 30 mg of sorbent and volume of 1 ml were used for solid phase extraction (SPE). Vacuum pressure about 5 mmHg was applied. Columns ware activated by 1 ml of methanol and conditioned by 1 ml of 0.1M HCl. Then 250 µl of spiked acidified plasma was introduced on the column. Plasma proteins were washed out of the column using 1 ml of 5% methanol in water and 1 ml of 30% methanol in water. Analyzed compounds were eluted by 1.5 ml of pure methanol into the glass tubes.

3.4 HPCL/MS/MS parameters

➢ HPLC

- Mobile phase: 41 % of ACN + 59 % of 5mM NH_4Ac with 0.1 % of acetic acid and 0.1 % of ACN
- Column: Phenomenex Synergi Max-RP
- Analysis time: 5.50 min
- Mobile phase flow: 0.5 ml/min
- Injection volume: 70.0 µl
- Column temperature: 40.0 °C

➢ Mass spectrometer

- Scan type: SRM table
- Polarity: positive
- m/z ratios: see table 6
- 2 segments – 0.00-4.00 min and 4.01-5.50
- Source CID collision energy: 1st segment – 10.0 eV, 2nd segment – 1.0 eV
- Source type: ESI
- ESI capillary temperature: 350.0 °C
- ESI spray voltage: 4.5 kV

Table 6. Mass-to-charge ratios and collision energies (SRM table)

	Precursor m/z	Fragment m/z	Collision energy [eV]
ABT	379.3	303.1	18.0
ABT-d$_3$	382.3	125.0	30.0
MDL	326.0	291.0	45.0
MDL-d$_5$	331.3	296.2	45.0
OH-MDL	342.0	203.0	44.0
^{13}C$_3$-OH-MDL	345.3	206.2	44.0

3.5 Validation parameters

➢ Calibration curve

The calibration curve method was used for quantification of compounds of interest. The influence of possible errors caused by the sample preparation and extraction or instability of HPLC injection volume was eliminated by using internal standards. Each calibration concentration was prepared twice. The equation of calibration curve was acquired from the dependence of peak area on the concentration using the method of the least squares.

➢ Accuracy

Accuracy is defined as a difference between true (calculated) and practically measured concentration of the sample. Accuracy of the method was determined in percents as a mean of absolute values of accuracies of QC and LOQ samples (n=18).

$$R(\%) = \frac{x - y}{x} * 100$$

(x = calculated concentration of the sample, y = measured concentration of the sample)

➢ Precision

Precision expresses a variation in measured concentration of separately prepared samples with the same concentration. Precision was calculated as relative standard deviation (RSD) of QC and LOQ samples (n=18).

$$SD = \sqrt{\frac{\sum(x_i - AVG)^2}{n - 1}}$$

$$RDS = \frac{SD}{AVG} * 100$$

(SD = standard deviation, x_i = concentration of sample i, AVG = average concentration of all samples, n = number of samples)

➢ Recovery

Recovery represents the loss of analytes during separation procedure. Recovery was measured for three different concentrations. Plasma samples were spiked with analyte,

processed by SPE and consequently spiked with internal standard. Ratio between peak area of analyte and IS was compared to the same ratio in control sample, which was prepared by spiking mobile phase with analyte and IS in corresponding concentration.

$$R(\%) = \frac{\dfrac{A_{an-sample}}{A_{IS-sample}}}{\dfrac{A_{an-100\%}}{A_{IS-100\%}}} * 100$$

($A_{an-sample}$ = *peak area of analyte in sample processed by SPE*, $A_{IS-sample}$ = *peak area of IS in sample processed by SPE*, $A_{an-100\%}$ = *peak area of analyte in spiked mobile phase*, $A_{an-100\%}$ = *peak area of IS in spiked mobile phase*)

➢ **Freeze and thaw stability**

An aliquot of the sample of known concentration was measured. Rest of the sample was put into a freezer maintaining the temperature around − 23°C and thawed again after four hour. This procedure was repeated three times, then the sample was measured and the results were compared with the values obtained before freezing.

3.6 Application of the method on pharmacokinetics of midazolam and 1-hydroxymidazolam

The validated analytical method was used for determination of MDL and OH-MDL in plasma samples from healthy volunteers from a clinical study. Unfortunately any clinical samples with ambrisentan were not yet available.

➢ Study population

12 healthy men and women participated on the original study. Plasma samples from 6 of them were processed by the new analytical method.

All participants were of the age between 18 and 50 years, mentally and physically healthy, especially with normal renal function and normal liver blood tests. The main exclusion criteria of the study were any drug treatment, smoking, excessive alcohol drinking, pregnancy and lactation.

➢ Study design, medication and blood sampling

Original study lasted 29 days. Only samples from day 1 were now used, when only midazolam was administrated. Midazolam (Dormicum®) was administrated orally – 4 mg and intravenously – 2 mg. 2.7 ml of blood ware taken 0, 0.16, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5 hours after oral administration of 4 mg midazolam and 0, 0.25, 0.5, 0.67, 0.83, 1, 1.25, 1.5, 2, 3, 4, 6, 8 hours after intravenous administration of 2 mg midazolam. An anticoagulant was added, blood was centrifuged and separated plasma was frozen.

➢ Sample preparation

Plasma samples were thawed under the laboratory temperature. 100 µl of plasma was transferred into a glass tube and spiked with 25 µl of IS solution. 100 µl of 0.1M HCl was added and mixture was vortexed properly. Samples were introduced into SPE tubes, which were first activated and preconditioned by 1 ml of methanol and 1 ml of 0.1M HCl. SPE tubes were washed by 1 ml of 5% methanol in water and 1 ml of 30% methanol in water. Analytes were finally eluted by 1.5 ml of pure methanol. Methanol was evaporated under the stream of nitrogen and samples were reconstituted by 200 µl of HPLC mobile phase (ACN/buffer 41:59) and injected into HPLC/MS/MS system.

4 AIMS OF THE DIPLOMA THESIS

Main aims of this diploma thesis are:

1. Development of HPLC/MS/MS method for simultaneous determination of ambrisentan, midazolam and 1-hydroxymidazolam in human plasma.

2. Validation of this method.

3. Application of the method on real clinical plasma samples.

5 RESULTS

5.1 Extraction, separation and detection conditions

5.1.1 Solid phase extraction

It was found out, that the biggest loss of analytes during SPE was due to their breakthrough during loading of spiked plasma to the SPE column. The retention of compounds can be influenced by changing the pH of the sample, its volume or dilution.

The best recoveries were achieved by setting the pH to value about 4 using 0.1M hydrochloric acid (Fig. 11). Using higher pH, rapid decrease of ABT recovery was observed. Various dilutions of the plasma by 0.1M HCl were applied (Fig. 12). Ratio 1:1 appeared to be optimal.
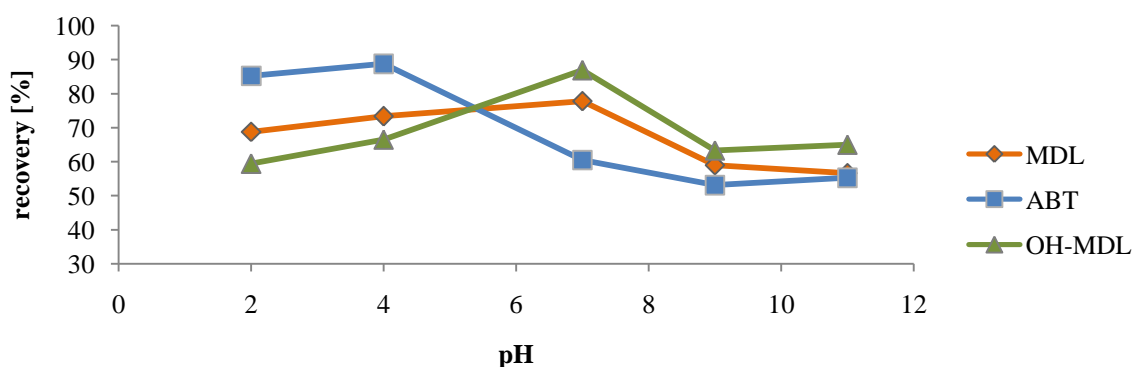
Fig. 11. Recoveries of analytes under various pHs
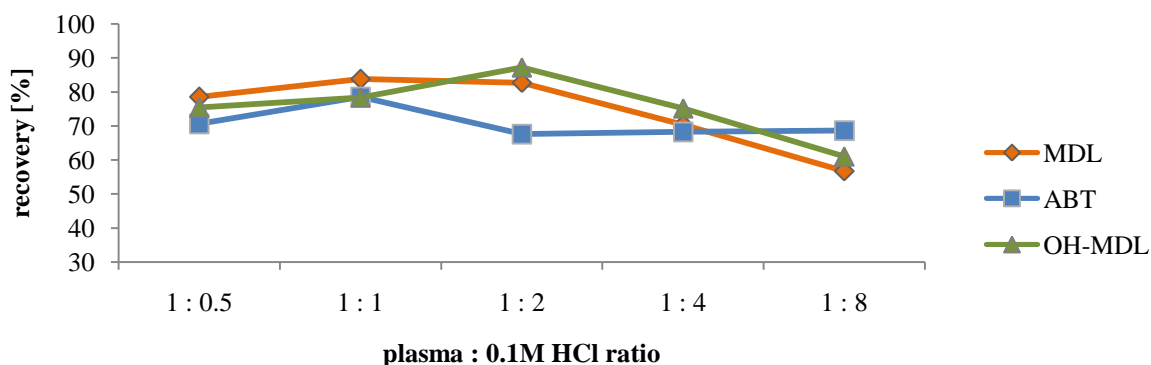
Fig. 12. Recoveries of analytes under various sample dilution

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5.1.2 HPLC mobile phase

The best composition of mobile phase, i.e. the ratio between acetonitrile and 5mM NH$_4$Ac with 0.1 % of acetic acid and 0.1 % of ACN was determined (table 7). Using 41 % of ACN in the mobile phase has given the shortest retention times of compounds, while all peaks were still well separated. Chromatogram under the final conditions is shown in Fig. 13.

Table 7. Retention times [min]

% of ACN	39	41	42	45	50
OH-MDL	2.97	2.53	2.44	2.00	1.65
MDL	4.02	3.45	3.30	2.77	2.29
ABT	5.58	4.63	4.15	3.27	2.37
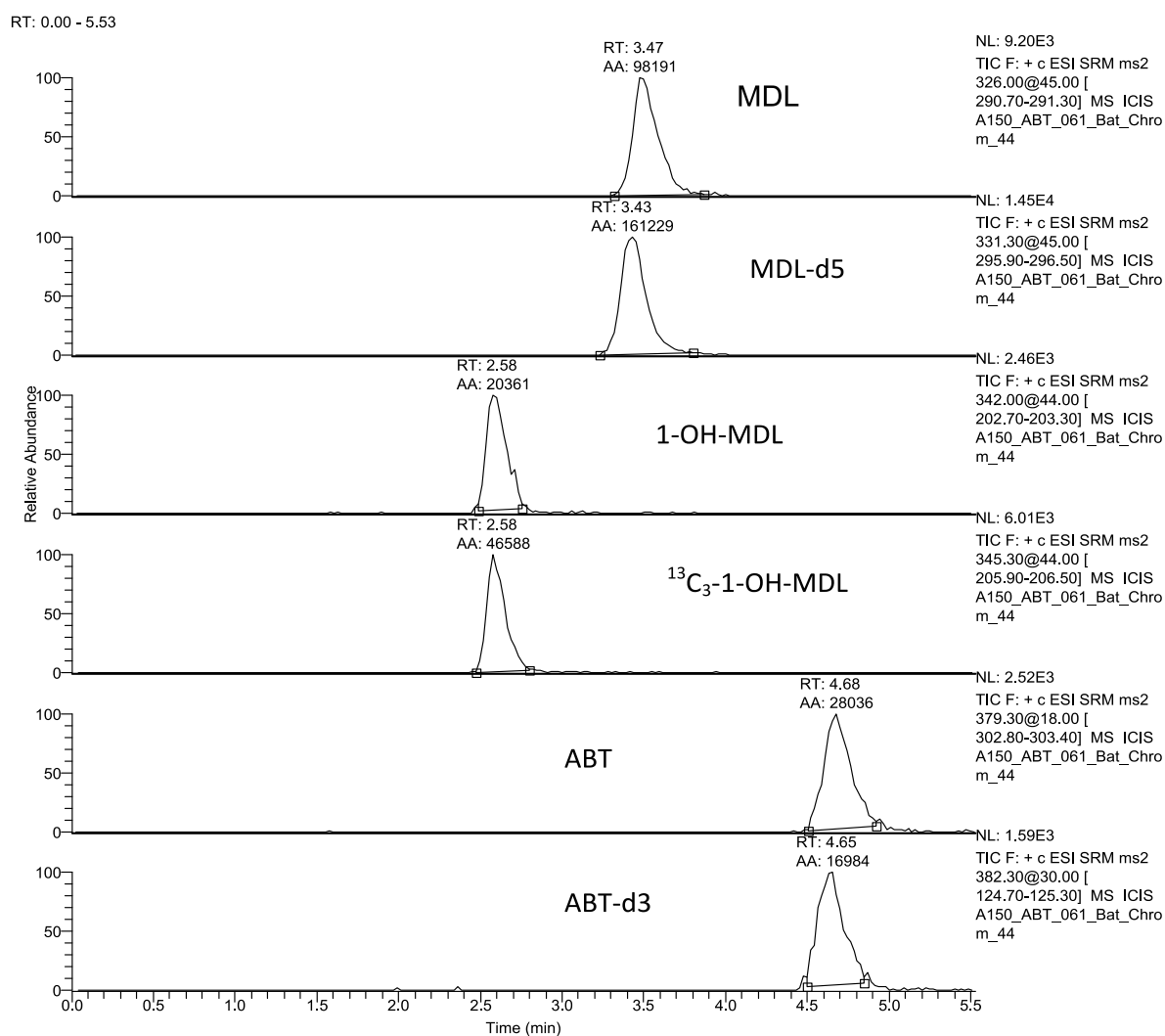
Fig. 13. Chromatogram under final conditions

5.1.3 Mass spectrometry

Mass-to-charge ratios of precursor and fragment ions of MDL, OH-MDL and their internal standards and corresponding collision energies were borrowed from an older analytical method developed by our department.

MS spectra of ABT and d_3-ABT were acquired. Molecular ions (m/z 379 and 382) were chosen as precursors in both cases, because they had the highest signal. Fragments of these ions were then analysed by TSQ − Tune software to find the most advantageous one and optimal collision energy (Fig. 14, 15). Fragment with m/z 303.1 using collision energy 18 eV and fragment with m/z 125 using collision energy 30 eV were chosen for ABT and ABT-d_3, respectively.

MS and MS/MS spectra of all analytes are shown in the Supplement.
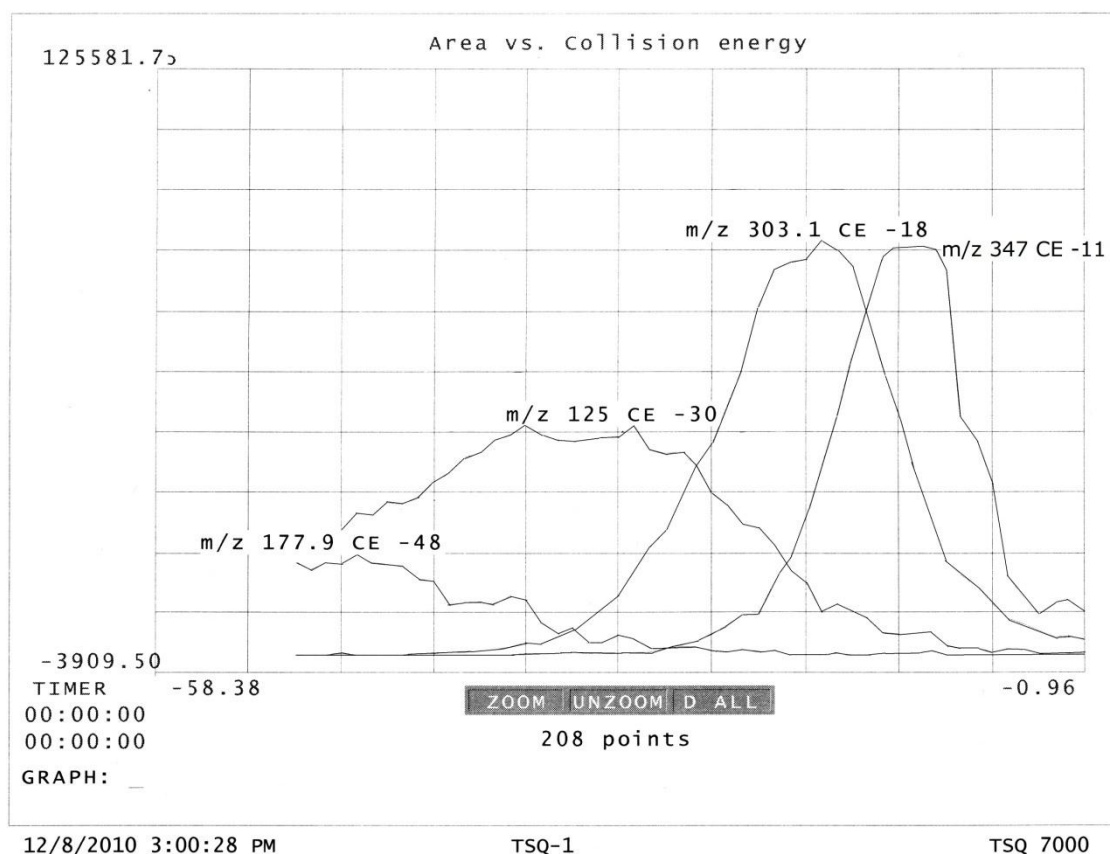
Fig. 14. Fragments collision energy optimalization for ABT m/z 379

50
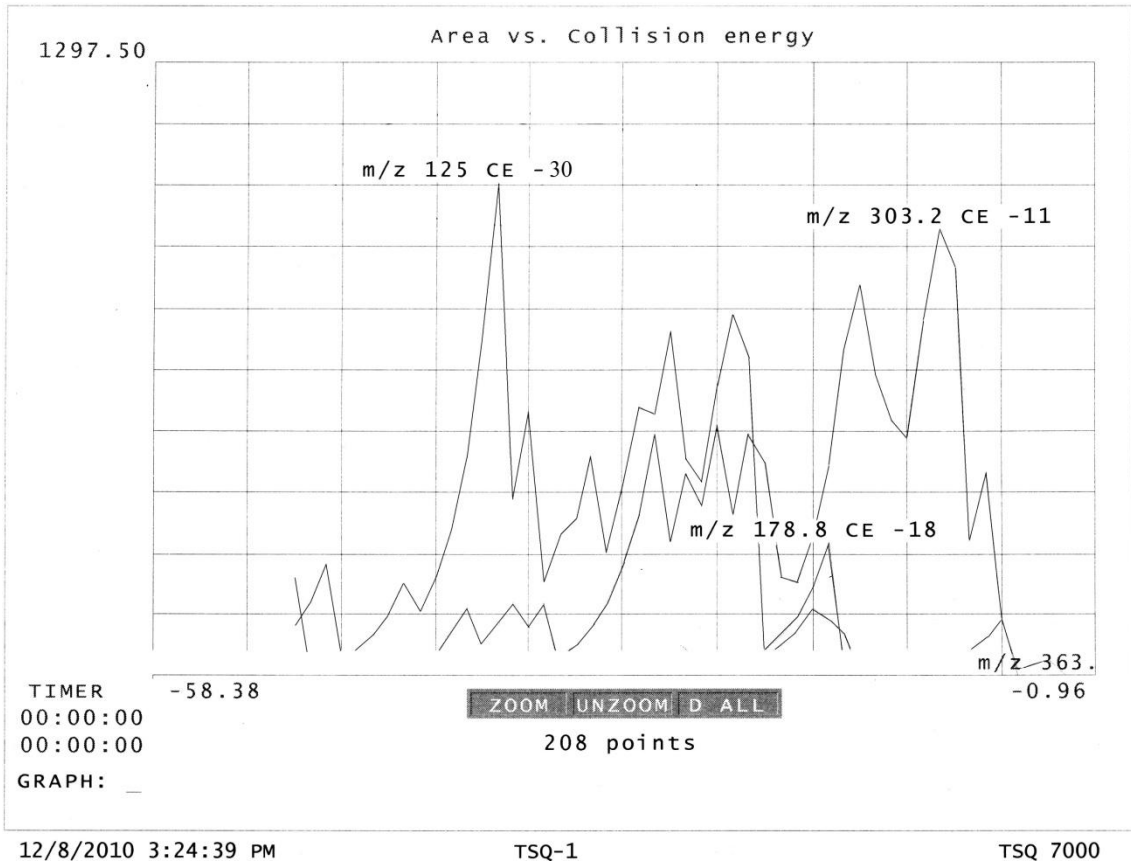
Fig. 15. Fragments collision energy optimalization for ABT-d$_3$ m/z 382

5.2 Validation parameters

➢ Calibration curve

Calibration samples were prepared as stated in chapter 3.3. Calibration range was set to 0.5–200 µg/ml for MDL and OH-MDL and 1–400 µg/ml for ABT. Calibration curves are shown in Fig. 16, 17, 18.

$$y = 0.0138x + 0.0155$$
$$R^2 = 0.9993$$
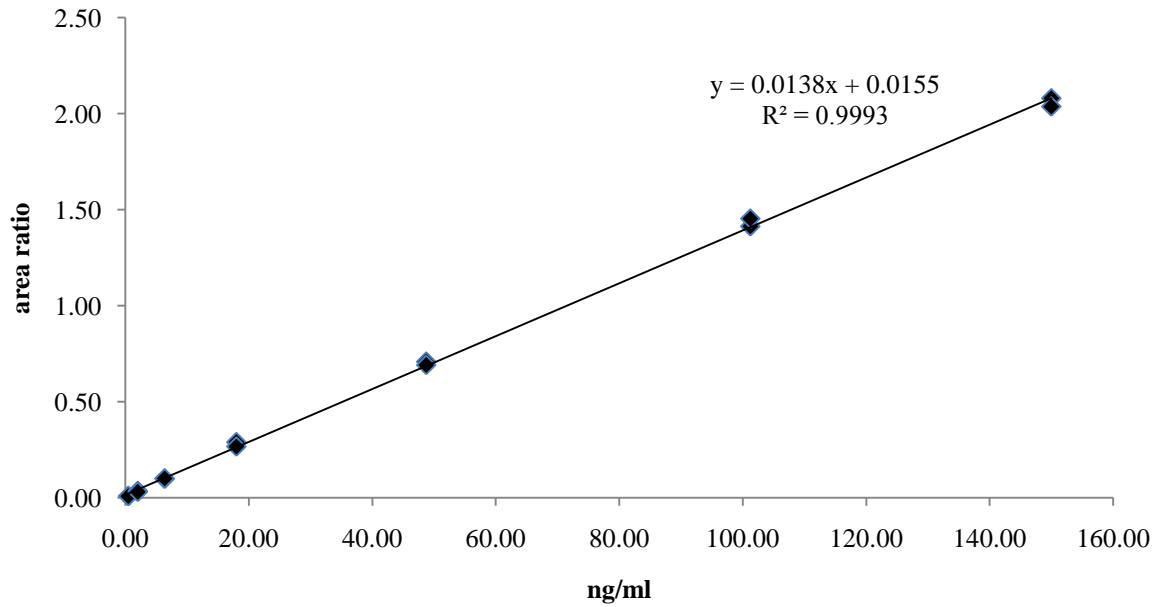
Fig. 16. Midazolam calibration curve

$$y = 0.0103x + 0.0057$$
$$R^2 = 0.9987$$
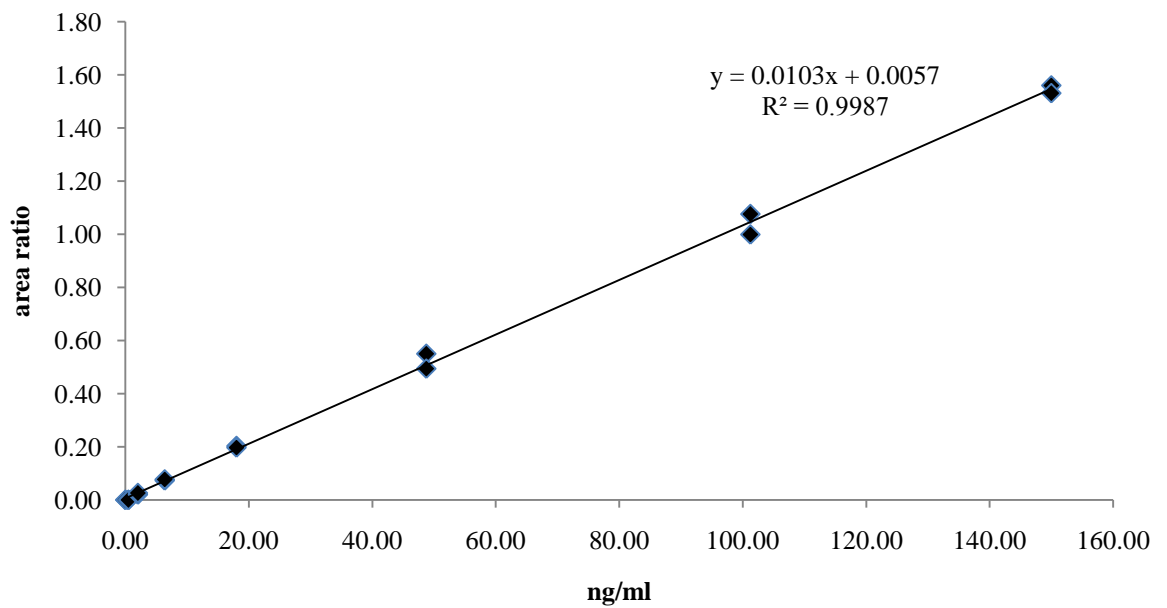
Fig. 17. OH-midazolam calibration curve

$$y = 0.0164x + 0.0067$$
$$R^2 = 0.9974$$
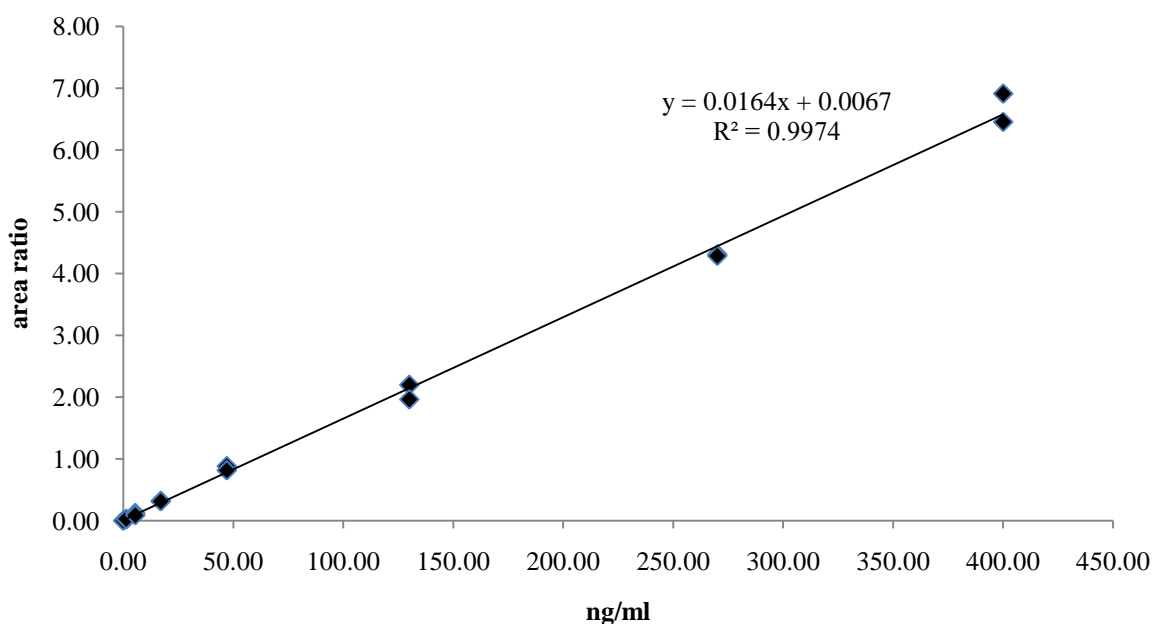
Fig. 18. Ambrisentan calibration curve

➤ Accuracy

Accuracy was counted from 18 samples in 3 separate batches for LOQ and each quality control concentration. All values passed the validation limit.

Table 8. Accuracy [%]				
	LOQ	**QC A**	**QC B**	**QC C**
ABT	9.30	10.58	5.54	7.59
MDL	12.24	8.33	4.43	3.04
OH-MDL	12.58	11.55	9.97	5.61
Validation limit	20.00	15.00	15.00	15.00

➤ Precision

Precision was counted from 18 samples in 3 separate batches for LOQ and each quality control concentration. All values passed the validation limit.

Table 9. Precision [%]				
	LOQ	**QC A**	**QC B**	**QC C**
ABT	11.13	13.43	7.17	9.23
MDL	13.26	9.68	5.83	4.25
OH-MDL	15.78	10.69	7.25	5.52
Validation limit	20.00	15.00	15.00	15.00

➤ Recovery

Recovery was calculated for each QC concentration and for the internal standard. Each value is a mean from two determinations.

Table 10. Recovery [%]

	QC A	QC B	QC C	IS
ABT	81.60	87.86	93.07	84.64
MDL	88.69	92.52	90.32	81.33
OH-MDL	93.45	80.84	91.30	83.69

➤ Freeze and thaw stability

Freeze and thaw stability was calculated for QC B concentration. Each value is a mean from two determinations and represents the loss of analyte during freezing and thawing.

Table 11. Freeze and thaw stability [%]

ABT	-2.82
MDL	-4.31
OH-MDL	-11.17

5.3 Midazolam and 1-hydroxymidazolam pharmacokinetic profile

Plasma concentrations of midazolam and 1-hydroxymidazolam of 6 healthy participants were determined. Plasma samples were taken 11 times after oral administration and 13 times after intravenous administration. Acquired concentrations are shown in Fig. 19-26. Although some values for orally administrated OH-MDL are below our intended quantification limit, they correlate well with higher values. Concentrations of OH-MDL after its intravenous administration were detectable, but practically not quantifiable.

➤ **Oral administration**
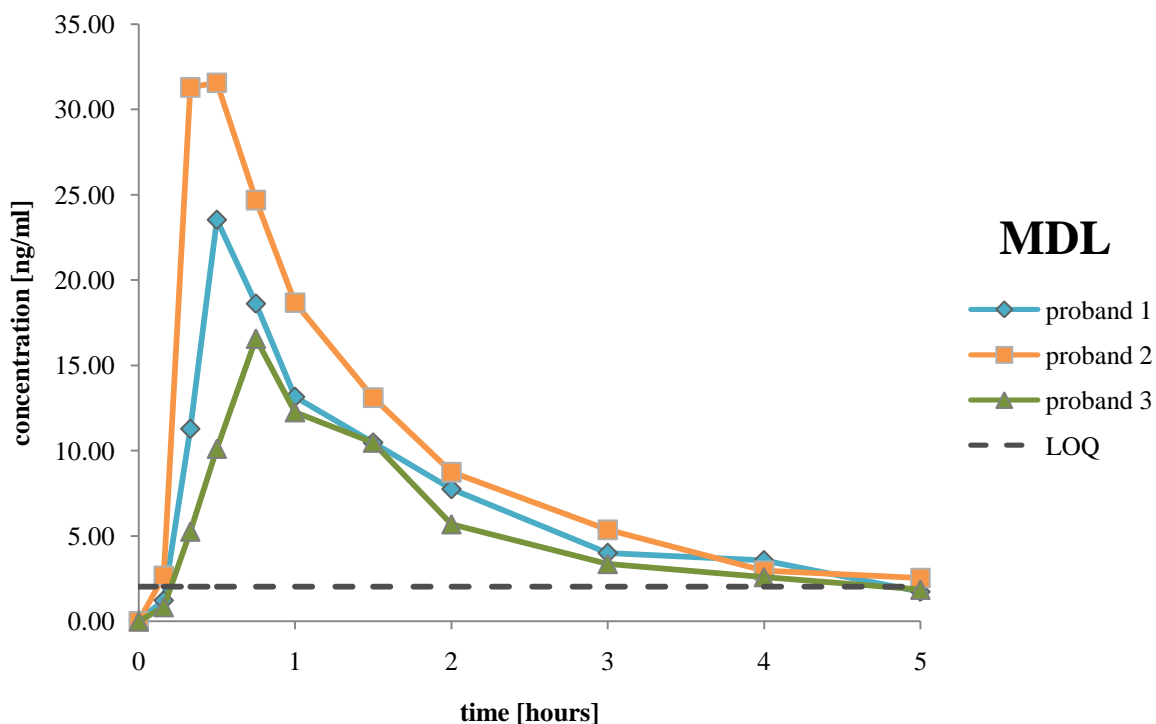
Fig. 19. MDL plasma concentration after oral administration
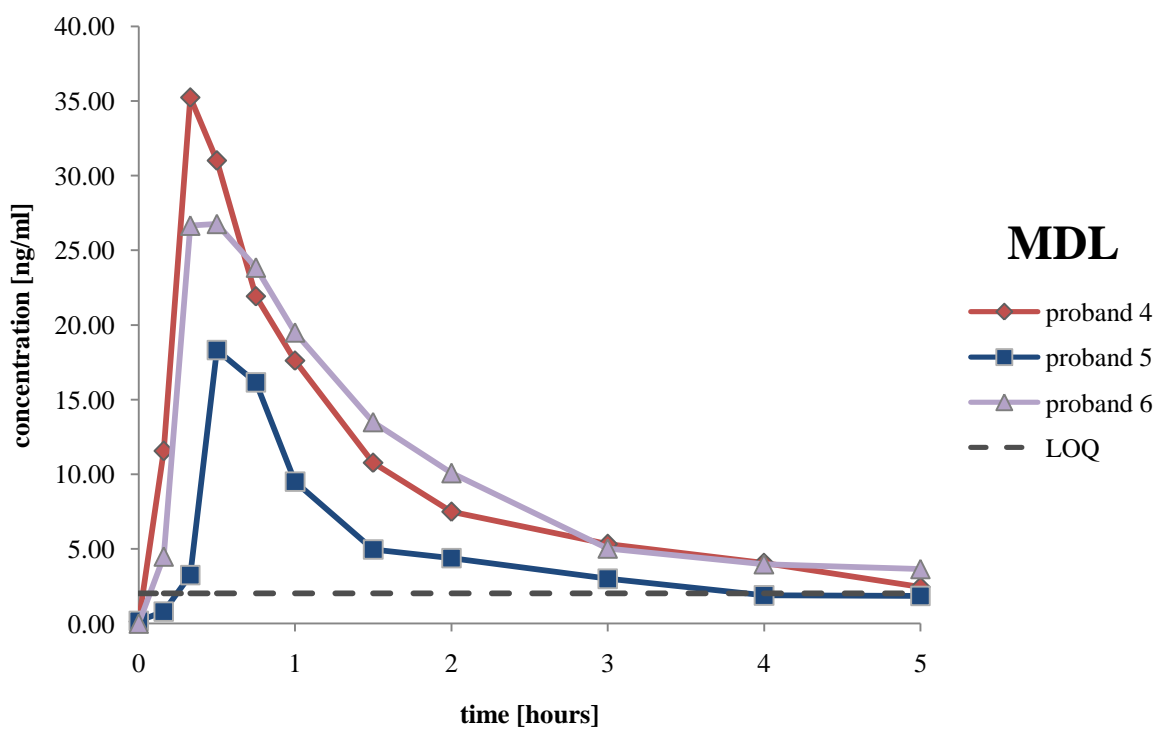
Fig. 20. MDL plasma concentration after oral administration
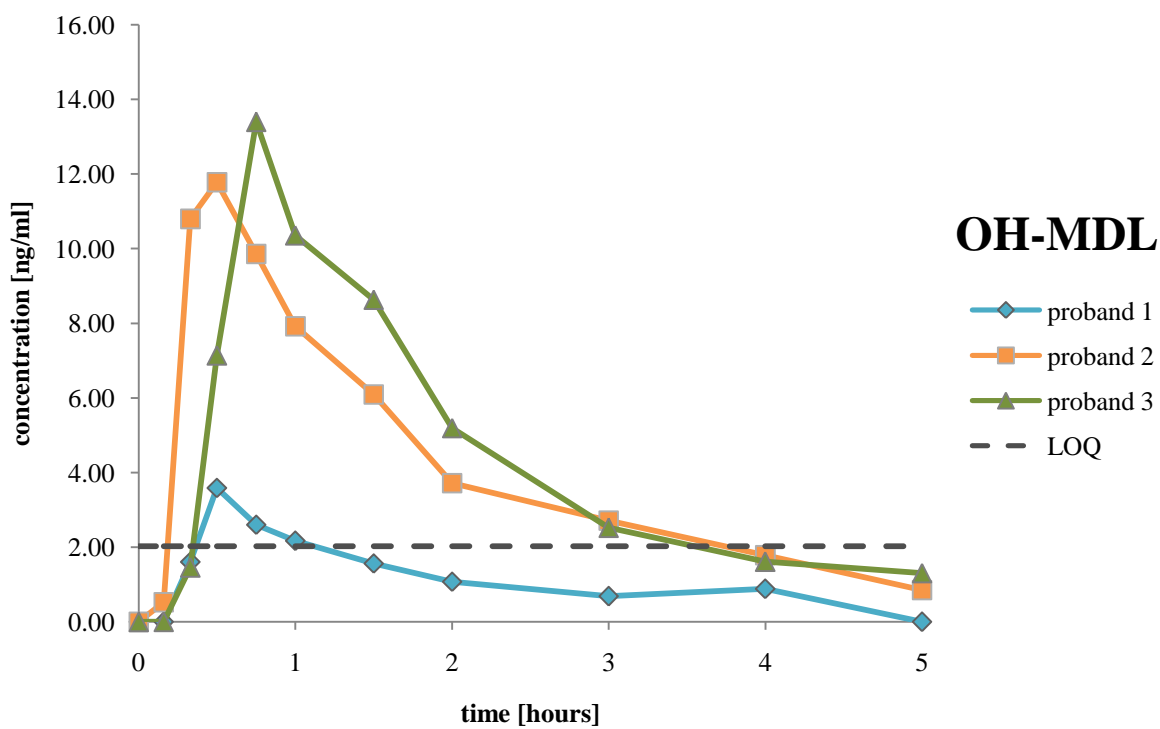
Fig. 21. OH-MDL plasma concentration after oral administration
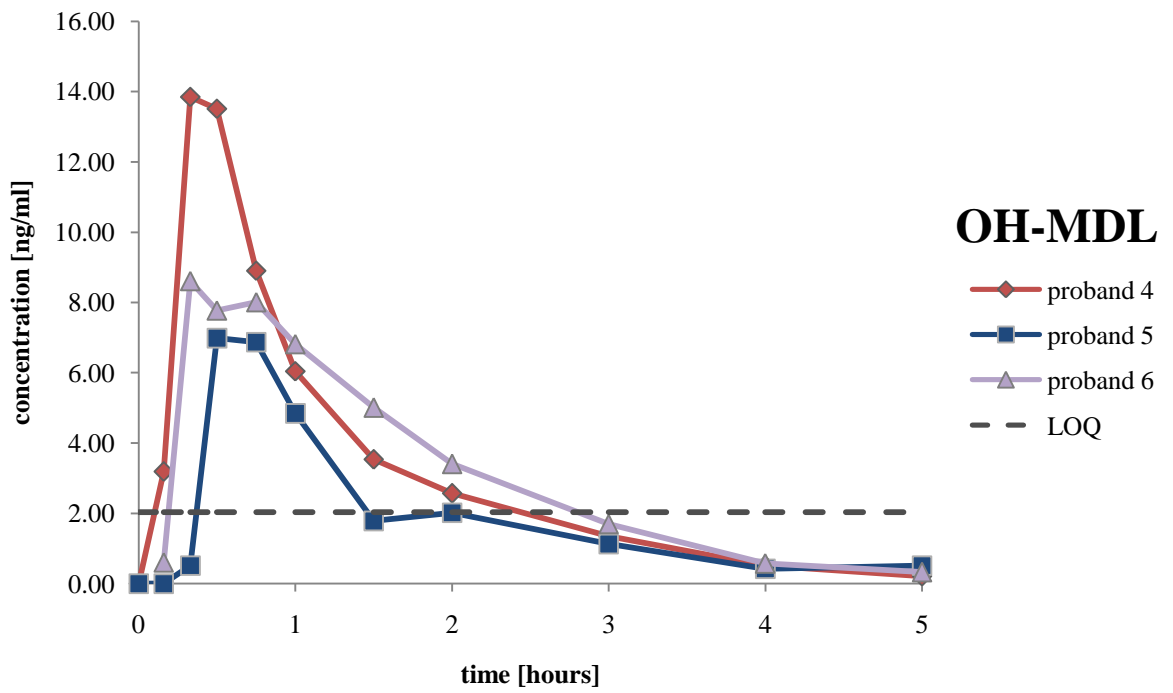
Fig. 22. OH-MDL plasma concentration after oral administration

➢ **Intravenous administration**
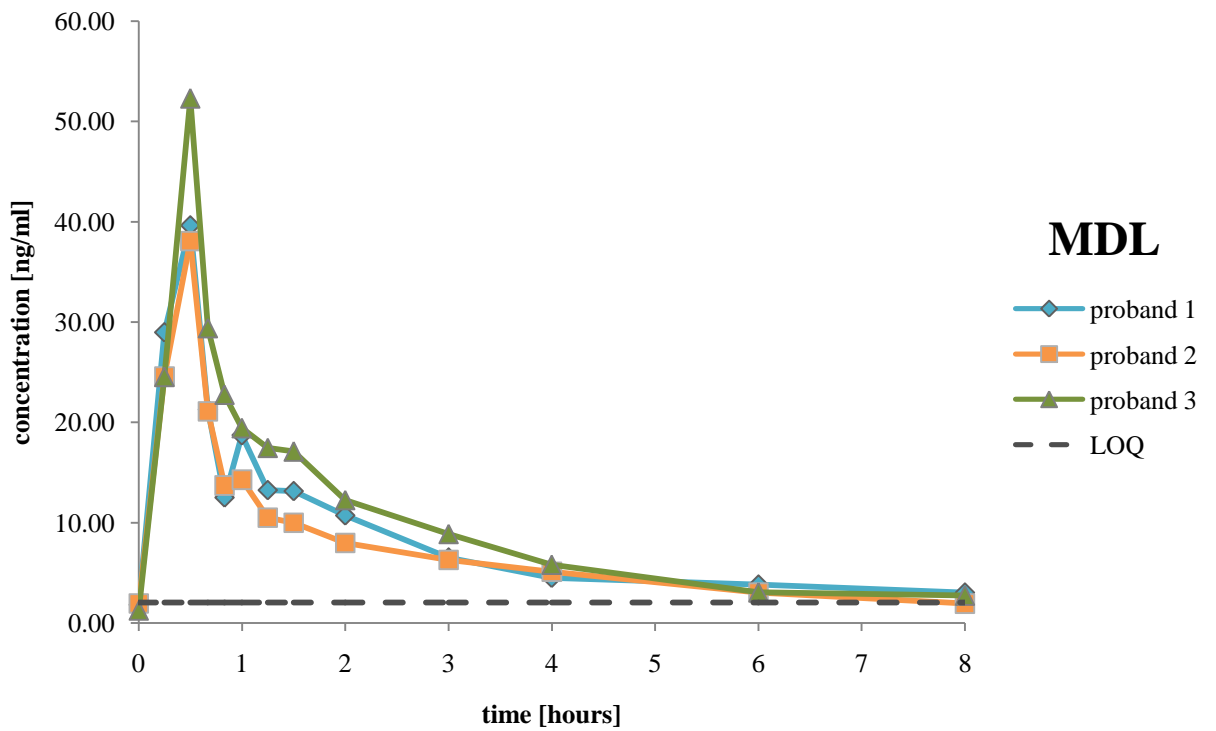
Fig. 23. MDL plasma concentration after intravenous administration
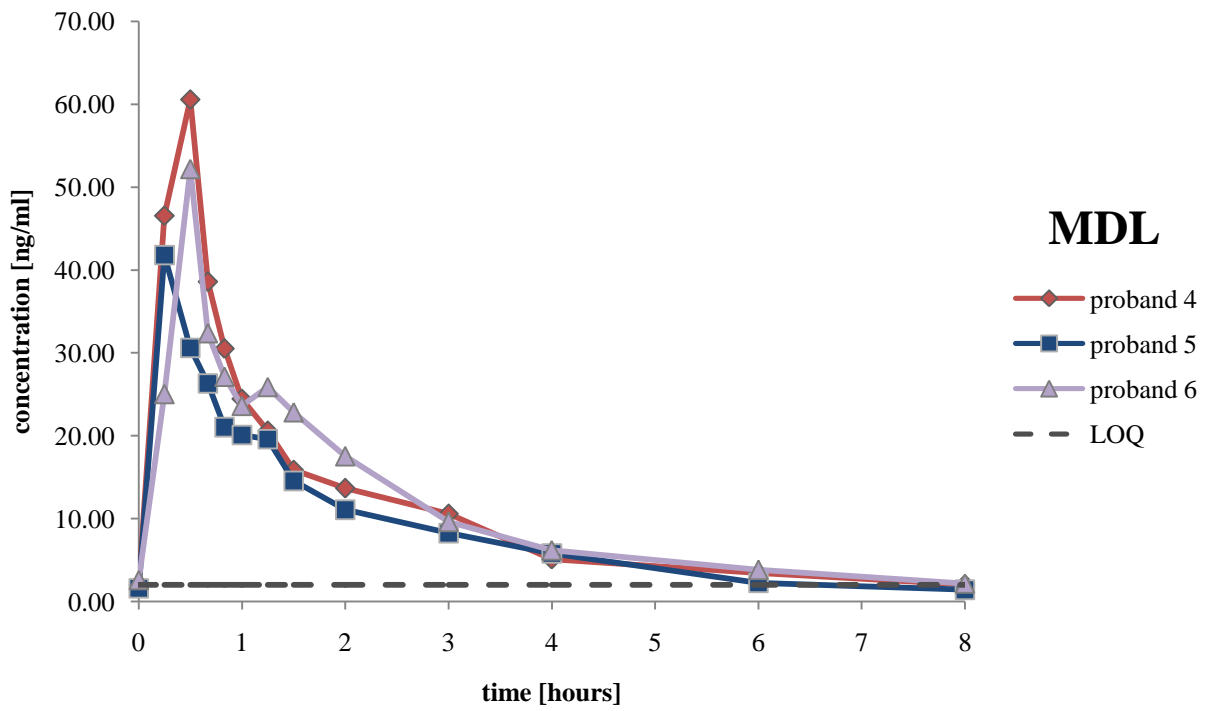
Fig. 24. MDL plasma concentration after intravenous administration
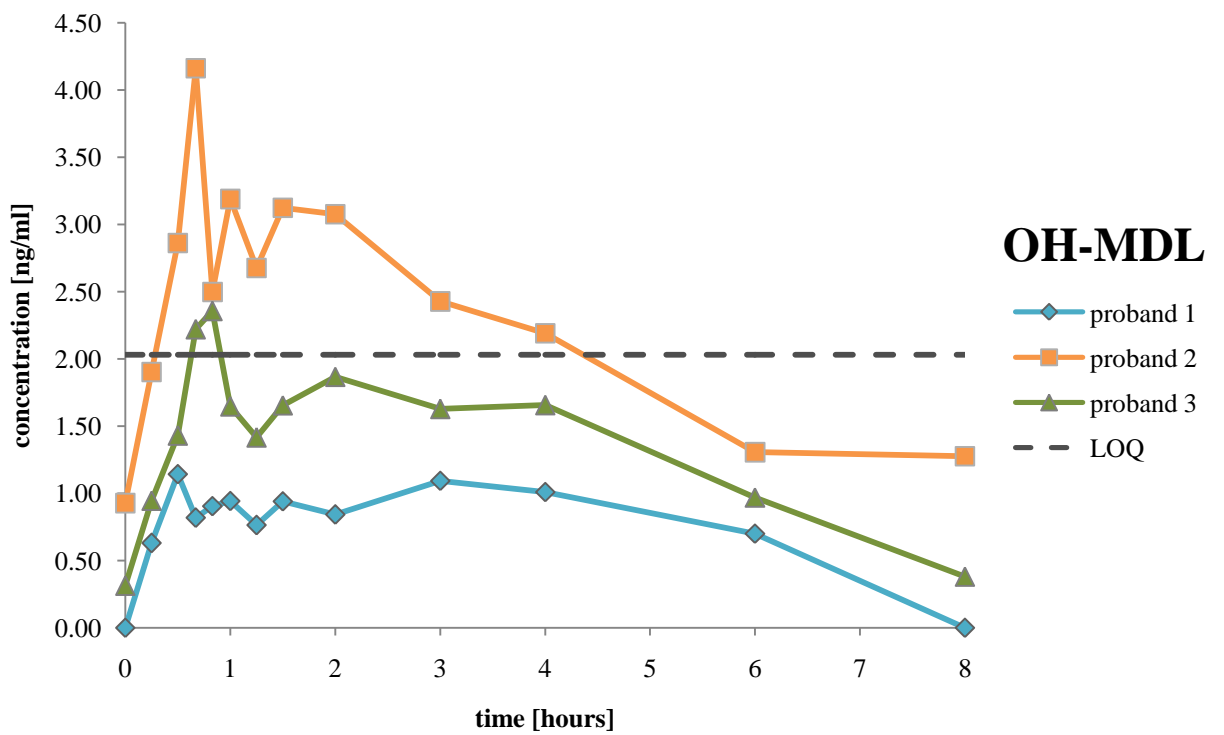
Fig. 25. OH-MDL plasma concentration after intravenous administration
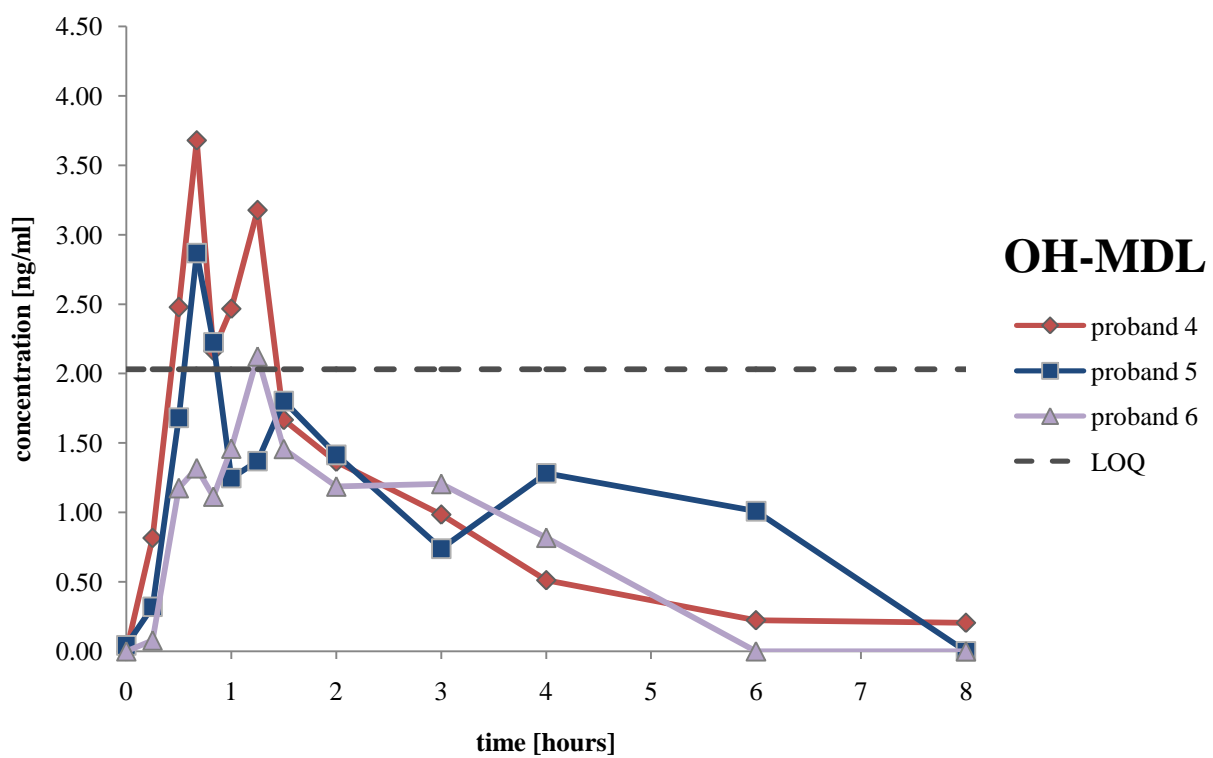
Fig. 26. OH-MDL plasma concentration after intravenous administration

6 DISCUSSION

The clinical study "Influence of CYP3A4-induction by St. John's wort (SJW) on the steady state pharmacokinetics of ambrisentan" held at University Hospital of Heidelberg set its goals among others as evaluating the impact of St. John's wort administration on steady state ambrisentan and evaluating the time course of CYP3A4 activity changes during ambrisentan treatment using midazolam as CYP3A4 activity marker.

Our task was to develop of analytical method that will be useful for simultaneous determination of ambrisentan, midazolam and 1-hydroxymidazolam in human plasma. The method should be extended in the future to SJW and the main metabolite of ambrisentan that was not available in the time of working on this thesis.

High performance liquid chromatography with tandem mass spectrometry was chosen as the most accurate and sensitive available method. It is often used for determination of both ambrisentan and midazolam. Besides high sensitivity, big advantage of tandem mass spectrometry is the possibility to use deuterated or ^{13}C derivates of parent molecules as internal standards. They have the same physical and chemical properties, therefore their behaviour during extraction and chromatography is practically the same too, which is the most important requirement on the internal standard.

HPCL columns Synergi Max-RP and Synergi Polar-RP (Phenomenex) were tried. Better peak shapes were achieved on Synergi Max-RP. Mobile phase was composed of ammonium acetate buffer and acetonitrile. Addition of methanol led to worse peak shapes. The ratio between ACN and buffer was chosen with regard to the shortest time of analysis and sufficient separation of analytes. Although complete separation is not necessary in MS/MS, it enabled to change mass spectrometer settings for each analyte. Mass-to-charge ratios of precursor and fragment ions and collision energies in the collision cell were determined as mentioned above.

The influence of the source CID collision energy on the MS signal was studied. Source CID collision energy is an additional energy applied at the ESI to fragment the molecules of analytes. Although molecular ions were used as precursors and therefore the fragmentation of molecules in the ion source was unwanted, it was found

that the application of certain energy leads to significant increase of the signal of MDL and OH-MDL. A possible explanation is that the extra energy is able to destroy adducts formed in the ion source but not the molecule of analyte itself. ABT, on the other hand, showed rapid decrease of the signal, if the additional energy was applied. It was possible to divide the MS method to two segments according to the time of analysis and so to set different source CID energies for MDL with OH-MDL and for ABT. However ABT signal was still dependable on the energy used in MDL segment, so an increase of source CID energy led to increase of OH-MDL/MDL signal, but to decrease of ABT signal. Moreover, switching off of the source CID energy in the ABT segment led to massive decrease of the ABT signal in comparison with application of the lowest possible energy, i.e. 1eV. The source CID collision energies were eventually set at 10 eV for MDL and OH-MDL and at 1 eV for ABT as the best reachable compromise.

Liquid-liquid extraction is the most often used method for separation of both midazolam and ambrisentan from plasma matrix. However simultaneous extraction of both analytes was not possible, because midazolam is a weak base whereas ambrisentan is a weak acid. Therefore they need opposite pH for entering the organic phase in their non-ionized form. Some experiments were made using two consecutive extractions with the change of pH. The best obtained recoveries were 90 % for ABT and 81 % for MDL. Despite relatively good recoveries, liquid-liquid extraction was abandoned, because the process was quite complicated and time-consuming. Solid phase extraction was found the most advantageous method. Different SPE columns with various types of sorbent were tried: Strata-X (Phenomenex), Strata-X AW (Phenomenex), Bond Elut (Varian), Oasis (Waters) and Evolute (Biotage). Usually the particular column was good for one or two analytes, but not for all of them. The best compromise was achieved on Strata-X SPE column. On this column, the highest recoveries were observed using 0.1M hydrochloric acid for acidification of the plasma. The most suitable washing liquids and eluent volume were found.

Analytical method was validated according to FDA validation parameters. Calibration curves were made with parameters mentioned in table 12.

Table 12. Calibration curves parameters

	Equation	r^2 value
ABT	y = 0.0164x + 0.0067	0.9974
MDL	y = 0.0138x + 0.0155	0.9993
OH-MDL	y = 0.0103x + 0.0057	0.9987

Accuracies and precisions variabilities of all analytes were within the 15% limit for QC and the 20% limit for LOQ samples. Recoveries of all analytes were over 80 %. The lower limits of quantification were set at first as the lowest calibration concentration. However accuracy and precision variabilities were over the 20% limit at these concentrations and therefore the LOQs were increased to 2.03 ng/ml for MDL and OH-MDL and 5.40 ng/ml for ABT. MDL and OH-MDL LOQs are comparable to values achieved in other published methods (49, 50, 51). Ambrisentan LOQ should be also sufficient, since its average trough plasma concentration is 63 ng/ml after administration of 5 mg (26), which will be also the dose used in our clinical study.

Validated method was applied to determination of MDL and OH-MDL concentrations in plasma of human volunteers. Any clinical samples containing ambrisentan were unfortunately not yet available. Measured concentrations correlated well with values acquired by an older validated LL/HPLC/MS/MS method for MDL and OH-MDL during unpublished original clinical study, where the plasma samples were taken from. New method is sensitive enough for detection of midazolam plasma concentrations after administration of 4 mg orally or 2 mg intravenously. After oral administration of midazolam, hydroxymidazolam is quantifiable in most cases. Although some of the measured concentrations are under the LOQ they correlate still well with higher values. OH-MDL concentrations are practically not quantifiable after intravenous administration of MDL.

7 CONCLUSION

HPLC/MS/MS method for simultaneous determination of ambrisentan, midazolam and 1-hydroxymidazolam in human plasma was developed and successfully validated. Solid phase extraction was used for extraction of analytes from plasma matrix.

The method was applied on 144 plasma samples containing midazolam and 1-hydroxymidazolam in expected concentrations.

The method is reliable and sensitive enough to be used for measuring of plasma concentrations of the drugs in the clinical study "Influence of CYP3A4-induction by St. John's wort (SJW) on the steady state pharmacokinetics of ambrisentan" held at University Hospital of Heidelberg.

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9 SUPPLEMENTS

➢ Mass spectra of ABT

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T: + c ESI Q1MS [150.00-500.00]
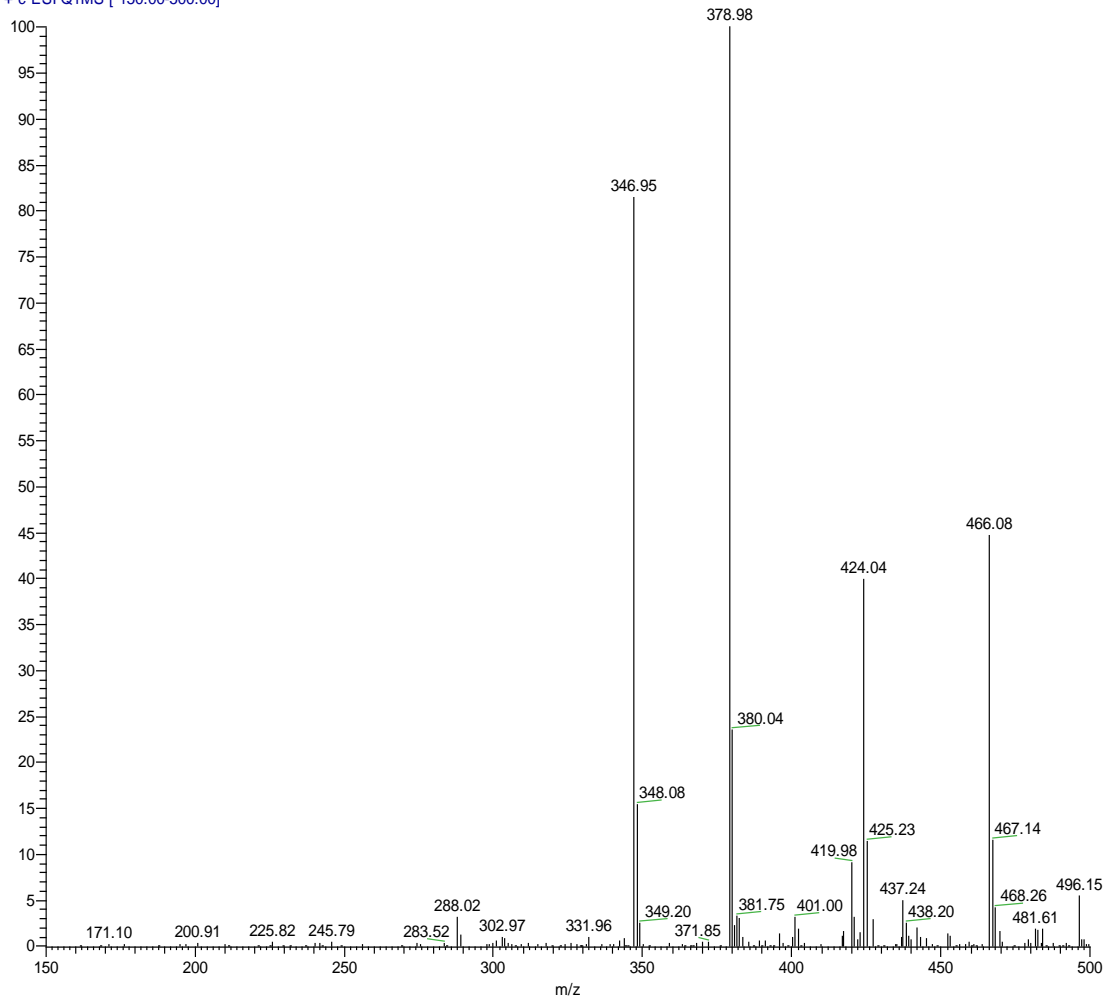
Supplement 1. MS spectrum of ABT
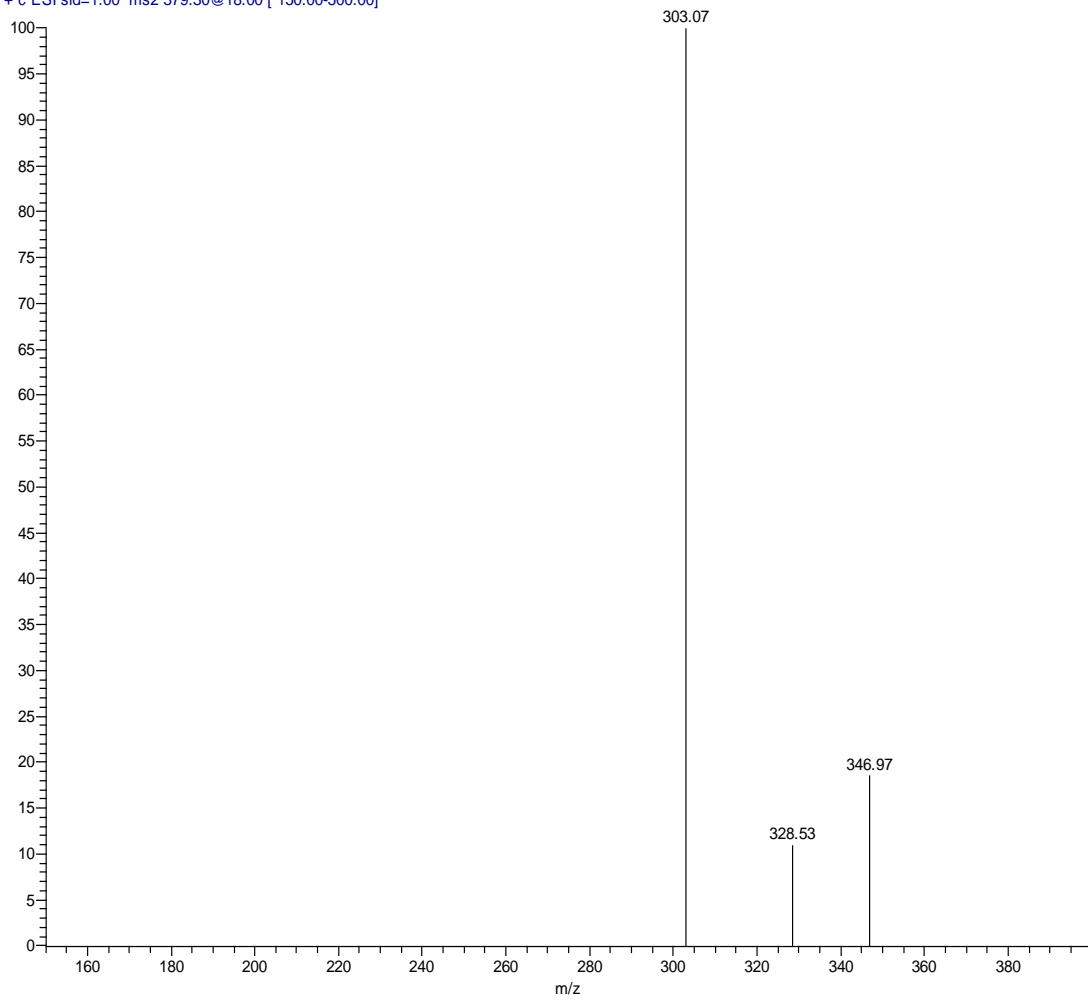
Supplement 2. MS/MS spectrum of ABT with 379.30 as the precursor mass
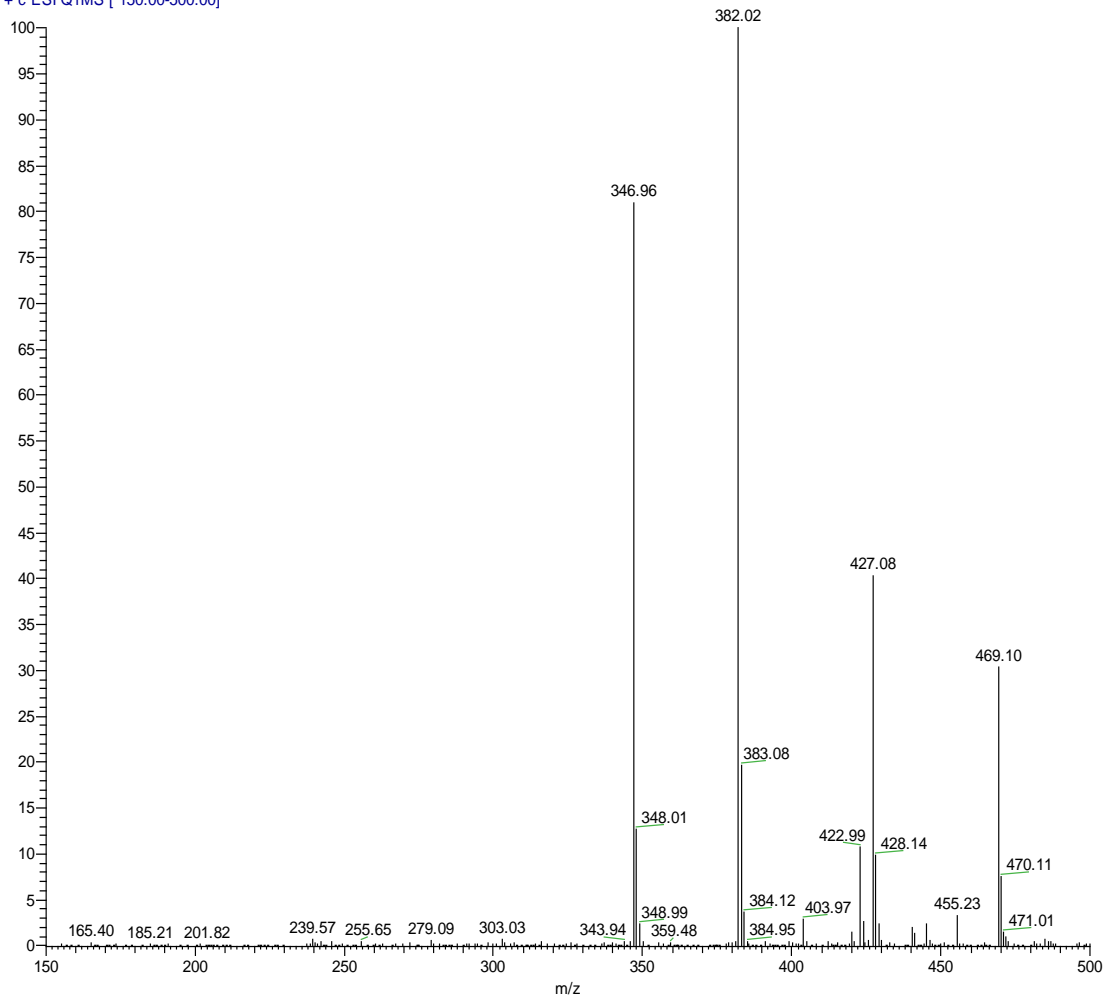
Supplement 3. MS spectrum of ABT-d3
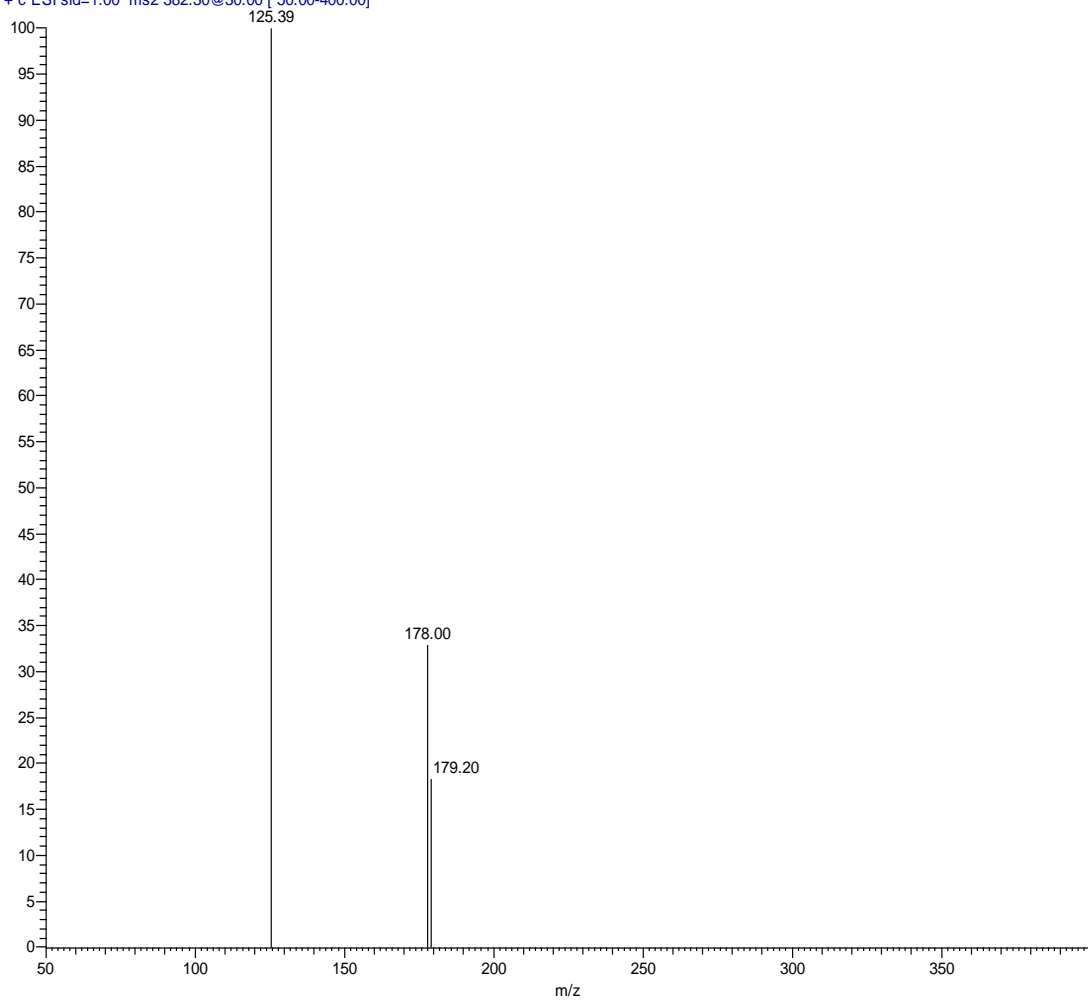
Supplement 4. MS/MS spectrum of ABT-d3 with 382.30 as the precursor mass

➤ **Mass spectra of MDL**
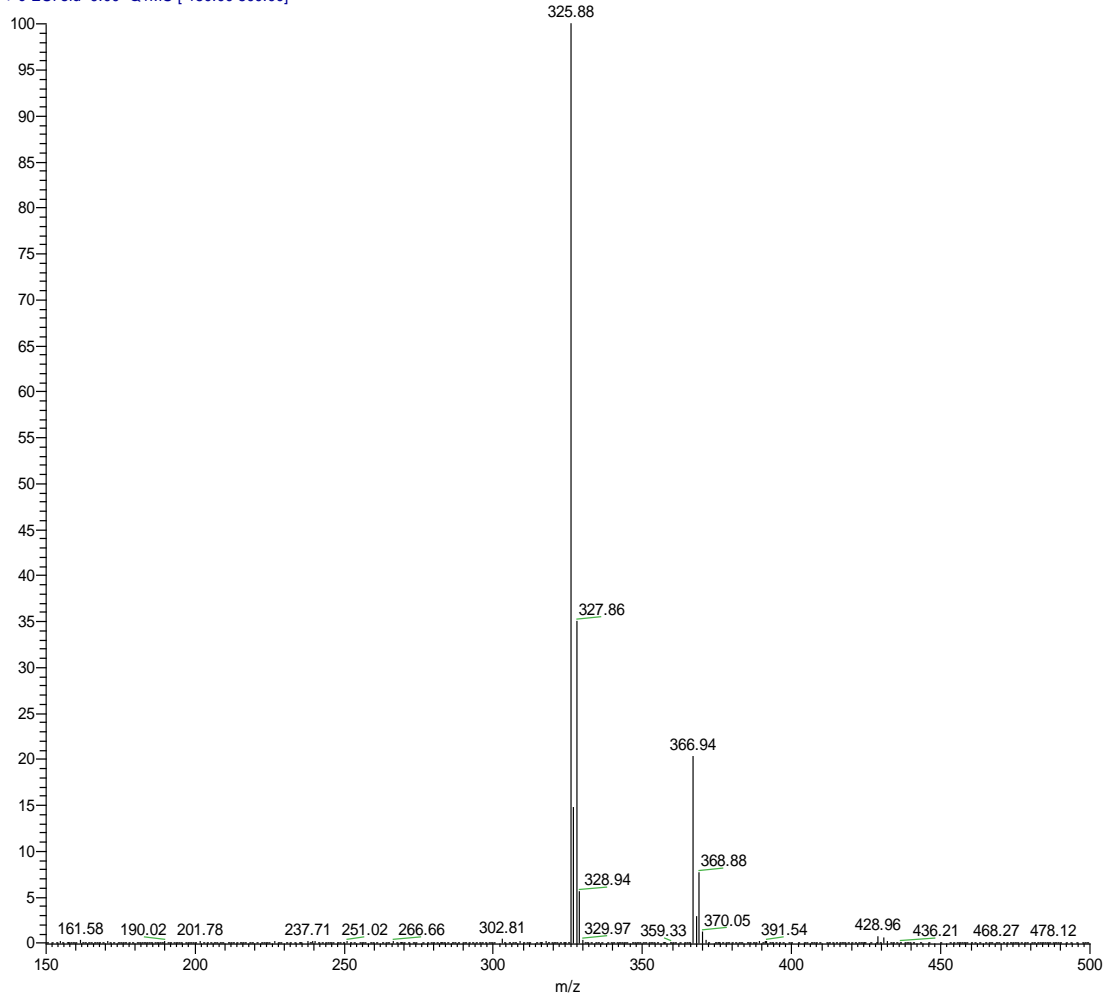
Supplement 5. MS spectrum of MDL
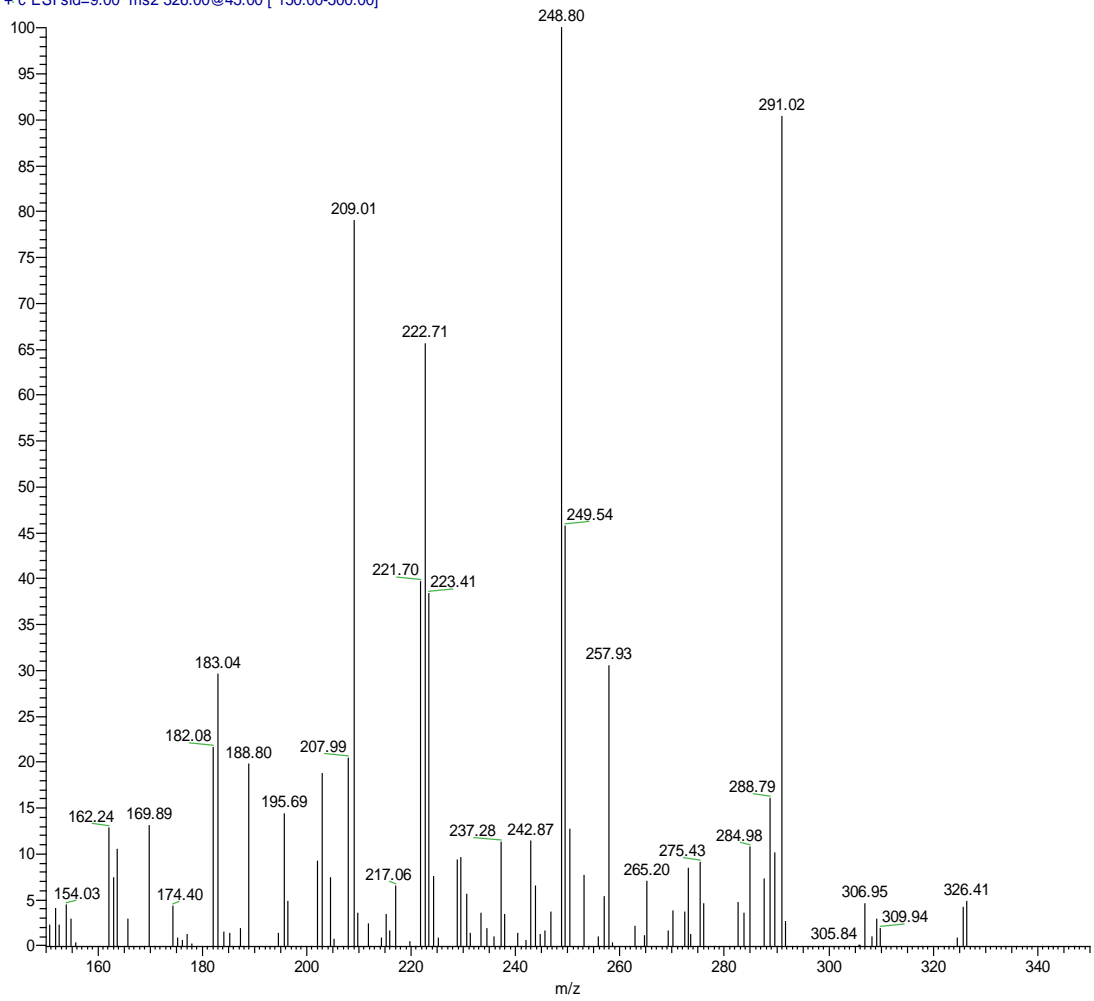
Supplement 6. MS/MS spectrum of MDL with 326.00 as the precursor mass
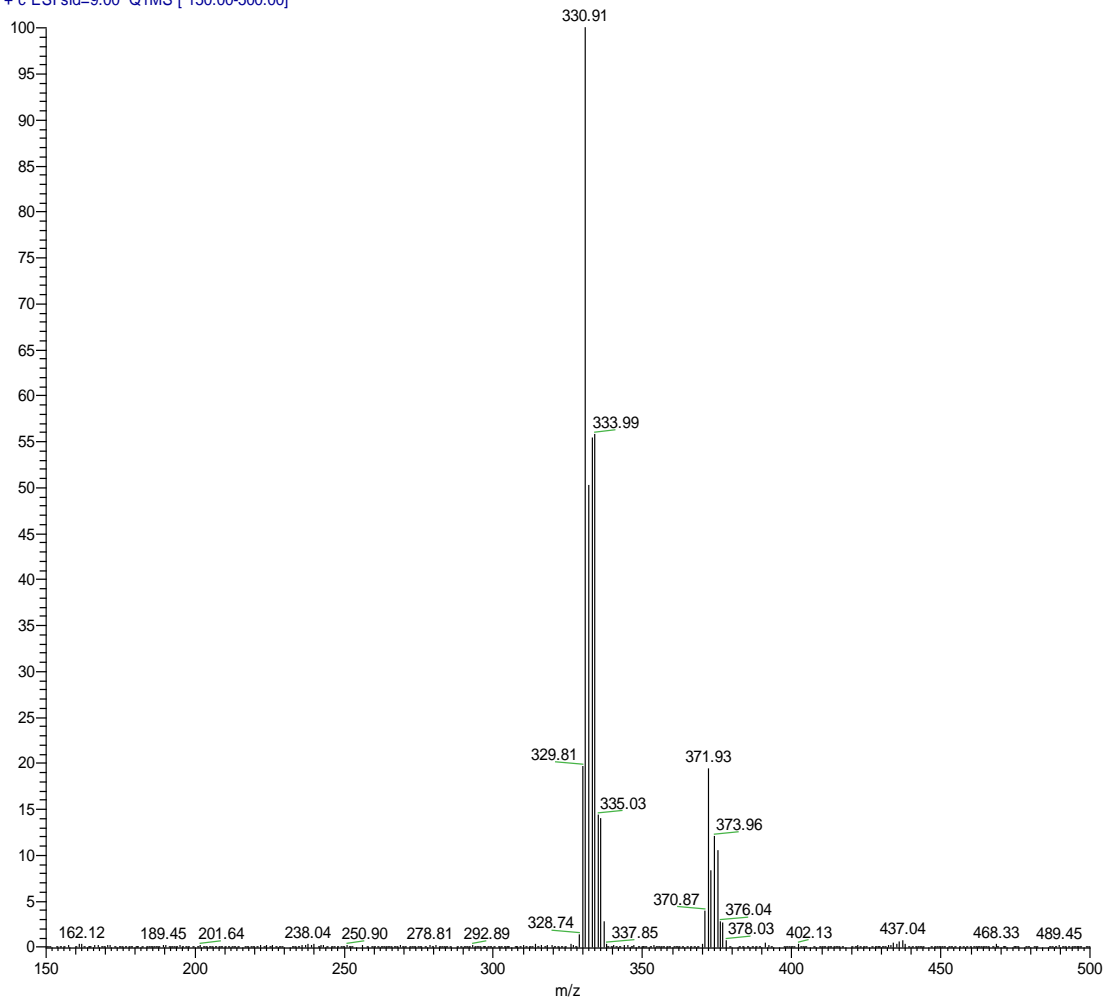
Supplement 7. MS spectrum of MDL-d5
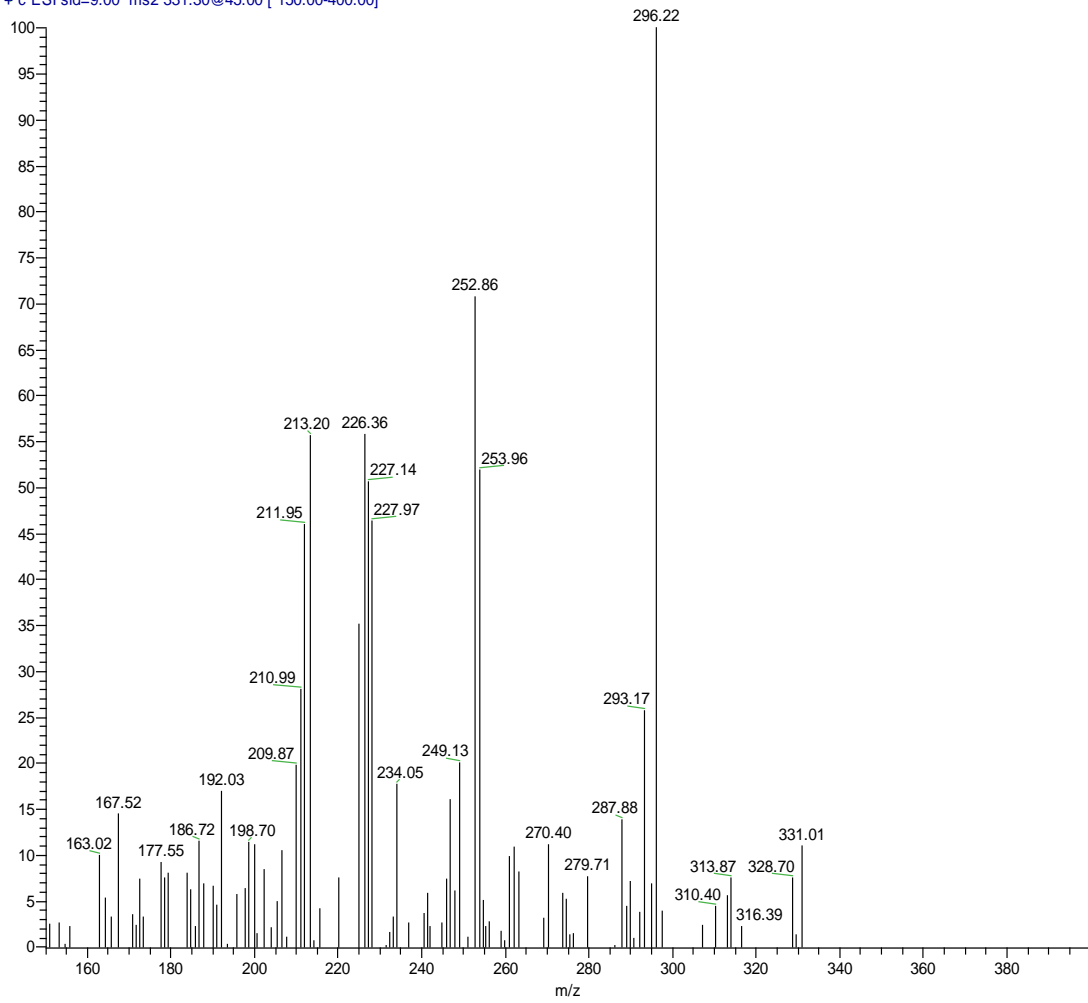
Supplement 8. MS/MS spectrum of MDL-d5 with 331.30 as the precursor mass

➢ **Mass spectra of OH-MDL**
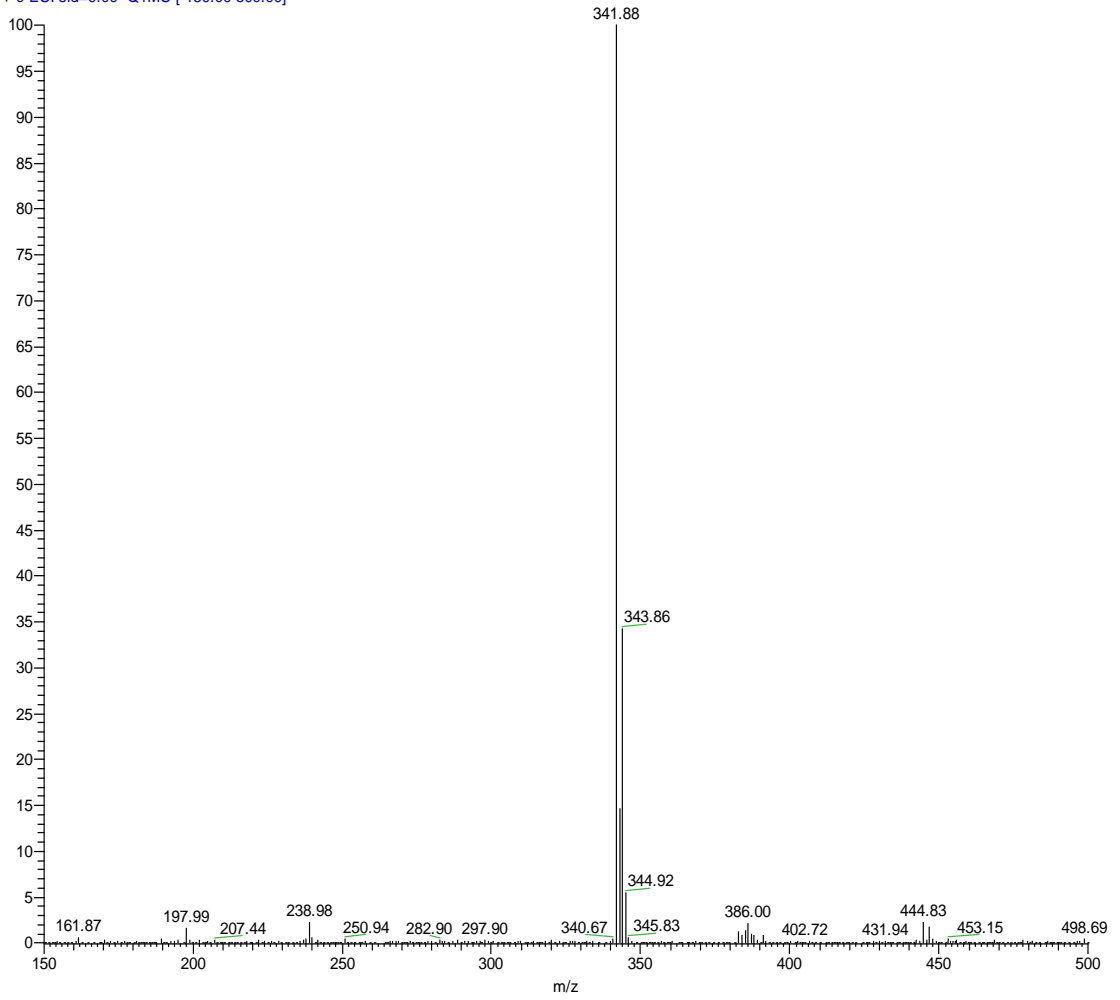
Supplement 9. MS spectrum of OH-MDL
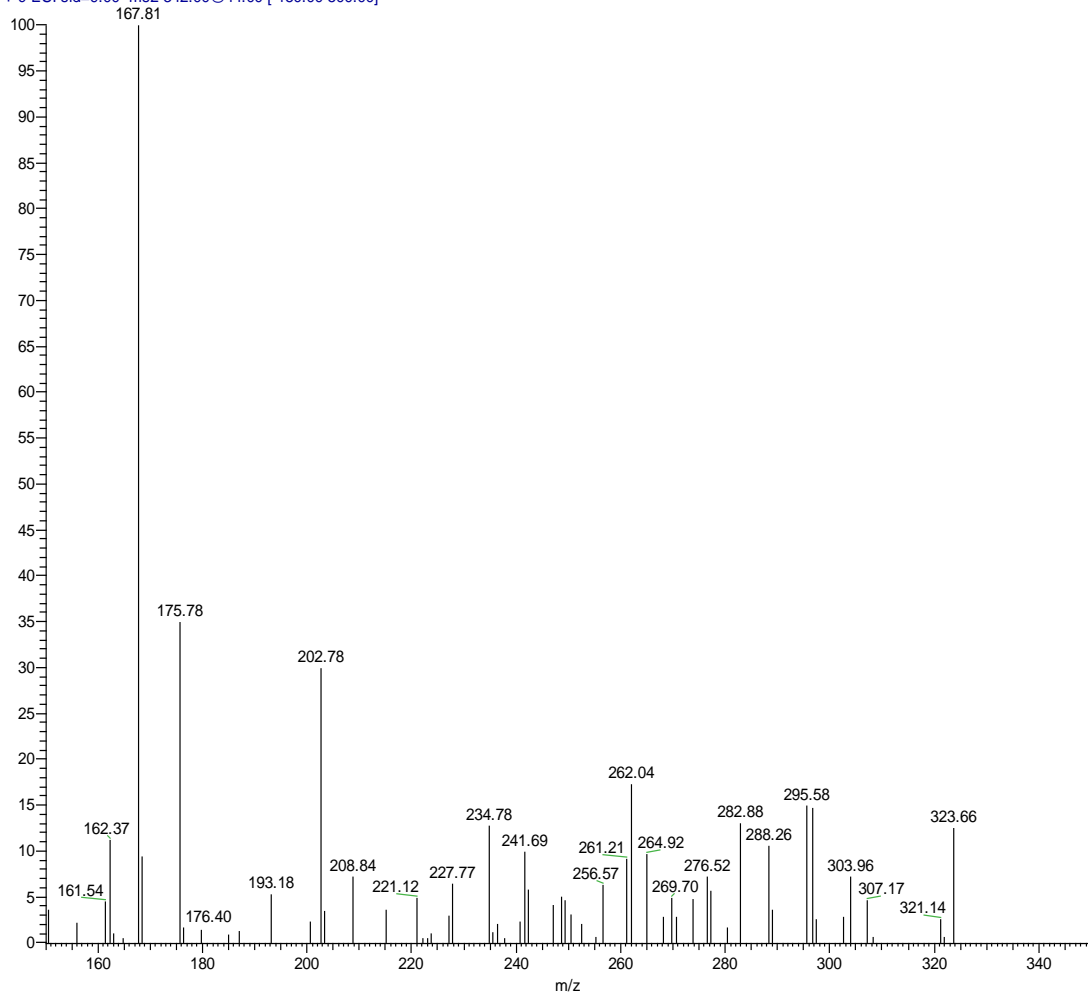
Supplement 10. MS/MS spectrum of OH-MDL with 342.00 as the precursor mass
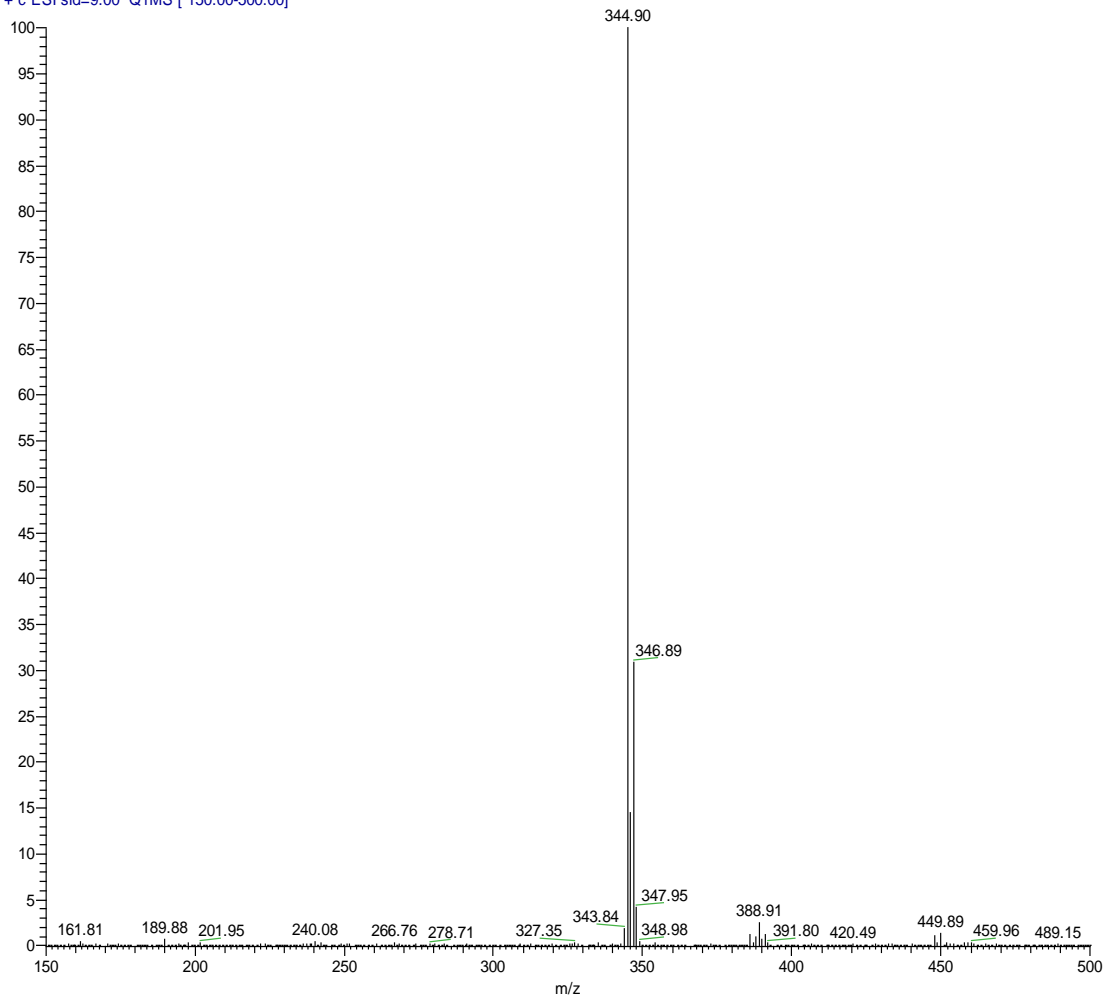
Supplement 11. MS spectrum of $^{13}C_3$-OH-MDL
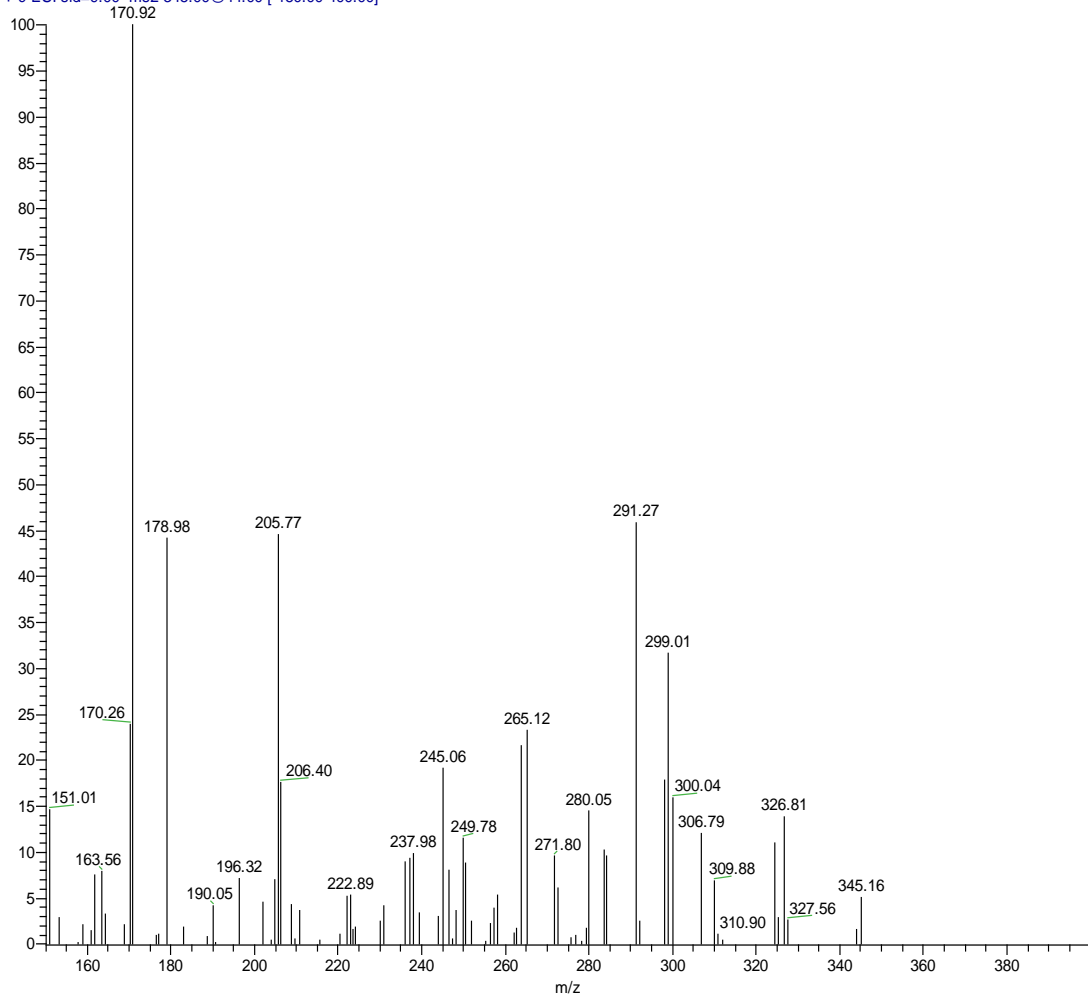
Supplement 12. MS/MS spectrum of 13C3-OH-MDL with 345.00 as the precursor mass