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DEVELOPMENT OF AN LC-MS METHOD FOR DETERMINATION OF NEW ANTIMALARIAL DRUGS IN BIOLOGICAL MATRICES

(Master thesis)

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I declare, this thesis is my own original work. It is my own intellectual property and I worked it out on my own.

All articles, literature and other information sources, that I gathered the information used in my thesis from, are mentioned in the list of literature. These are properly cited.

Jiří Klimeš

Thesis Content

LIST OF ABREVIATION USED

ACN	Acetonitril
APCI	Atmospheric pressure chemical ionisation
AR	Artemisinins
ARST	Artesunate
cps	Counts per seconds (intensity)
DHA	Dihydroatemisinin
DMDCS	Dimethyl-dichlorsulfoxid
ESI	Electrospray ionization
FT – ICR	Fourier Transform Ion Cyclotron Resonance
I.S.	Internal standard
HP	Haemolysed plasma (plasma with some portion of haemolysed whole blood)
LC/HPLC	High performance liquid chromatography
LLE	Liquid-liquid-extraction
LLOQ	Lower limit of quantification
Met-OH	Methanol
MF	Mobile phase
MS	Mass spectrometry/ Mass spectrometer
PHEN	Phenantroline
PP	Protein precipitation
QHS	Artemisinin
SPE	Solid-phase extraction
T_R	Retention time
WHO	World health organization

ABSTRACT

Artemisinin a representative of Endoperoxide class of drugs and its derivatives particularly artesunate are the most important class of antimalarial drugs used in clinical practice. They are recommended as the first-line treatment of malaria in combination with other longer-acting antimalarial drugs (lumefantrine, piperaquine).

As the main skeleton of these compounds lacks UV visible or fluorescent chromophore, earlier methods of detection have used post-column on-line derivatisation or electrochemical detection in the reductive mode. However, these methods suffer from poor sensitivity and selectivity.

Within this thesis the whole LC-MS method development for the analysis of artesunate and its major metabolite dihydroartemisin in biological samples from the very beginning was performed. It included tuning of ESI - triple quadrupole MS detector and optimization of chromatographic conditions, particularly mobile phase pH.

Artesunate (ARST) and dihydroartemisin (DHA) were assayed in human plasma using artemisisnin as an internal standard. Different approaches of plasma sample treatment, (protein precipitation and liquid-liquid extraction) were optimized and compared. Pre-validation data for liquid-liquid extraction revealed LLOQ as 2.5 and 3.0 ng/ml for DHA and ARST, respectively using 400 µl of plasma. Pre-validation data for protein precipitation revealed LLOQ for both DHA and ARST 8.0 ng/ml using 300 µl of plasma.

Further study focused on influence of free iron and haem iron as the source of vast degradation of compounds within process of assay was performed and possible solutions of this issue were suggested. This study is essential to overcome difficulties regarding analysis of these compounds in blood/plasma samples taken from patients infected by malaria parasite, where products of haemolysis are presented in large amount. Moreover, this study was of great importance for the analysis of homogenized rat´s embryo samples which contain large amount of haemoglobin and myoglobin.

The assay of these compounds in rat´s embryo is crucial for further investigation of potential risks associated with the using of artemisinin derivatives in pregnancy, particularly within the first trimester. (The safety of usage Artemisinins during pregnancy is EU collaborative project; Coordinator: Professor S. A. Ward; Liverpool School of Tropical Medicine).

ABSTRAKT

Artemisin a jeho deriváty, především artesunat, jsou nejdůležitější skupinou léků používaných v klinické praxi v léčbě malárie. Významná a charakteristická je pro ně endoperoxidická skupina. WHO doporučuje tyto látky v kombinaci s jinými déle působícími antimalariky (lumefantrinem, piperaquinem) jako léky volby.

Jelikož ve struktuře těchto látek chybí UV nebo fluorescentně detekovatelné chromofory, dřívější metody pro stanovení těchto látek využívaly post-column on line derivatizaci nebo elektrochemickou detekci v redukujícím módu. Tyto metody ovšem vykazovaly malou selektivitu a sensitivitu.

V rámci této práce byl proveden kompletní vývoj LC-MS metody pro stanovení artesunatu a jeho hlavního metabolitu dihydroartemisinu v biologických vzorcích. Vývoj zahrnoval tyto části: ladění ESI – triple quadrupole hmotového spektrometru a optimalizaci chromatografických podmínek, především pak pH a složení mobilní fáze.

Artesunat (ARST) a dihydroartemisin (DHA) byly stanovovány v lidské plasmě za použití artemisinu jako vnitřního standardu. Různé úpravy vzorků plasmy, a to liquid-liquid extrakce a protein precipitace, byly optimalizovány a porovnány. Na základě prevalidačních údajů byl zjištěn spodní limit kvantifikace při úpravě liqud-liquid extrakcí 2,5 a 3,0 ng/ml pro DHA a ARST za použití 400 µl plasmy. Při úpravě vzorků protein precipitací dosahuje spodní limit kvantifikace 8,0 ng/ml pro ARST i DHA za použití 300 µl plasmy.

Další studie v této práci byla zaměřena na vliv železa hemu a volného železa jako zdroje odpovědného za degradaci těchto látek v průběhu jejich stanovení v biologickém materiálu. Možná řešení, kterými lze předejít degradaci těchto látek v průběhu jejich analýzy, jsou v této práci navrženy. Studie tohoto typu je nepostradatelná, má-li být vyřešen problém při stanovení těchto látek ve vzorcích krve/plasmy od pacientů infikovaných malárií, v jejichž krvi jsou produkty hemolýzy přítomny ve velkém množství. Důležitost této studie spočívá především pak v uplatnění poznatků pro LC-MS stanovení artemisinových derivátů v (homogenizovaném) potkaním embryu, které obsahuje velké množství hemoglobinu a také myoglobinu.

V rámci studie případných rizik spojených s podáváním těchto látek v těhotenství, především pak v prvním trimestru, je jejich stanovení v potkaním embryu nepostradatelné. („The safety of usage Artemisinins during pregnacy“ je projektem EU, koordinováno Prof. S. A. Ward; Liverpool School of Tropical Medicine).

1. Theoretical Part

INTRODUCTION

Malaria is one of the leading causes of morbidity and death worldwide, infecting 300 – 500 million people and killing 1.5 to 2.7 million people (usually children) per year[1].

Malaria is caused by obligate intracellular protozoan parasites of the genus Plasmodium, Sporozoa class. There are four species of human malarial parasites; *P. vivax, P. malariae, P. ovale and P. falciparum*, last named causes the most severe and fatal disease[2].

Most cases and deaths are in Sub-Saharan Africa. However, Asia, Latin America, the Middle East and parts of Europe are also affected. Additionally, travelers from malaria-free regions going to areas where is malaria transmission are even more highly vulnerable – they have little or no immunity and are often exposed to delayed or wrong malaria diagnosis when returning to their home country[3].

Artemisinin and its derivatives have become essential component in antimalarial treatment. These plant-derived peroxides are unique among antimalarial drugs in killing the young intraerythrocytic parasites, thereby preventing their development to more pathological mature stages. Artemisinin combination treatments are now first line drugs for uncomplicated falciparum malaria, the multi-drug resistant strain[4,5].

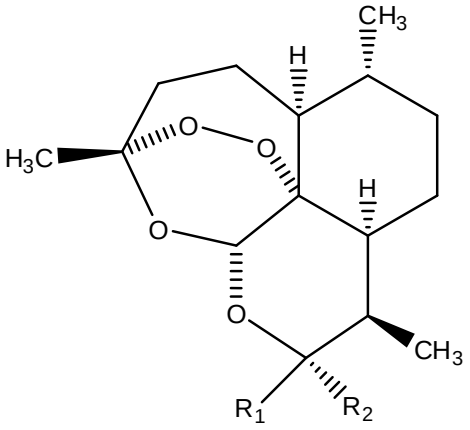
1.1 ARTEMISININ DERIVATIVES

1.1.1 History, General Information

Artemisinin is extracted from herb *qinghao,* which has been documented in traditional Chinese pharmacopoeia for treatment of fevers for over two thousands of years. Once a structure of artemisinin was solved in 1972, a lot of derivatives were prepared to improve its properties, e.g. solubility in oil and water [6].

These derivatives *(Fig.1)* now comprise the most important class of antimalarial and have a profound impact on modern antimalarial treatment regimens[6].

Chemically, Artemisinin is a sesquiterpene – a natural product, containing 15 carbon atoms, which is biosynthesized in *Artemisia annua* from mevalonic acid via dimethylallyl and isopentenyl pyrophosphates.[6] It contains a peroxide bridge, which is essential to its antimalarial activity[7].



R_1	R_2	
	=O	QHS
H	-OH	DHA
H	$-OCOCH_2CH_2COOH$	ARST

Fig 1.; Structure of artemisinin derivatives

1.1.2 Physical chemical properties

1.1.2.1 Artemisinin

Synonyms: Arteannuin; Artemisinina; Artémisinine; Artemisinine; Artemisininum; Huanghuahaosu; Qinghaosu

Chemical Name: (3R,5aS,6R,8aS,9R,12S,12aR)-Octahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4,3-j]-1,2-benzodioxepin-10(3*H*)-one

Molecular Formula: $C_{15}H_{22}O_5$

Molecular Weight: 282.3

CAS Registry: 63968-64-9

ATC: P01BE01[8]

Melting point: 154 – 157°C [9, 10, 11]

1.1.2.2 Artesunate

Synonyms: Artésunate; Artesunato; Artesunatum

Chemical Name: (3R,5aS,6R,8aS,9R,10S,12R,12aR)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano-[4,3-j]-1,2-benzodioxepin-10-ol hydrogen succinate

Molecular Formula: $C_{19}H_{28}O_8$

Molecular Weight: 384.4

CAS Registry: 83507-69-1; 88495-63-0; 182824-33-5

ATC: P01BE03[8]

1.1.2.3 Dihydroartemisin

Chemical Name: (3S,3aR,4S,5aR,9bS)- Naphtho[1,2-b]furan-2,8(3*H*,4*H*)-dione, 3a,5,5a,6,7,9b-hexahydro-4-hydroxy-3,5a,9-trimethyl

Molecular Formula: $C_{15}H_{20}O_4$

Molecular Weight: 284.3

CAS Registry: 108739-44-2

1.1.3. Usage and different combination of AR derivatives

Beyond those compounds named above, current literature discuss another artemisinin derivatives, e.g. Artemisone (which can be even more effective against multi-drug resistant *P. falciparum*[12]) and also "The second generation artemisinin analogs" [13].

Due to rapid elimination (short half – lifes), it is convenient to combine these compounds with other antimalarial drugs with slower onset of action, to prevent recrudescence and resistance[14].

The combination with methylene blue, the first synthetic antimalarial drug, was tried with succes in randomized control trial[15]. Also the artemisinin – quinine hybrid joint by ester linkage prove a good activity[16].

Different combinations with azitromycin,[14] amidoquine and lumefantrine have been already using with good results in many medical preparations. The combination of artemether and lumefantrine is also registred in Czech Republic and Sweden[8].

1.1.4 Pharmacokinetic and pharmacodynamic properties

Artemisinins act more rapidly than other types of antimalarials, both in killing parasites and inhibiting their metabolic processes. Additionally, they attack the broadest age range of parasites[6].

Artemisinins can be administered by several routes, oral, intramuscular, intravenous and even intra-rectal routs are possible. Common oral dose of artesunate is 200 mg, intravenous dose is 120 mg[17,18].

Artemether and artesunate are rapidly metabolized to dihydroartemisinin, the active metabolite, which could undergo rapid glucuronidation. And after that, the conjugated polar product is excreted in bile[6, 7].

Elimination half-life differs from about 2 hours (h) for artemisinin, 3-11 h for artemether, less than 1 h for artesunate and 3 h for dihydroartemisinin[7].

Oral artemisinins are rapidly however incompletely absorbed within uncomplicated malaria. Bioavailability differs from about 10% for artemisinin, 54% for artemether, 82% for artesunate and 85% for dihydroartemisinin. These derivatives are also highly protein-bounded (more than 80%) and suffer from extensive first-pass effect[6, 7].

1.1.5. Mechanisms of action

Artemisinins act via distinct mechanisms from other antimalarial classes. The exact mechanism of action is still a subject of debate. Nevertheless, it is known that peroxide linkage within 1,2,4-trioxane system is essential for antimalarial activity. It is responsible for generation of reactive oxygen species (ROS). The formation of ROS is enhanced through the Fe^{2+}- dependent Fenton process[6].

These radicals can be responsible for alkylation of essential proteins of *Plasmodia* and also for inhibition of hemin polymerisation into for parasite non-toxic hemozoin[19].

1.1.6 Possible toxicity

Although, artemisinins are clinically effective and safe antimalarials, they are not recommended during the first trimester of pregnancy because of the abnormalities observed in animal reproduction studies [20].

Study on both rat and frog embryo[21] showed that primitive red blood cells from yolk sac are the target of artemisinins, which results anemia. So that oxidative stress (mechanisms of action) may be involved in artemisinins toxicity[20].

Additionally, it should be considered differences between rodents and humans. In particular, early hematopoesis takes only 7 days in rats compared with 6 weeks in humans[20]. The susceptibility and duration of exposure must be considered to evaluate the clinical relevance of these toxicity data[21].

Some articles also discuss possible neurotoxicity of these compounds[22] and embryotoxicity that seems to be connected also with defective angiogenesis and vasculogenesis[23].

Despite of these side effects, there are many clinical trials which proved safety and efficiency of these compounds[24]. However, there are not enough data about effects of these compounds during early pregnancy. Extensive investigations aimed particularly at determination of tissue distribution are essential to gather more information about safety of this class of antimalarial drugs.

1.1.7 Alternative usage of AR derivatives

Artemisinins also possess an activity against helminth trematode infections. It is discussed the potential impact that artemisinin-based combination therapy might be used on trematode infections in settings where malaria and helminthiases are co-endemic[25].

The anti-angiogenesic activity of artemisinins derivatives has also been using for inhibition of tumor cell growth *in vitro*[26,27].

1.1.8 Stability of AR derivatives within process of isolation from biological matrices

AR derivatives undergo rapid degradation in contact with products of haemolysis. [28] This was also proved in article[29] where two sources of degradation were expressed; haemolytic plasma and haemolysed products in sample (sourced by malaria infection).

It is crucial to solve the problem caused by effect of haemolysis when an LC-MS method should be used for investigation in rat`s embryo. (This tissue would be probably homogenized because of its tinny size that does not allow isolation of pure plasma).

Lindegardh et al.[29] suggested the way how to avoid AR degradation in plasma samples caused by haemolysis. They postulated a hypothesis that degradation occurs after addition of organic solvent, particularly those used to precipitate proteins. Organic solvent free SPE method described in Lindegardh`s article[29] gave good results with percentage of haemolysis up to 2 %. Moreover, addition of acetic acid that decreases the pH of sample extensively was in that method skipped. This step was not discussed at all, even though it plays very important role in degradation of AR derivatives.

It is also reported that AR derivatives undergo rapid degradation in the presence of ferrous iron (Fe^{2+}) *in vitro* but are relatively stable in the presence of ferric iron (Fe^{3+}) (i.e. in absence of reducing agents[29, 30]).

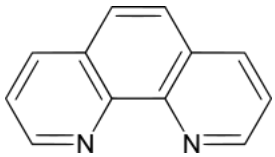
Solution of ferrous iron (Fe^{2+}) are unstable, because these ions are easily oxidized to ferric iron (Fe^{3+}) even with air oxygen, whereas acid solutions of ferrous iron (Fe^{2+}) are stable. Oxidation reduction potential for pair Fe^{3+}/Fe^{2+} is + 0,771[31].

Additionally, it is known that iron plays essential role in mechanisms of the antimalarial action.

1.1.8.1. Iron chelators

Iron chelators such as desferioxamine, deferipone, drugs used in clinical practice to bond iron have shown inhibition of AR derivatives antimalarial activity *in vitro*[29, 30, 32]. These chelators and EDTA form stable complexes with ferric ions, however not with ferrous ions.

Steady complexes with ferrous ions (Fe^{2+}) are formed with phenantroline (PHEN), the stability constant of complex $[Fe^{3+}(phen)_3]^{3+}/[Fe^{2+}(phen)_3]^{2+}$ is 1,12[31]. As it is apparent from formula, Fe^{2+} made exclusively tris-complexes.



Structure of PHEN

1.2 METHODS FOR ARTEMISININS DERIVATIVES DETERMINATION

Sensitive methods for determining AR derivatives in biological fluids are needed in therapeutic drug monitoring, pharmacokinetic, metabolic and tissue distribution studies. Gas chromatography (GC) as the analytical approach has proven to be problematic since these compounds are thermally labile[33]. Despite this fact, one GC/MS method had been developed and validated[34], however with less satisfying validation data. High-performance liquid chromatography (HPLC) seems to be method of choice. Various type of detection using this approach are discussed below.

As the main skeleton of these compounds does not contain an ultraviolet (UV), visible or fluorescent chromophore, post-column derivatisation is needed prior to the UV detection[35], however this approach lacks sensitivity[33].

Another approach, however with poor sensitivity, utilized supercritical fluid chromatography with electron-capture detection[33].

The technique that best meets the sensitivity and specificity requirements is HPLC with electrochemical detection in reductive mode system (HPLC-ECD) [36, 37]. The limitation of

this approach is expensive and dedicated equipment. In addition, the mobile phase and flow path must be strictly maintained oxygen-free due to the reductive operation mode[33].

The high sensitivity and selectivity of mass spectrometry have opened the way to analytical methods based on the LC/MS. LC/MS methods either based on atmospheric pressure chemical ionization (APCI)[38,39] or electrospray ionization (ESI) for determination of AR have been already published [35].

Radiochromatographic detection was exploited as well in a recent HPLC study aimed particularly at determinating the ratio of α/β dihydroartemisinin in vivo and evaluating the protein binding ability of this compound[35].

1.3 MASS SPECTROMETRY/ LIQUID CHROMATOGRAPHY; HPLC/MS

Mass spectrometr (MS) is a powerful detector for separation techniques like gas chromatography (GC), high performance liquid chromatography (HPLC), capillary electrophoresis and supercritical fluid chromatography because of its high sensitivity and ability to identify chemical compounds positively. The challenge in interfacing the mass spectrometer to a separation system is maintaining the required vacuum in the mass spectrometer while introducing flow from the chromatograph. Interfaces containing restrict or reduce the gas flow have made the combination of gas chromatography and mass spectrometry (GC/MS) a widely used technique for years. Now, the use of capillary GC permits even direct connection to MS[40].

In 1973, there was the first attempt of connection liquid chromatography and mass spectrometry; HPLC/MS (Baldwin, McLafferty)[41]. This technique is more demanding. When vaporized the solvent from a liquid chromatography represent a volume of 100 – 1000 times greater than of carries gas in gas chromatography[40]. In addition, there are different modes under these two instruments work, once there is high pressure in HPLC, in difference to high vacuum in MS.

The primacy advantage of LC/MS compare to GC/MS is the capability to analyze much wider range of components[42], including thermally labile ones.

When LC/MS method is used, it is very important to define the type of instrument, ionization technique and the conditions within the process of analysis. Hence the spectrums reached by LC/MS cannot be compared with the library of spectrums of the same compound, as it is possible using GC/MS[41].

The schema of HPLC/MS connection is shown at *Fig. 2.*

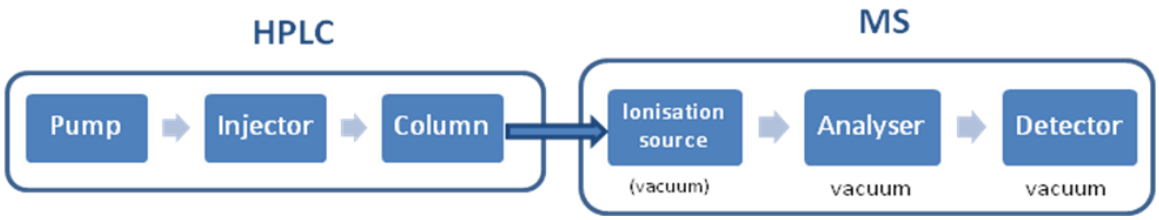


Fig. 2

1.4 MASS SPECTROMETRY; MS

Mass spectrometer is an analytical tool based on measuring molecular mass of a sample[43]. MS analysis does not require any previous chemical modification of the analyte, any unique or specific chemical properties. In theory, MS is capable of measuring any gas-phase molecule that carries a charge, ranging in size from H^+ to macromolecules. This technique is essential for identification of molecular mass, determination of structure, determination of elemental composition and isotopic composition and for quantification[44].

1.4.1 Mass spectrometer

A mass spectrometer is an instrument that measures the masses of individual molecules that have been converted into ions, i.e., molecules that have been electrically charged. The instrument does not actually measure the molecular mass directly, but rather the mass-to-charge (m/z) ratio of gas-phase ions by subjecting them to known electric or magnetic fields and analyzing their resultant motion.

The ionization source imparts energy into the sample in order to drive conversion to gas - phase (if necessary) and ionization of molecule. The sample is evaporated or sublimed into the gas phase, usually by heating.

The mass analyzer manipulates the motion of the ions. The separated ions are detected and this signal is sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of a m/z spectrum.

The analyzer and detector of the mass spectrometer are maintained under high vacuum in order to the ions can easily travel from one end of the instrument to the other without any collision with molecules of air[40,43,44].

As it is apparent from the *Fig. 2* above, MS consists of three main parts:

- *Ionization source*
- *Analyzer*
- *Detector*

1.4.1.1 Ionization source

Many ionization methods are available and each has its own advantages and disadvantages. Individual method used should depend on the type of sample under investigation and the mass spectrometer available.

Within the act of ionization, both positive and negative ions are generated in the ion source at the same time, only one polarity is recorded at a time. Molecules that are not ionized and neutral fragments are pumped away[40,43].

Ionisation methods generally used are listed bellow:

- Chemical Ionisation (CI)
- Electrospray Ionisation (ESI)
- Fast Atom Bombardment (FAB)
- Matrix Assisted Laser Desorption Ionisation (MALDI)
- Atmospheric Pressure Photo Ionisation (APPI)
- Electron ionization (EI)
- Field Desorption / Field Ionisation (FD/FI)
- Thermospray Ionisation (TSP)
- Atmospheric Pressure Chemical Ionisation (APCI)

Two most common ionization techniques used for HPLC/MS as interface, are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) [42].

Electrospray ionization; ESI

It is an atmospheric pressure ionization that enables MS detection even of large, non-volatile molecules with no fragmentation (Nobel Prize 2002). It is conveniently coupled to liquid separations[44].

This interface must to move ions from solution to the gas phase and after that to transfer the gas-phase ions from atmospheric pressure to vacuum. It is supposed to yield ion beam with maximum current and minimum kinetic energy distribution [42].

Mechanisms of ESI ionisation

The analyte is introduced to the source at flow rates up to 1ml min^{-1}. But usually the flow rates about 0.1-0.4 ml/min are used. Higher flow rates may require nebulizing gas (N_2) to form droplets. The analyte solution flow passes through the electrospray needle that has a high potential difference (with respect to the counter electrode) applied to it (2.5 - 4 kV), that induces a strong electric field ($10^6 – 10^7$ Vm^{-1}). These forces spray charged droplets from the needle with a surface charge of the same polarity. Repulsions between adjacent cations combined with the pull of the cations towards the grounded MS inlet cause the surface to expand into a so-called 'Taylor cone.'

As the droplets traverse the space between the needle tip and the cone, solvent evaporation occurs. This is circled on the *Fig.3* and in higher resolution in *Fig.4*.

As the solvent evaporation occurs, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge (*the Rayleigh limit*) at which point a "Coulombic explosion" occurs and the droplet is ripped apart. This produces smaller droplets that can repeat the process as well as naked charged analyte molecules. These charged analyte molecules (not strictly ions) can be singly or multiply charged.

This method of ionization is very soft (very little, usually no fragmentation is produced) as very little residual energy is retained by the analyte upon ionization[42, 44].

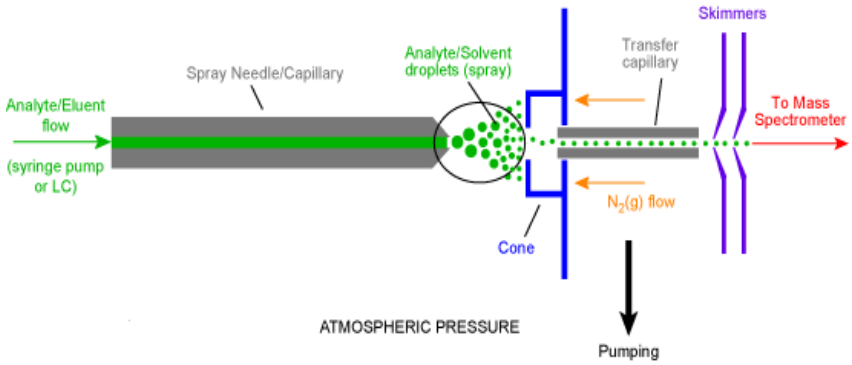


Fig. 3; Schema of an ESI interface[42]

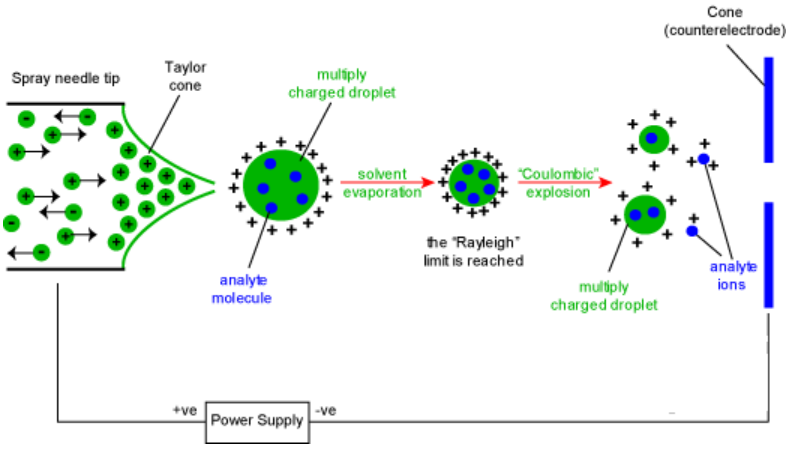


Fig. 4; Schema of the mechanisms of ion formation[42]

Atmospheric pressure chemical ionization; APCI

In contrast to chemical ionization (CI), this method is achieved at atmospheric pressure and it is primary applied for ionization of low mass compounds, but it is not suitable for thermally labile compounds. It is also a soft but more intensive technique than ESI. The general source set-up shares a strong resemblance to ESI.

APCI uses flow of N_2 to convert eluate into a fine aerosol mist from which solvent and analyte evaporate. The high voltage is applied to a metal needle in the path of the aerosol that caused an electric corona around the needle and during sequence of reactions ions are created[38,41,42,45].

1.4.1.2 Analyzer

The analyzer uses dispersion or filtering to sort ions according to their mass-to-charge ratios or a related property.

The most widely used analyzers are:

- Magnetic Sectors
- Time-of-flight Mass Analyzers
- Trapped-Ion Mass Analyzers like Fourier Transform Ion Cyclotron Resonance (FT - ICR) and Quadrupole Ion Traps
- Quadrupole mass Filters[40]

Each of the analyzers has different features, like the m/z range that can be covered, the mass accuracy, and the achievable resolution. Additionally, compatibility of particular analyzer with different ionization methods varies. For example, all of the analysers listed above can be used in conjunction with electrospray ionisation, whereas MALDI is not usually coupled to a quadrupole analyser[43].

Quadrupole/ Quadrupole mass filter analyzer

Quadrupole mass filter, consists of four parallel poles/rods. In this device (*Fig. 5*), mass sorting depends on ion motion resulting from simultaneously applied constant (dc) and radio frequency electric (rf) electric fields. Scanning is accomplished by systematically changing the field strengths, thereby changing the m/z value that is transmitted through the analyzer[40].

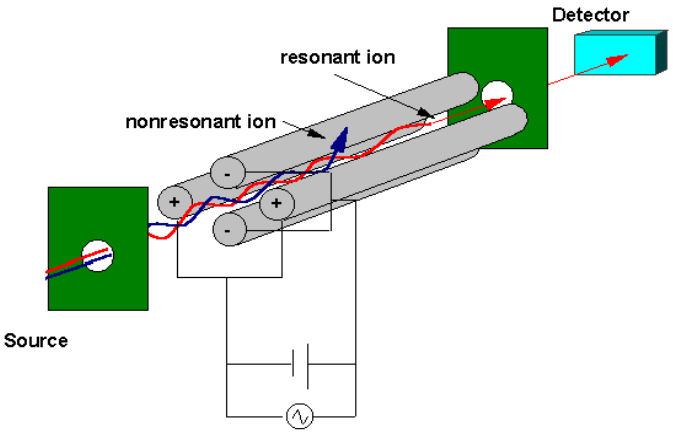


***Fig. 5*:** Ions with the selected mass-to-charge ratio pass through the analyzer to be collected at the detector (resonant ions), while ions with other m/z values collide with or escape between the rods (non-resonant ions) [40, 46].

The quadrupole provides classical mass spectra with good reproducibility and relatively small and low-cost systems.

However, there is limited resolution and peak heights variability as a function of mass (mass discrimination).

This device is applied in majority of benchtop GC/MS and LC/MS systems, sector/quadrupole hybrid MS/MS systems and triple quadrupole MS/MS systems[40, 46].

Tandem (MS/MS) mass spectrometers

Tandem (MS/MS) mass spectrometers are instruments that have more than one analyzer and so can be used for structural and sequencing studies. Two, three and four analyzers have all been incorporated into commercially available tandem instruments. The analyzers do not necessarily have to be of the same type, in this case the instrument is a hybrid one. More popular tandem mass spectrometers include those of the quadrupole-quadrupole, magnetic sector-quadrupole , and more recently, the quadrupole-time-of-flight geometries[43].

Coupling two stages of mass analysis (MS/MS) can be very useful in identifying compounds in complex mixtures and in determining structures of unknown substances[40].

Additionally, modern soft ionization techniques, like ESI or MALDI provide spectra containing only the ionized molecules with very little fragmentation. In these cases, induced fragmentation is required using collision induced dissociation (CID). There are also other methods of fragmentation within tandem mass spectrometry (MS/MS)[42, 47].

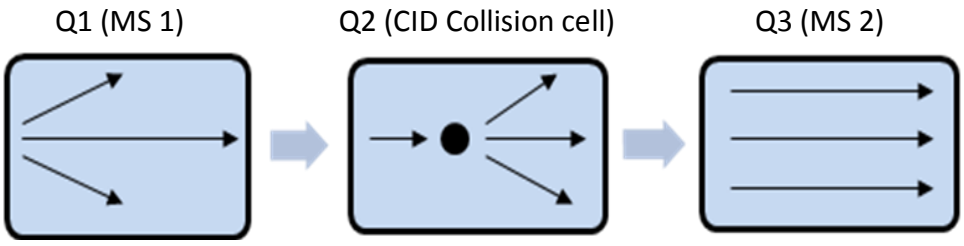
Triple quadruplole

Triple quadrupole (QQQ) is one of the most commonly available tandem mass spectrometers. It is a linear series of three quadrupoles, the first (Q1) and third (Q3) quadrupoles act as mass filters, and the middle (Q2) quadrupole is employed as a collision cell[42].

There are several types of experiments that can be performed using QQQ.

Fig. 6 represents a schema of QQQ and its mechanisms[46].

Fig. 6: Product ion scan[46]; the precursor ion is focused in Q1 and transferred into Q2 - the collision cell - where it interacts with a collision gas and fragments. The fragments are then measured by scanning Q3. This results in the typical MS/MS spectrum.



1.4.1.3 Detector

The mass analyzer sorts the ions according to m/z and the task of detector is to record the abundance of each m/z[48].

In all mass spectrometers except of FT-ICR instruments, the ions are detected after mass analysis by converting the detector-surface collision energy of the ions into emitted ions, electrons, or photons that are then sensed with various light or charge detectors. In FT-ICR mass spectrometers the oscillating signal induced by orbiting ions in the detect plates is used for ion detection[40].

Following detector properties are preferred:

High amplification, fast time response, low noise, high collection efficiency, low cost, narrow distribution of responses, same response for all masses, large dynamic range, long term stability, long life and mounted outside of the vacuum if possible[48].

The most frequent used detectors are listed below:

- Faraday Cup (Direct Current Detection)
- Electron Multiplier Notes
- Continuous Dynode Electron multiplier
- Array Detector
- Conversion Dynode[48]
- Postacceleration (Daly Knob) Detector
- Photomultiplier Detector Notes
- Microchannel Plate Detector
- Multichannel Plate (MCP) Detector

1.5 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography is nowadays the most frequently used analytical technique for drug analysis. It is a separation method that enables qualitative and quantitative assay of mixture of analytes. This method is commonly used for research and development of new drugs and medical preparation as well as in drug control and quality assurance. It is the method of choice for assay of drugs and their metabolites in biological matrices for investigation of pharmacokinetic data[45, 49].

In contrast to GC, HPLC is capable to asses wider range of analytes particularly ones that are not volatile [45, 49].

HPLC methods are widely used for qualitative and quantitative assay.

qualitative assay of drugs using HPLC is based on the same retention times (T_R) of analyte and referential standard. Uniformity of retention times do not define the analyte positively, usually further information like spectra from diode array or infrared or mass spectrometer are needed[45, 49, 50].

For **quantitative assay** of drugs and other related compounds is usually used the method based on comparison of peaks areas (or peaks heights) of analyte assayed with referential compound. There are several approaches, the ones that are used most often are method of internal and external standard[45, 49, 50].

1.5.1. HPLC Instrumentation

The modern HPLC instruments consist from following parts: pump, mobile phase degasser, autosampler with injection valve, column thermostat, detector. These parts are like a jigsaw built into HPLC instrument[49].

1.5.1.1. Pump

The quality of a pump for HPLC is measured by how steady and reproducible a flow can produce. A fluctuating flow rate leading to a detector noise that obscures weak signals. Standard pumps for HPLC systems have these properties: constant flow rate up to 10 ml/ min at pressures up to 40 MPa (400 bar) and increments up to 1 % (usually electronically

controlled in 0,1 incerments). The gradients made up from two or more solvents of mobile phase can be made either at low pressure or high pressure of the pump[45, 49].

1.5.1.2. Mobile phase degasser

Gas bubbles in the mobile phase disturb a fluent flow rate and thus it has a great impact on the process of separation and it can deteriorate a signal of detectors. Therefore just degassed mobile phase can lead to reproducible results[45, 49].

1.5.1.3. Autosampler

This part of HPLC system enables injection of variable numbers of samples (usually up to 100 pcs.). The samples in the autosampler are placed in the try which can be temperature-controlled (i.e. cold to prevent samples degradation).
The samples are injected via the injection valve [45, 49].

1.5.1.4. Detector

The function of detector is to monitor compounds eluted from chromatographic column and transfer their quantitative values on proportional dependent signal. Detector used has a great impact on sensitivity and selectivity of analysis.
An ideal detector of any type is sensitive to low concentrations, selective, provides linear response and does not broaden the eluted peaks. It is also resistant to changes in temperature and solvent composition. It is valuable if it is able to provide qualitative information about analyte[45, 49].

a) Spectrophotometric detector

Spectrophotometric detector is the most common HPLC detector, because many analytes absorb ultraviolet light. Requirement for this type of detection is presence of chromophore.
The concentration is assayed according to Lamber-Beer law.
More versatile instruments (nowadays standards instrument) enable to choose the optimL wavelength for the analyte detection from the whole UV/ViS range. The specific type of

spectrophotometric detector is diode array detector which enables to record complete ultraviolet spectrum during whole run.

The sensitivity which can be reached by this detector is about $10^{-9} – 10^{-10}$ g/ml[45, 49].

b) Fluorescence detector

Fluorescence detector excites the eluate and measure the fluorescence emitted by the analytes.

These detectors are sensitive and selective, it can be reached the limits up to 10^{-12} g/ml.

However, only few analytes respond to fluorescent, this problem can be issued by the process of derivatization[45, 49].

c) Electrochemical detector

An electrochemical detectors responds to analytes that can be oxidized or reduced. They reach the sensitivity between $10^{-9} – 10^{-12}$ g/ml.

There are several restriction that narrow the usage of this type of detectors[45, 49].

d) Refractive index detector

It measures the difference between refractive index of pure mobile phase and mobile phase with analyte.

A refractive index detector responds to almost every solute, but its detection limit is relatively low (10^{-6} g/ml). There are further disadvantages of this type of detector, like incompatibilty with gradient elution and great sensitivity to temperature changes[45, 49].

e) Conductivity detector

The presence of analyte is measured by a change of conductivity in the conductivity cell. These detectors are used for ion-exchange chromatography[45, 49].

f) Evaporating Ligh-Scattering detector

An evaporating ligh-scattering detector responds to any analyte that is significantly less volatile than the mobile phase. The eluate from column is forced to form a uniform

dispersion of droplets. Solvent evaporates and the solid particles are detected by the light that they scatter from a diode laser to a photodiode[45, 49].

g) Mass spectrometer detectors

This type of detector is discussed in paragraph 1.4.

1.5.1.5. HPLC sorbents and columns

The HPLC columns are usually made of steel. The common analytical columns have 5 – 25 cm in length and inner diameter of 1 – 4,6 mm. The entrance to the main column is usually protected by a short guard column. There is the stationary phase inside the column. The most common stationary phase is highly pure, spherical, microporous particles of silica with common diameter of 3 – 5 μm. By silica silanol groups are covalently attached various chemical groups, therefore bounded stationary phases are made (these are commonly used for HPLC) [45, 49, 50].

These chemical groups mostly are:

- Amino (-NH_2), cyano (-CN), diol (as representatives for common polar phases)
- Octadecyl, octyl, phenyl (as representatives for common nonpolar phases)

Another example of a different kind of stationary phase is a porous graphitic carbon deposited on silica.

For separation of optical isomers, many optical active groups bonded to silica are available[45, 49, 50].

Besides the silica-based stationary phase, columns built from other materials (e. g ZrO_2 or polymers) are also available on the market[49].

Chromatography on bare silica is an example of **normal-phase chromatography** (on polar stationary phases)[45, 49, 50].

Reversed-phase chromatography is the more common scheme in which the stationary phase is nonpolar or weakly polar. In this case, less polar solvent has a higher eluent strength. The most common organic solvent used are methanol, acetonitril and tetrahydrofuran (THF).Their eluent strength decreases in succession THF > ACN > Met OH[45, 49, 50].

HPLC analysis can be performed using the constant content of mobile phase – **isocratic elution**. If one solvent does not provide sufficiently rapid elution of all compounds, then **gradient elution** can be used. In this case an other solvent is added to mobile phase according to default program in order to create a continuous gradient[45, 49, 50].

1.6 SAMPLE PREPARATION

Sample preparation prior to HPLC analysis is an essential part of bioanalysis. It enables to get homogenous solution of assayed compounds which can be injected to chromatographic column. The most commonly used biological materials are plasma, serum, blood, erythrocytes, urine or feces. Main reasons why sample preparation is so important are[49, 51, 52]:

- To remove all interferences and impurities from sample
- To prevent column damage
- To make assayed sample compatible with HPLC method used
- To enable concentration or derivatisation of sample (if necessary)[49, 51]

The ideal method of sample preparation should be fast, easily automatized, cheap and compatible with wide range of analytical techniques.

The most common sample pretreatment techniques used in the drug bioanalysis are deproteination, liquid-liquid extraction and solid-phase extraction[49, 51].

Among other sample pretreatment methods belong solid phase microextraction, technique of column switching, supercritical fluid extraction and liquid phase microextraction[49, 51].

Protein precipitation and liquid-liquid extraction are further described below, these methods were used for experiments.

1.6.1. Deproteination

This technique is the easiest sample pretreatment method. Deproteination can be done by protein precipitation, enzymatic deproteination and ultrafiltration[49, 52].

Protein precipitation

In protein precipitation various agents are used to remove the proteins by denaturation and precipitation. These agents are water-miscible organic solvents, strong acids and salts of heavy metals or their combination. The most commonly used precipitating agents are organic solvents. Precipitative ability decreases in following order: acetonitrile > acetone > ethanol > methanol. Possible negative influence to analyte or interaction between analyte and precipitative agent (e.g. limited stability in acid pH, ability of complexes formation with metals) must be taken into account when particular agent is chosen[49, 51, 52].

1.6.2. Liquid-liquid Extraction

Liquid-liquid extraction is the direct extraction of the biological material with a water-immiscible solvent. The analyte is isolated by partitioning between the organic phase and the aqueous phase (biological material). The most important factor is the relative lipophilicity or hydrophilicity of the analyte, more lipophilic compounds can be transferred into organic solvent more easily. The organic solvent is then evaporated to dryness and the rest is usually dissolved in mobile phase and injected on the column.

The organic solvent used for LLE should have following properties:

- Low solubility in water (up to 10 %)
- Volatility
- Compatibility with HPLC detection
- Proper polarity for maximal recovery
- High purity to minimize contamination[49, 52]

According to Nernst distribution law, the compound is distributed between two immiscible solvents according to its distribution constant:

$$K_D = \frac{C_0}{C_{aq}}$$

K_D - distribution constant

C_0 - Concentration of the analyte in the organic phase

C_{aq} - Concentration of the analyte in the aqueous phase[49, 51]

A more useful expression is the fraction of analyte extracted (E) into organic phase:

$$E = \frac{K_D V}{1 + K_D V}$$

V_0 – The volume of organic phase

V_{aq} - The volume of aqueous phase

V – The phase ration $V_{0/}\ V_{aq}$[49, 51]

For one-step extractions K_D must be large (> 10) for the quantitative recovery and the phase ratio V must be also maintained within values of $0{,}1 < V < 10$.

To increase the value of K_D hence efficiency of extraction can be performed by:

- Change of organic solvent used
- Suppressing analyte`s ionization by adjustment of pH (according to Henderson–Hasselbalch equation), usage of ion-pair extraction reagent
- *Salting out* decreases an analyte`s concentration in the aqueous phase by addition of an inert salt (sodium sulfate) to the aqueous phase.
- (Adjustment of extraction time) [49, 51].

Typical extraction solvents for LLE are aliphatic hydrocarbons (hexane), ethers, Methylene chloride, chloroform, aliphatic alcohols, halogen hydrocarbons, etc. Apart from miscibility consideration, the main selection criteria is the polarity of the solvent in relation to that of the analyte. The maximum K_D values occur when the polarity of the extraction solvent matches that of the analyte. In some case *back extraction* is used to reduce interferences (from biological matrices)[49, 51].

Another form of the LLE is *microextraction* where ratio of organic/aqueous phases is 0,001 - 0,01. Analyte recovery may suffer compare to conventional LLE, but analyte is greatly concentrated in the organic phase[49, 51].

Common practical problems associated with LLE include:

- Emulsion formation
- Analyte`s strongly sorbed to particulates
- Analyte bound to high-molecular-weight compounds (proteins)
- Mutual solubility of the two phases
- Big consumption of solvents[49, 51]

2. Work Aim

I. The first aim of this thesis was to develop a novel sensitive and selective LC/MS method for determination of ARST and its major metabolite DHA in human plasma using QHS as an internal standard. The development involves tuning of MS detector and optimization of chromatographic conditions.

II. The second aim of thesis was to investigate the source of degradation of assayed compounds in plasma containing certain portion of haemolysed whole blood or Fe^{2+} cation; free iron.

The results of this part are of particular importance for LC/MS method development for analysis investigated compounds in rat`s embryo.

This work is part of EU collaborative project; The Safety Pharmacology of Artemisinins When Used to Reverse Pathophysiology of Malaria in Pregnancy (Coordinator: Professor S. A. Ward; Liverpool School of Tropical Medicine).

3. Experimental part

3.1. INSTRUMENTATION AND REAGENTS

3.1.1. The List of Instrumentation Used

- PE Sciex, API 365 LC/MS/MS system (Thurbospray with triple quadrupole) with Analyst 1.4.2 softaware
- Perkin Elmer, Series 200 Autosampler with Peltier tray
- Perkin Elmer, Series 200 Micro Pump
- Thermo Scientific Column, BETASIL Phenyl-Hexyl, 50 X 2,1, 5µm (with pre-column filter 2µm)
- Syringe pump; Harvard apparatus, using with Hamilton-Bonaduz, Schweiz, 100µml
- Millipore, ultrapure water system, Milli-Q plus, PF
- Nitrogen generator; Peak Scientific Instrumnet Ltd, 2008; 99,99 % purity
- ULTRAsonik 28x, for degassing
- EBA 20, Hettich Zentrigugen
- Eppendorf centrifuge, Mini Spin
- Vortex Gene2, Scientific industries
- DADE, multi-tube Vortexer
- pH meter, Denver instrument, Model 225
- Analytical scale; Mettler Toledo 8 (d=1 µg)
- Eppendorf pipets, Reference; 5000, 1000, 100
- Dissposible glass Pasteur pippets, 150 mm

3.1.2. The List of Reagents and Standard Compound Used

Name	*Supplier*	*Quality/ Batch*
Ammonium acetate	Fischer scientific	HPLC grade
EDTA dinatrium salt	Merck	HPLC grade
Ammonium bicarbonate	Fluka analytical	LC-MS grade
O-phenanthrolin hydrochloride monohydrate	Fluka analytical, Sigma-Aldrich	puriss .p.a, reag. Ph. Eur
Sodium fluoride	Fluka analytical, Sigma-Aldrich	HPLC grade
Iron(II) sulfate heptahydrate	Fluka analytical, Sigma-Aldrich	ACS reagent, ≥99.0%
Potassium oxalate monohydrate	Fluka analytical, Sigma-Aldrich	puriss .p.a.; >99,5% (RT)
Acetic acid glacial	Fisher scientific	HPLC grade
Acetonitrile	Fisher scientific	HPLC Far UV
Methanole	Fisher scientific	HPLC
1-chlorbutane	Fisher scientific	HPLC
Toluene	Fisher scientific	HPLC
5 % DMDCS in Toluene (Sylico CT)	Supelco Analytical	HPLC
Water	Produced by Millipore Milli-Q plus, PF	HPLC
Nitrogen	Nitrogen generator; Peak Scientific Instrumnet Ltd, 2008	>99,99 %
Hydrogen peroxide solution; 30-32 wt. % in water	Sigma-Aldrich	99.99%
Artemisinin	DK Pharma-Hanoi-Vietnam	DHN 120905
Dihydroartemisinin	DK Pharma-Hanoi-Vietnam	DHN 280905
Artesunate	DK Pharma-Hanoi-Vietnam	DHN 210905

3.2. LC/MS METHOD DEVELOPMENT, METHODS AND PROCEDURES

3.2.1. Instrumentation; Mass spectrometer

PE Sciex, API 365 triple quadrupole mass spectrometer (Applied Biosystems /MDS SCIEX, USA) with TurboIonSpray Ion Source (TIS) interface operated in positive ion mode, was used for multiple reaction monitoring (MRM) LC-MS/MS. All acquired data were processed by Analyst 1.4.2. software.

The mass spectrometric conditions were optimized for each assayed compound by infusion 10 μM standard solution in mobile phase (ACN – Ammonium acetate buffer 10 mM pH4, 50:50, v/v) at a flow rate of 10μl/min (using Hamilton syringe pump) connected directly to mass spectrometer.

An additional tuning optimization was performed by continuously infusing the same standard solution with same flow rate via "T" connector into the post-column mobile phase with final flow (150μl/min). The TIS temperature was finally set on 350°C and as TIS voltage was used value of 5000 V. The flow of curtain gas was set on 10 liters/min, nebulizer gas was set on 15 liters/min and the value of collision gas was 5 liters/min.

Final mass transition for each compound and additional instrument settings are mentioned in *Tab.1* below.

Tab.1:

compound	Q1 (mass) amu	Q3 (mass) amu	Time (msec)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
DHA	302.400	267.300	200.0	20.0	170.0	3.0	11.0	8.0
ARTS	402.500	267.100	200.0	12.0	124.0	2.5	12.0	7.0
QHS (IS)	300.360	209.150	200.0	14.0	135.0	1.5	14.0	7.0

Time; one-scan time

DP; Declustering potential

FP; Focusing potential

EP; Entrance potential

CE; Collision energy

CXP; Collision cell exit potential

3.2.2. HPLC Instrumentation and Conditions

The HPLC system consisted from Perkin Elmer, Series 200 Autosampler with Peltier tray set on 4°C, Perkin Elmer, Series 200 Micro Pump. The compounds were analyzed on Thermo Scientific Column, BETASIL Phenyl-Hexyl, 50 X 2.1, 5µm (with pre-column filter; 2µm). The final mobile phase used in this study consisted of ACN – ammonium acetate buffer 10 mM pH4 (50:50, v/v) with flow rate of 150µl/min (on isocratic program). Mobile phase was degassed in ultrasonic bath before used.

Injected volume was 20 µl (whole loop), washing solution used for pre and post-injection wash (1000 µl) consisted of methanol-water (50:50, v/v).

There was performed study using different pH of mobile phase; The T_r and probably even signal of ARST as a weak acid (pKa = 4.6) is extremely dependent on pH[53].

3.2.3. Preparation of Plasma Standards and I.S. (QHS)

Combined stock solution of DHA and ARST (1000 µg/ml) was prepared in methanol. This stock solution was diluted with a mixture of methanol-water (50:50, v/v) to concentration of 10 µg/ml in order to prepare working solution. 200 µl of this working solution were added to 9800 µl of blank human plasma in silanised tube, in order to prepare plasma standard solution of concentration 200 ng/ml, this solution was diluted with blank human plasma down to concentrations of 100, 10, 5, 2.5 ng/ml. Plasma solutions were prepared on and kept on ice until use, or aliquoted of 1500 µl into glass silanised bottles and stored at -80°C until use. Both stock and working solutions were stored in silanised glass at -80°C.

Blank human plasma was treated with CPS (anticoagulant based on citrate), plasma from two healthy donors was mixed in ratio 50:50.

Three solutions of I.S. (QHS) were prepared. The first one (for LLE) of concentration 600 ng/ml in methanol-water (50:50, v/v) was prepared by dilution of working solution (1000 µg/ml QHS in methanol) by mixture of methanol-water (50:50, v/v). The second and the third one (for PP) of concentration 150 ng/ml in ACN and 150 ng/ml in ACN-Met-OH (50:50, v/v) were prepared from working solution of 1000 µg/ml QHS in ACN

by serial dilution with ACN and ACN-Met-OH (50:50, v/v), respectively. Both working solutions and all the solutions of I.S. were stored in silanised glass at -80°C.

Glass silanisation was performed with 5 % DMDCS in toluene according to process described by its supplier - Supelco Analytical, in order to prevent binding of assayed compounds onto glass walls.

3.2.4. Preparation of Human Plasma Samples; Protein Precipitation

All plasma standards and blank plasma were defrosted and kept on ice within the process of sample preparation.

An eppendorf pipettes were used to place 300 µl of human plasma into 1.5 ml eppendorf tube. 100 µl of 1% acetic acid in water were added and finally 500 µl of I.S. (QHS) of concentration 150 ng/ml in acetonitrile-methanol (50:50, v/v) were added.

5 min of agitation were followed by 5 min of centrifugation at 13400 rpm using Eppendorf centrifuge. After that 300 µl of supernatant was put into glass vial which was caped and placed into autosampler with peltier tray, the temperature of tray was set on 4°C. The samples were analyzed after 16 hours in order to set desirable equilibrium between α and β-DHA (Just α-DHA is more dispose to form adducts with NH_4^+ ions).

3.2.5. Preparation of Human Plasma Samples; Liquid-liquid-Extraction

All plasma standards and blank plasma were defrosted and kept on ice within the process of sample preparation.

An eppendorf pipettes were used to place 400 µl of human plasma into 10 ml silanised glass test tube, 100 µl of I.S. (QHS) of concentration 600 ng/ml in Met-OH/H_2O (50:50, v/v) and 1000 µl of 1% acetic acid in water and 4000 µl of 1-chlorbutane as the extraction solvent were added.

10 min of agitation were followed by 10 min of centrifugation at 4000 rpm.

The organic (top, clear) layer was then removed with a glass pasteur pipette into a new silanised glass test tube. The organic phase was evaporated to dryness under steady stream of nitrogen gas, which was performed in a fume hood.

250 µl of mixture ACN-water (50:50, v/v) was added to dried sample and mixed for 20 seconds to ensure dissolution of the sample. The reconstituted sample was then transferred into glass vial and capped. The vials were placed into autosampler with peltier tray, the temperature of tray was set on 4°C. The samples were analyzed after 16 hours in order to enable epimerisataion to desirable equilibrium between α and β-DHA.

3.2.6. Pre-validation Data

This thesis was mainly focused on LC-MS method development, complete validation was not performed. Only selected "pre-validation" data have been acquiring within four days in order to check the ability of the method to enter full validation. LLOQ were estimated according to signal to noise ratio.

3.3. INFLUENCE OF HAMOLYSIS; IRON HAEM OR FREE IRON-METHODS AND PROCEDURES

Within this thesis it was assumed that in plasma samples with a portion of haemolysed blood is the most responsible agent for degradation ferrous iron originated from haem, particularly when pH is decreased. The proposed process of degradation (degradation on endoperoxide bridge of AR) could be expressed by this equation:

$$O_2^{2-} + 2Fe^{2+} + 4H^{+} \rightarrow 2Fe^{3+} + 2H_2O$$

It was performed study on plasma samples with some defined portions of haemolysed whole blood, on plasma samples with some defined portions of ferrous sulfate ($FeSO_4$. $7H_2O$) and on standard solutions of assayed compounds in ACN/H_2O using iron chelators in all mentioned cases.

3.3.1. Analytical procedure

All the studies mentioned bellow were performed with sample containing ARST and DHA at the concentration of 100 ng/ml. Plasma samples with 2% and 20% of haemolysis were prepared by mixture of plasma sample of ARST and DHA of concentration 200

ng/ml with blank human plasma containing 4% and 40 % of haemolysed whole blood respectively, in ratio 50:50 (v/v). Whole blood was obtained from healthy donor, blood was treated with citrate as an anticoagulant. Haemolysed blood was prepared by repeated freeze and thaw circle with vigorous mixing when blood was thawed.

In order to study influence of free iron on the drugs stability, plasma samples containing ARST and DHA (100 ng/ml each) and Fe^{2+} at the concentration of either 0.03 or 0.15 mg/ml, respectively were used. This amount of iron represent 5% and 25% of iron respectively, which is likely presented in whole blood (1 ml of whole blood contains 0,6 mg of iron, which is mainly built in haem[54]).

These plasma samples were prepared by mixture of plasma sample containing ARST and DHA (200 ng/ml each) and blank plasma spiked with $FeSO_4$. $7H_2O$ at concentration either 1.5 or 0.03 mg/ml in a ratio of 50:50 (v/v).

In the study focused on investigation of free iron influence on the drugs standard solution stability (part 3.3.4.Tab.4.), following standard solution was used. This solution contains ARST and DHA (100 ng/ml each) and free iron (0.03 and 0.15 mg/ml respectively) dissolved in ACN/H_2O (50:50, v/v). It was prepared by the same dilution like that used for plasma samples (see first paragraph).

3.3.2. Influence of Haemolysis and Free Iron; Protein Precipitation

All samples were treated with protein precipitation method as previously described. All samples (plasma, haemolysed plasma and plasma with free iron samples) were analyzed in triplicate and average of their signal is presented. As 100% signal intensity was taken the signal of plasma solution containing ARST, DHA (100 ng/ml each) only.

In experiment focused on effect of phennatroline, the solution of this compound was added to the plasma samples at first, mixed for 10 seconds using vortex and kept stand for 5 min. Thereafter, in the first part of the study 100 µl of 1% acetic acid of was added (according to protein precipitation procedure described in 3.2.4.), in the second part of the study acetic acid was replaced by 100 µl of water in order to sustain the same volume.

3.3.3. Influence of Haemolysis and Free Iron; Liquid-liquid Extraction

All samples were treated with liquid-liquid extraction as previously described. All samples (plasma, haemolysed plasma and plasma with free iron samples) were analyzed in triplicate and average of their signals is presented. As 100% signal intensity was taken the signal of plasma solution containing ARST, DHA (100 ng/ml each) only.

In experiment focused on effect of phennatroline, the solution of this compound was added to the plasma samples at first, mixed for 10 seconds using vortex and kept stand for 5 min. Thereafter, in the first part of the study100 µl of 1% acetic acid of was added (according to liquid-liquid extraction procedure described in 3.2.5.), in the second part of the study acetic acid was replaced by 1 ml of water in order to sustain the same volume.

3.3.4. Influence of Free Iron to Standard Solution of ARST and DHA

Influence of free iron to standard solution of ARST and DHA (100 ng/ml each) in ACN/H_2O is presented in Tab. 4. Reagents were added to standard solution in 1.5 ml eppendorf tube in succession as it is mentioned in Tab. 4., agitation after each addition was performed.

All samples (standards and standards with free iron) were analyzed in triplicate and average of their signals is presented. As 100 % signal is taken signal of standard solution of ARST, DHA (100 ng/ml each) with a small portion of acetic acid.

4. Results and Discussions

4.1. LC/MS METHOD DEVELOPMENT

4.1.1. Mass spectrometer; Tuning parameters

4.1.1.1. Results

Mass spectra of Q1 range and fragmentation of the most intensive ion are shown in *Fig.7.* (a) and (b) for QHS, (c) and (d) for DHA, (e) and (f) for ARST.

Ion with molecular mass 267 is formed frequently. Mass transition of ARST and DHA is particularly to this value of m/z. Proposed fragmentation pattern and structure of this ion is mentioned in article[39]; *Fig. 8.*

4.1.1.2. Discussion

Ammonium ion is essential since adducts with ammonium $[M+NH_4]^+$ reveals higher intensity than protonated molecular ion $[M+H]^+$. Formation of ammonium adducts is extremely stereo-selective regarding DHA. Just α-DHA is much more dispose to form ions with NH_4^+, particularly when ESI as an interface is used.

Equations below further explain formation of adducts with ammonium ions for each compound, it is mentioned molecular weigh in cycle brackets for illustration.

- $QHS_{(Mw=282)} + NH_4^+{}_{(Mw=18)} = [DHA + NH_4]^+{}_{(Mw = 300)}$
- $DHA_{(Mw=284)} + NH_4^+{}_{(Mw=18)} = [DHA + NH_4]^+{}_{(Mw = 302)}$

  $[DHA + NH_4]^+{}_{(Mw = 302)} - H_20{}_{(Mw=18)} = [XY]^+{}_{(Mw = Mw\ (DHA) = 284)}$
- $ARST_{(Mw=384)} + NH_4^+{}_{(Mw=18)} = [ARST + NH_4]^+{}_{(Mw = 402)}$

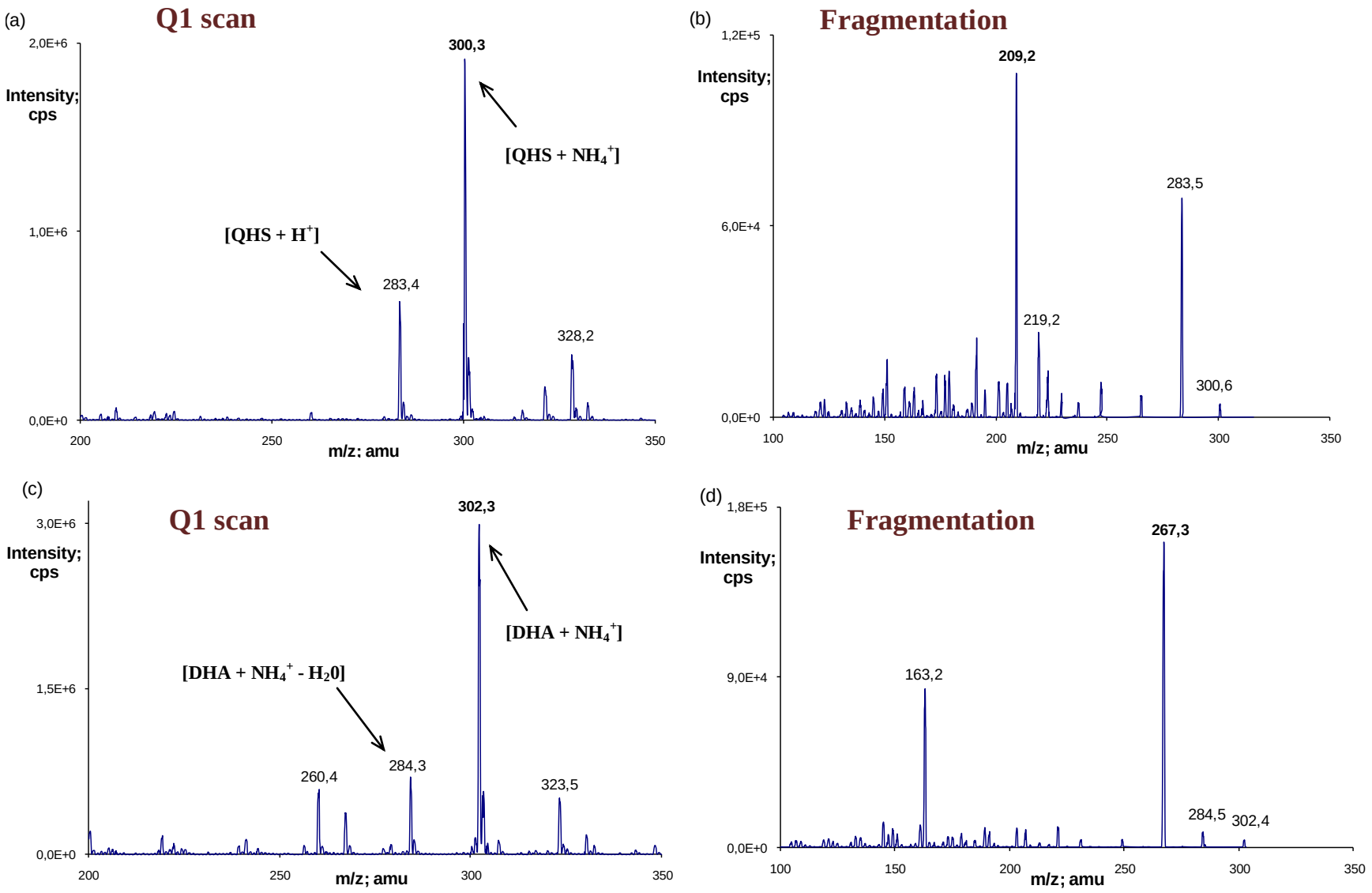

(a)
Q1 scan
2,0E+6
1,0E+6
0,0E+0
Intensity; cps
300,3
[QHS + NH4+]
[QHS + H+]
283,4
328,2
200
250
300
350
m/z; amu
(b)
Fragmentation
1,2E+5
6,0E+4
0,0E+0
Intensity; cps
209,2
283,5
219,2
300,6
100
150
200
250
300
350
m/z; amu
(c)
Q1 scan
3,0E+6
1,5E+6
0,0E+0
Intensity; cps
302,3
[DHA + NH4+]
[DHA + NH4+ - H20]
284,3
260,4
323,5
200
250
300
350
m/z; amu
(d)
Fragmentation
1,8E+5
9,0E+4
0,0E+0
Intensity; cps
267,3
163,2
284,5
302,4
100
150
200
250
300
350
m/z; amu

Fig. 7; (a), (b), (c), (d)

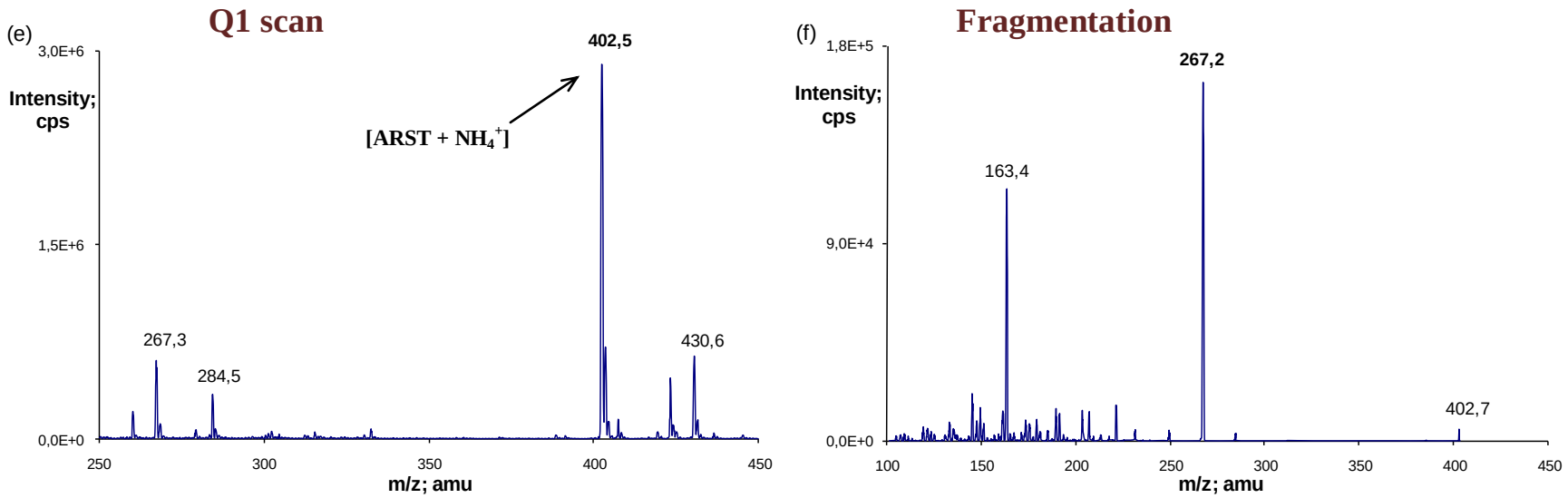


Fig.7; (e), (f)

ARST; Mw: 384 **DHA; Mw: 284** **Mw: 267**

Fig. 8: Proposed fragmentation pattern, structure of ion m/z = 267

4.1.2. HPLC Instrumentation and Conditions

4.1.2.1 Results

Final retention times were 2.7 min, 3.4 min and 4.7 min for DHA, ARST and QHS, respectively. Total time for analysis was 6.0 min.

4.1.2.2 Discussion

Fig. 9: Chromatogram of standard solution of DHA, ARST and QHS dissolved in acetonitril-water (50:50, v/v); blue: DHA; red: ARST; green; QHS

a) Mobile phase; Ammonium acetate buffer pH 5/ ACN (50:50, v/v). T_r of ARST and DHA are the same.
b) Mobile phase; Ammonium bicarbonate buffer pH 6.7/ ACN (50:50, v/v). ARST is eluated before DHA and also with higher signal. At this pH is ARST ionized. Smaller T_r makes sense taking into account that reverse phase column is used. However the signal at this pH was changeable.
c) Mobile phase; Ammonium acetate buffer pH 4/ ACN (50:50, v/v)
   It was also investigated if better signal would be reached using pH 3.5 as it is used in other methods[29, 53]. This value of pH leads just to lower signal (data not shown).

Just α-DHA is used for quantification.

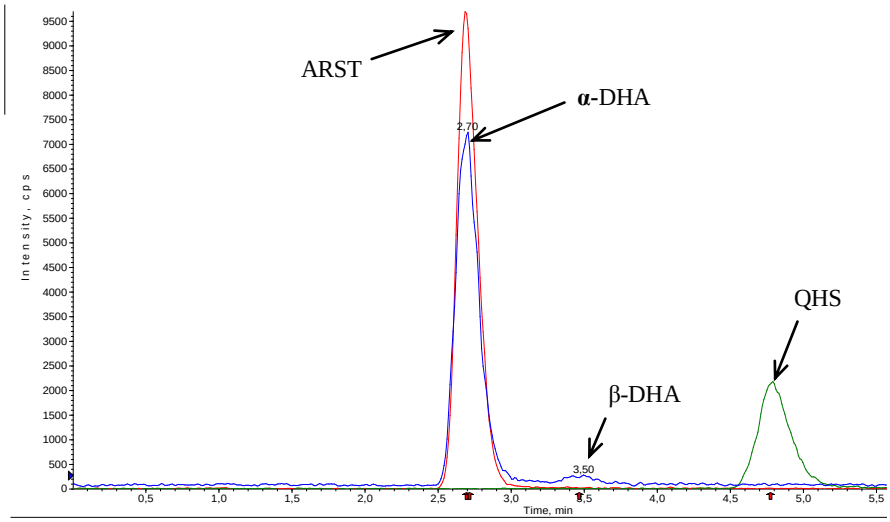


Fig 9. a)

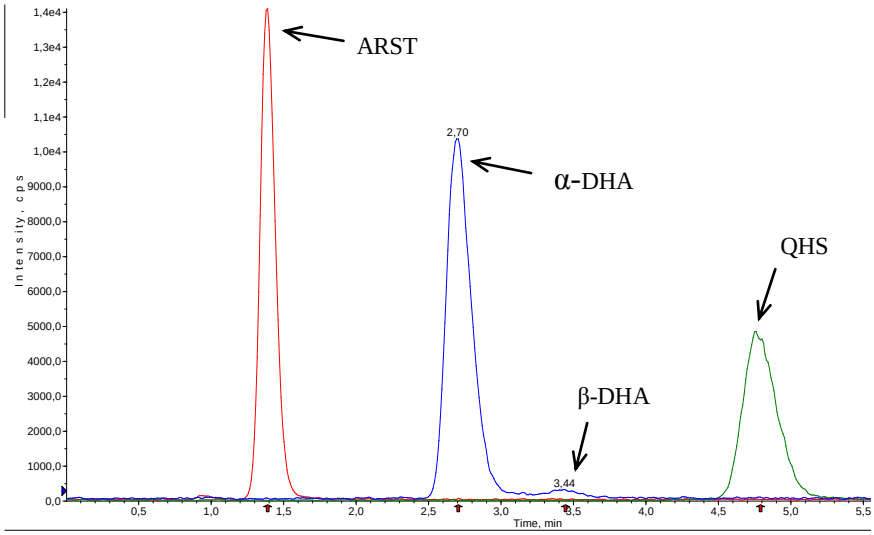


Fig 9. b)

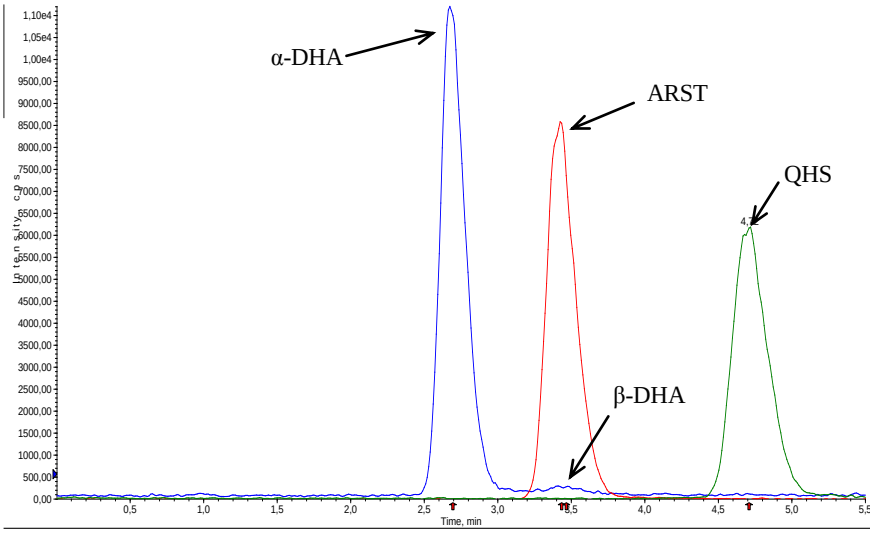


Fig 9. c)

4.1.3. Protein Precipitation; Results

Conditions of protein precipitation method are mentioned in paragraph 3.2.4.
Instead of usage 500 µl of I.S. (150 ng/ml in ACN-Met-OH) as precipitative agent it was also tried usage of 500 µl of I.S. (150 ng/ml in pure ACN).
The difference between using mixture of ACN/Met-OH and pure ACN in recovery, hence response of detector is shown in ***Fig. 10 c)***.

Fig. 10: *a)* Chromatogram of human plasma sample of concentration 200 ng/ml ARST and DHA (**blue**) and blank human plasma (**red**) treated with protein precipitation according to process mentioned in paragraph 3.2.4.

b) Chromatogram of human plasma sample of concentration 10 ng/ml ARST and DHA (**blue**) and blank human plasma (**red**) treated with protein precipitation according to process mentioned in paragraph 3.2.4.

c) Chromatogram of human plasma sample of concentration 200 ng/ml ARST and DHA treated with 500 µl of I.S. in ACN/Met-OH (50:50, v/v) (**blue**) and the same plasma concentration of ARST and DHA treated with 500 µl of I.S. in pure ACN (**red**).

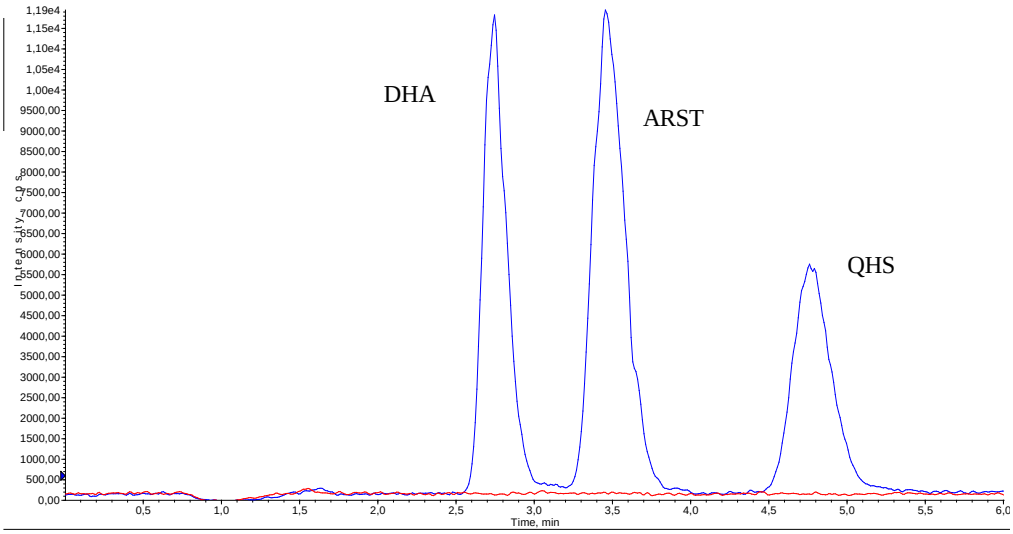


Fig. 10 a)

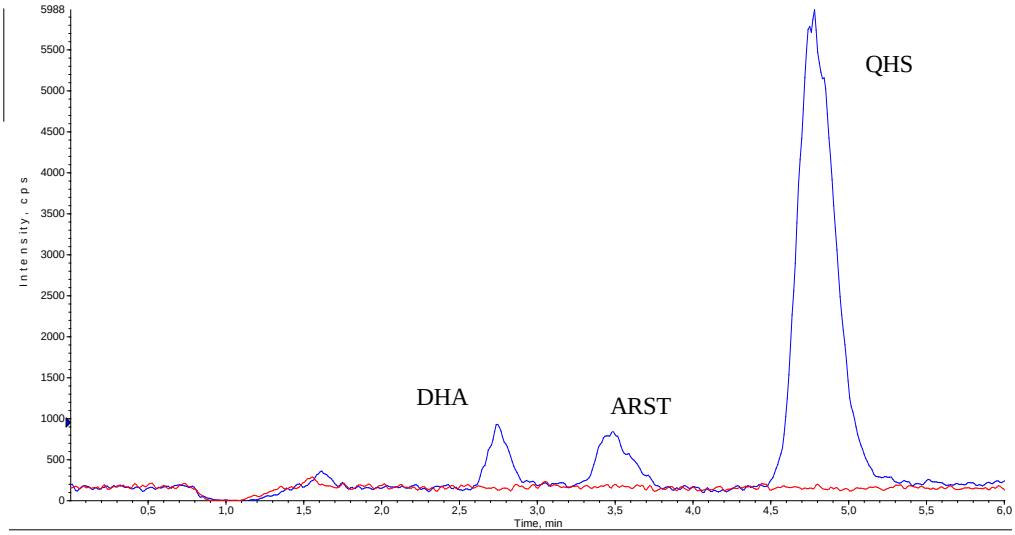


Fig. 10 b)

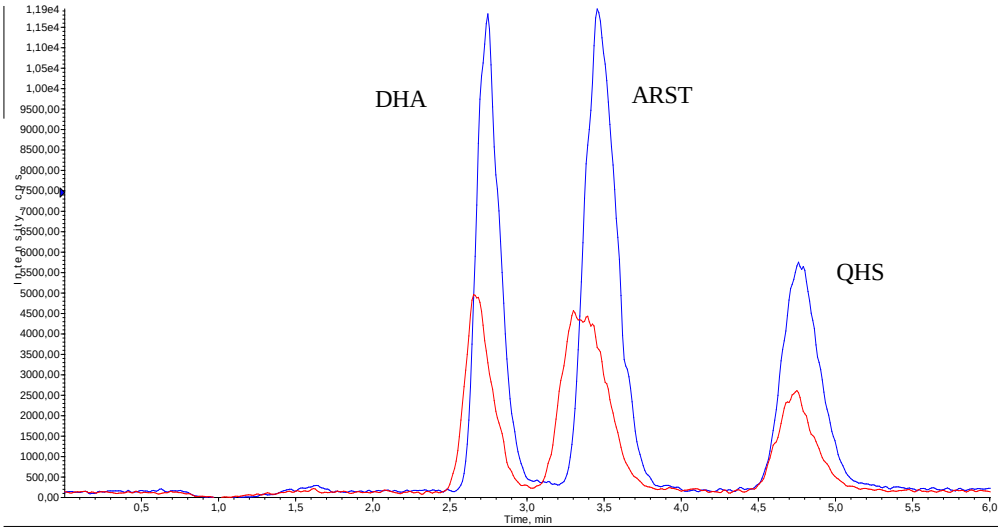


Fig. 10 c)

4.1.4. Liquid-liquid Extraction; Results

Conditions of liquid-liquid extraction method are mentioned in paragraph 3.2.5.

Fig. 11: *a)* Chromatogram of human plasma sample of concentration 200 ng/ml ARST and DHA (**blue**) and blank human plasma (**red**) treated with liquid-liquid-extraction according to process mentioned in paragraph 3.2.5.

b) Chromatogram of human plasma sample of concentration 2.5 ng/ml ARST and DHA (**blue**) and blank human plasma (**red**) treated with liquid-liquid-extraction according to process mentioned in paragraph 3.2.5.

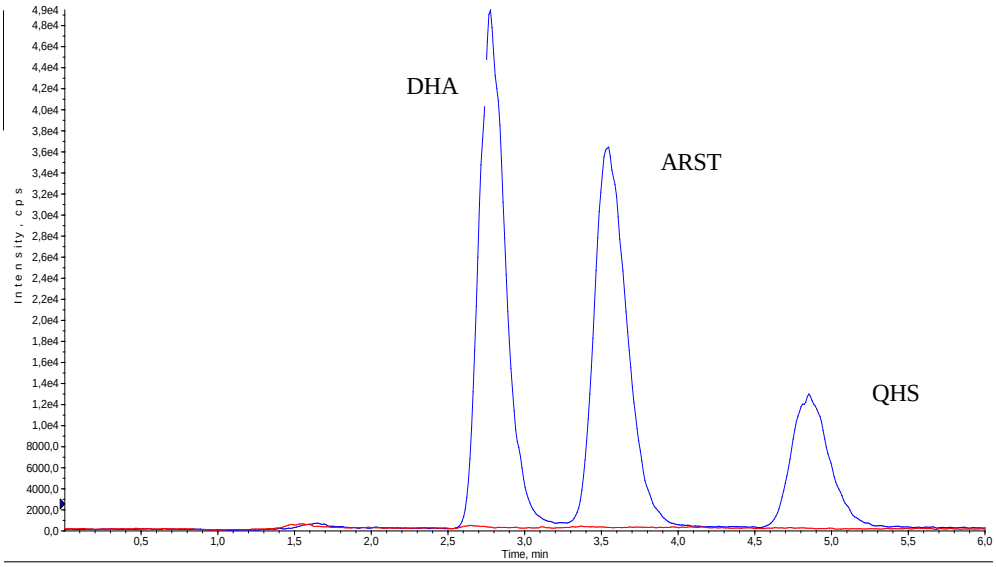


Fig. 11 a)

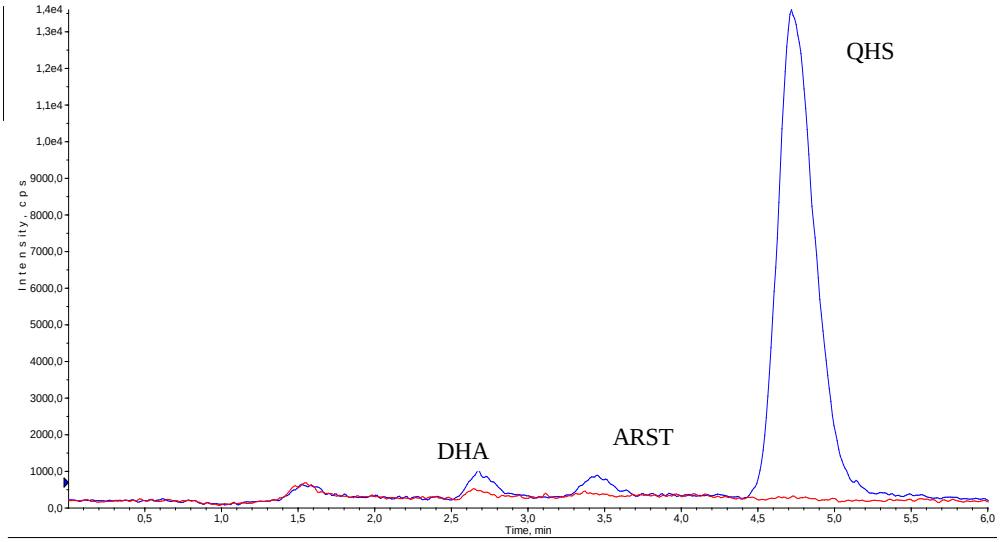


Fig. 11 b)

4.1.5. Pre-validation Data

LLOQ for plasma samples treated with liquid-liquid extraction was determine as 2.5 and 3.0 ng/ml for DHA and ARST respectively.
LLOQ for plasma samples treated with protein precipitation was calculated as 8.0 for both DHA and ARST.
Proposed range for calibration curves are for DHA; LLOQ – 2500 ng/ml and for ARST; LLOQ – 1000 ng/ml, which would correlate with therapeutic plasma levels of assayed compounds.

4.1.6. Discussion; Plasma samples treatment and LC/MS conditions

Protein precipitation using a mixture of ACN/Met-OH (50:50, v/v) as a precipitation agent gives the signal which was 40 % higher than using pure ACN. This is an improvement of method published in article[29].
Although pure ACN precipitate plasma proteins more readily and in a faster way, the recovery was lower than when mixture of ACN-Met-OH was used. This was probably due to higher affinity of AR to bond of precipitate when pure ACN was used. When pure Met-OH was used no further signal improvement was observed.
Also addition of 100 µl of 1% acetic acid in water plays a very important role to enhance signals of all assayed compounds.
Recovery of final protein precipitation procedure was about 75% for each compound.
Possible improvement of protein precipitation method could be done by concentrating of supernatant by evaporation of the sample to dryness and further dissolution in a small defined volume of solvent (i.e. 200 µl of mixture ACN-water (50:50, v/v))

In case of liquid-liquid-extraction recovery was about 70 % for each compound. Addition of 1000 µl of 1% acetic acid in water is essential for assay ARST which is a weak acid (pK_a = 4.6). According to Handerson - Hasselbach equation, ARST is in physiological plasma pH nearly completely ionized. Therefore, it is crucial to lower pH and to enhance extraction of ARST into organic solvent. At pH 3 nearly 98% of ARST is in unionized form.

Possible improvement of liquid-liquid-extraction method could be in introduction of an other step to whole procedure. After centrifugation the tubes can be placed into freezer. Thereby the organic (top, clear) layer could be removed more easily. Another way how to improve this procedure can be in utilizing of other extraction solvent. For example mixture of hexane-n-butanol-heptaflourobutanol[55] can be used, instead of 1-chlorbutane.

As it is apparent, LLE provides lower LLOQ. In contrast to PP, LLE enable to concentrate the sample. However, LLE is more complicated and time consuming procedure. The fact that sample is more concentrated within the process of preparation can be a big advantage when detector posses limited sensitivity.

Generally, no matter if plasma sample is treated with liquid-liquid-extraction or protein precipitation, it is essential to run the analysis after 16 hours of staying at 4°C. This is crucial to enable epimerisation to desirable equilibrium between α and β-DHA (Just α-DHA is more dispose to form adducts with NH_4^+ ions, probably spherical influence plays the most important role). If it is measured instantly after plasma sample treatment, the response of detector is just 40 % of a final one at time of 16 hours. In the article[56], it is mentioned response of detector even just 25 % of a final one at time 16 hours. But the usage of different solvent in that method must be taken into account.

In order to prevent formation of DHA from ARST (cleavage of ester bond) it is crucial to do all steps with plasma samples on ice. Low temperature can notably slow down cleavage of ester bond. This cleavage is mainly related to biological matrix. Beyond low temperature, usage of fluoride-oxalate as anticoagulant has also supportive effect.

4.2. INFLUENCE OF HAEMOLYSIS, IRON HAEM OR FREE IRON

4.2.1. Results

Results of the experiments focused on investigation of effects of heamolysis and free iron on the stability of ARST and DHA in plasma and standard solution are given in Tab.2-4. The results are presented as a decrease of signal intensity.

Tab. 2: Influence of Haemolysis and Free Iron; Protein Precipitation

Sample type	PHEN	Acetic ac. (100 µl)	Water (µl)	DHA (signal; %)	ARST (signal; %)	QHS (signal; %)
Plasma		***X***	***50***	***100***	***100***	***100***
HP 20%		X	50	0	0	0
HP 20%	X	X		0	0	0
HP 2%		X	50	0	0	0
HP 2%	X	X		0	0	0
HP 0.2%		X	50	40	45	40
Fe^{2+} Plasma		X	50	80	90	90
Fe^{2+} Plasma	X	X		70	90	80
Plasma			150	60	80	70
HP 20%			150	0	0	0
HP 20%	X		100	0	0	0
HP 2%			150	0	0	0
HP 2%	X		100	0	0	0
Fe^{2+} Plasma			150	50	70	60
Fe^{2+} Plasma	X		100	45	70	60

HP 0.2/2/20%; 300µl of plasma with 0.2/2/20% of heamolysed whole blood with concentration of DHA and ARST 100ng/ml each.

Fe^{2+} Plasma; 300µl of plasma with concentration of free iron 0,03 mg/ml (Which represent amount of iron like in 5% haemolysed whole blood in plasma), conc. of DHA and ARST (100ng/ml).

Plasma 300µl of plasma with conc. of DHA and ARST 100ng/ml.

PHEN addition of 50 µl phenantroline hydrochloride in water; conc; 25 mg/ml (enough to capture amount of iron like in 40% haemolysed whole blood).

Acetic ac.; 1% acetic acid in water.

Tab. 3: Influence of Haemolysis and Free Iron; Liquid-liquid Extraction

Sample type	PHEN	Acetic ac. (1000 µl)	Water (µl)	DHA (signal; %)	ARST (signal; %)	QHS (signal; %)
Plasma		***X***	***50***	***100***	***100***	***100***
HP 20%		X	50	0	0	0
HP 20%	X	X		0	0	0
HP 2%		X	50	0	0	0
HP 2%	X	X		0	0	0
Fe^{2+} Plasma		X	50	10	10	30
Fe^{2+} Plasma	X	X		25	30	65
Plasma			1050	95	0	85
HP 20%			1050	75	0	25
HP 20%	X		1000	30	25	25
HP 2%			1050	85	0	75
HP 2%	X		1000	40	35	35
Fe^{2+} Plasma			1050	35	0	80
Fe^{2+} Plasma	X		1000	40	30	70

HP 2/20%; 400µl of plasma with 2/20% of heamolysed whole blood with concentration of DHA and ARST 100ng/ml each.

Fe^{2+} Plasma; 400µl of plasma with concentration of free iron 0.15 mg/ml (which represent amount of iron like in 25% haemolysed whole blood in plasma), conc. of DHA and ARST (100ng/ml).

Plasma 400µl of plasma with conc. of DHA and ARST 100ng/ml.

PHEN Addition of 50 µl phenantroline hydrochloride in water; c = 50 mg/ml (enough to capture amount of iron like in 80% haemolysed whole blood).

Acetic ac.; 1% acetic acid in water.

Tab. 4: Influence of Free Iron on Standard Solution of ARST and DHA

Sample type	PHEN	Acetic ac.	Water (µl)	Signal ARST/ %	Signal DHA/ %
Standards		***X***	***50***	***100***	***100***
Standards			150	100	100
Fe^{2+} Standards [1]		X	50	90	90
Fe^{2+} Standards [1]			150	95	90
Fe^{2+} Standards [1]	X	X	0	60	50
Fe^{2+} Standards [1]	X		100	60	50
Fe^{2+} Standards [2]		X	50	65	25
Fe^{2+} Standards [2]			150	85	40

Standards; 400 µl of standard solution of ARST and DHA in ACN/H_2O (c = 100 ng/ml each).

Fe^{2+} Standards [1] 400 µl of standard solution of ARST and DHA in ACN/H_2O (c = 100ng/ml each) with concentration of free iron 0.03 mg/ml (Which represent amount of iron like in 5% haemolysed whole blood).

Fe^{2+} Standards [2] 400 µl of standard solution of ARST and DHA in ACN/H_2O (c = 100ng/ml each) with concentration of free iron 0.15 mg/ml (Which represent amount of iron like in 25% haemolysed whole blood).

PHEN Addition of 50 µl phenantroline hydrochloride in water; c = 7,5 mg/ml (enough to capture amount of iron like in 12% haemolysed whole blood).

Acetic ac.; Addition of 100 µl 1% Acetic acid in water.

Fig. 12: Chromatograms: Influence of Haemolysis and Free Iron using Liquid-liquid Extraction

a) ▪ Plasma with conc. of DHA and ARST 100ng/ml without addition of acetic acid; **blue**

▪ 2 % of haemolysate in plasma with conc. of DHA and ARST 100ng/ml without addition of acetic acid; **green**

▪ 20 % of haemolysate in plasma with conc. of DHA and ARST 100ng/ml without addition of acetic acid; **red**

b) ▪ 20 % of haemolysate in plasma with conc. of DHA and ARST 100ng/ml without addition of acetic acid; **blue**

▪ 20 % of haemolysate in plasma with conc. of DHA and ARST 100ng/ml with addition of iron chelator PHEN, without addition of acetic acid; **red**

▪ 2 % of haemolysate in plasma with conc. of DHA and ARST 100ng/ml with addition of iron chelator PHEN, without addition of acetic acid; **green**

c) ▪ Plasma with conc. of DHA and ARST 100ng/ml with portion of Fe^{2+} without addition of acetic acid; **blue**

▪ Plasma with conc. of DHA and ARST 100ng/ml with addition of acetic acid; **grey**

▪ Plasma with conc. of DHA and ARST 100ng/ml with portion of Fe^{2+} with addition of acetic acid; **red**

▪ Plasma with conc. of DHA and ARST 100ng/ml with portion of Fe^{2+} and with iron chelator PHEN with addition of acetic acid; **green**

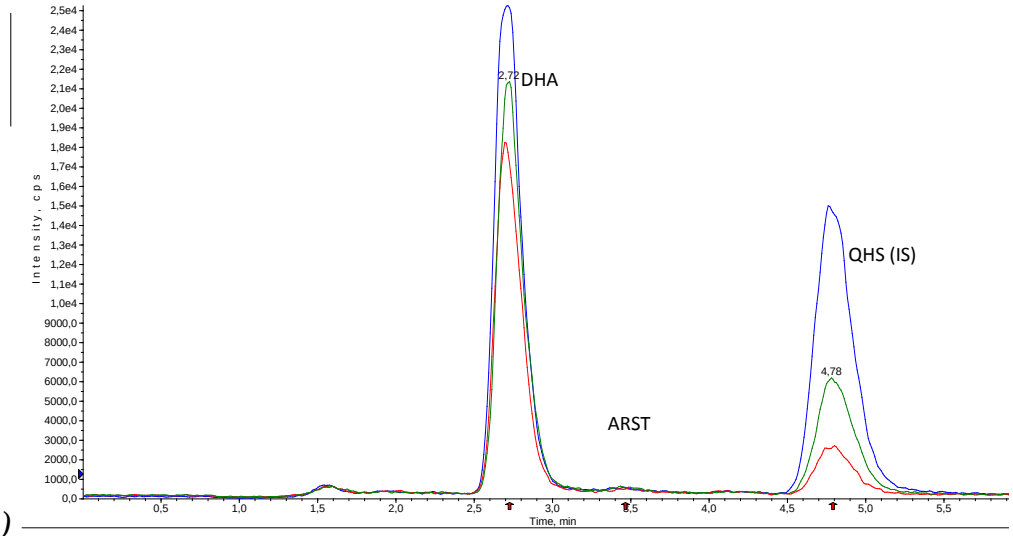


Fig. 12 a)

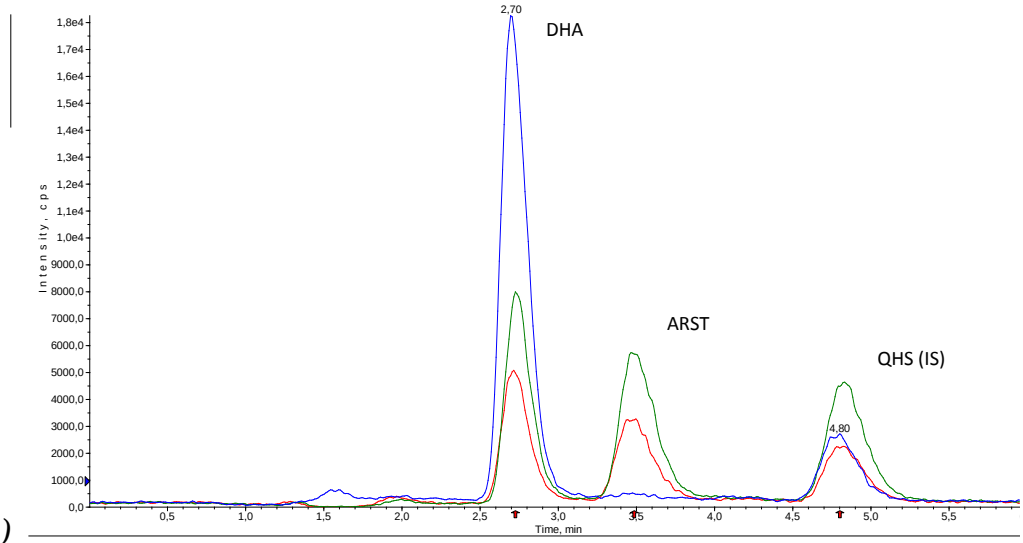


Fig. 12 b)

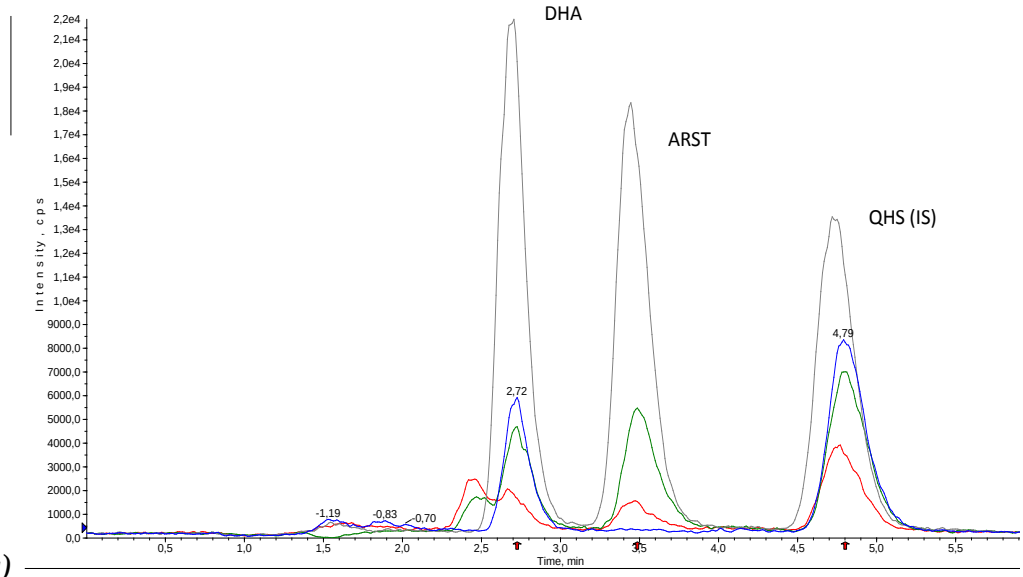


Fig. 12 c)

4.2.2. Discussion

When haemolysed plasma samples are treated with protein precipitation it does not matter if acetic acid was added, even 0.2 % of haemolysis cause reduction of responses more than 50 % for each compound. Hence, 2 % respectively 20 % of haemolysis cause total reduction of responses of each compound (zero signal). Addition of iron chelator PHEN did not prevent reduction of response in case of haemolysed plasma samples (Tab.2).

Plasma samples with free iron (amount of iron like in 5% haemolysed whole blood in plasma) showed reduction of responses between 10 and 20 % when acetic acid was added and between 30 and 50 % when acetic acid was replaced by water. (this phenomena was already described in paragraph 3.4. - addition of acetic acid increases response of detector). Addition of iron chelator PHEN did not prevent reduction of response in case of plasma samples with free iron (PHEN can have some protective role, however competition between ammonium ions from mobile phase and basic PHEN must be taken into account when some portion of PHEN is also injected to LC/MS system).

When haemolysed plasma samples are treated with liquid-liquid extraction and acetic acid is added, 2 % respectively 20 % of haemolysis cause total reduction of responses of each compound (zero signal). Addition of iron chelator PHEN does not prevent total loss of signal (Tab. 3).

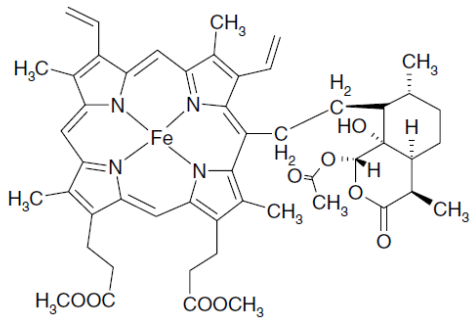
When addition of acetic acid was replaced by water even haemolysed plasma samples reveal relatively high responses of detector (reduction of responses is derived from percentage of haemolysis; 20% of haemolysis reveal higher reduction than 2 %). However, there was no signal of ARST which was not extracted to organic solvent because of its ionized state at this physiological plasma pH.). Addition of iron chelator PHEN did not prevent any reduction of response in case of haemolysed plasma samples, signals of each compound was even lower (Probably effect of competition between ammonium ions from mobile phase and basic PHEN when some portion of PHEN is injected to LC/MS system). Nevertheless, despite of preservation physiological plasma pH, significant signal of ARST was observed in this case – addition of PHEN (ion-pair extraction effect).

Plasma samples with free iron (amount of iron like in 25% haemolysed whole blood in plasma) reveal vast reduction (up to 90 %) of responses. This reduction of responses was higher when addition of acetic acid was performed (more extensive reduction properties of Fe^{2+}). Addition of iron chelator PHEN prevented such a vast reduction of signal and increased responses between 15 and 35% (Tab. 3).

Study with standard solution of ARST and DHA in ACN/H_2O (conc. 100 ng/ml each) with portion of iron Fe^{2+} showed that samples with higher amount of iron demonstrated lower detector response, this reduction of response is enhanced by addition of acetic acid. This study also showed that DHA is more sensitive towards Fe^{2+} ions then ARST. Addition of iron chelator PHEN, did not prevent any reduction of responses. (Reduction was even more extensive which is probably derived from competition between ammonium ions from mobile phase and basic PHEN in LC/MS system) (Tab. 4).

Nevertheless, even about 25% of free iron presented in the sample did not cause total loss of signal compare to 1% haemolysed blood in plasma samples. Effect to reduction of responses of free iron is evident, however is not the main reason for vast reduction of signal in haemolysed plasma samples. Hence, usage of iron chelators within assay in order to prevent reduction of signal (degradation of compounds) does not make any sense. The main reason for such a vast degradation of assayed compounds is probably associated with one aspect of AR mechanisms of action. This is a direct alkylation of haemoglobin which is described in article by Luigi Messori et al[57]. The proposed structure of hemin-artemisinin adduct is shown in *Fig. 13*.

Fig. 13. Haem-artemisinin adduct; Mw = 898 (≈ haem + QHS) analyzed by ESI MS[57]



It was assumed and proved by Luigi Messori at al. that haem alkylation by AR undergo as a relatively slow reaction, however the reaction rate can be extremely enhanced by protein precipitation. This phenomena can be explained by an importance of spherical effect to reaction rate. (Precipitation of proteins alters their conformation extensively).

This study showed that not only protein precipitation enhanced the process of alkylation. The pH decrease has also a great impact on this process. Decrease of pH can probably also change protein conformation and therefore haem is then more susceptible to alkylation. Moreover, addition of acetic acid (process of pH decrease) leads to reactivity increase of endo-peroxide group. However, alteration of protein conformation will be probably the leading mechanisms.

Formation of non-covalent complexes of AR with haem[58] was also reported.

Nevertheless, the articles which emphasis the crucial importance of free iron (non-haem iron) in mechanisms of activation of AR (which is connected with their degradation) were also published. However, this effect of free iron is probably related with activation inside *plasmodium* parasite[30, 32].

5. Conclusion

The LC/MS method for determination of ARST and DHA in human plasma was developed and is ready to be validated.

Tuning of triple quadrupole thurbospray ionization MS detector was performed. Mass transition used for QHS was 300 to 209 amu, for DHA 302 to 267 amu and for ARST 402 to 267 amu.

Chromatographic conditions were optimized, particularly mobile phase pH and its ratio of organic (ACN) and inorganic phase (ammonium acetate buffer). Final retention times 2.8, 3.6 and 4.7 min. were obtained for DHA, ARST and QHS, respectively. Total time for analysis was extremely short – 6.0 min.

Using mixture of ACN/Met-OH as precipitative agent represent an improvement of protein precipitation procedure published in paper[29]. In order to prevent decomposition of ARST to DHA it is crucial to perform all steps with plasma samples on ice. It is very important to perform analysis of treated plasma samples using LC/MS after 16 hours of staying at 4°C, to enable epimerization of β-DHA to α-DHA (which is much more disposed to form adducts with NH_4^+ ions; the adducts which the MS detector is tuned on).

LLOQ using 400 µl of plasma treated with LLE were 2.5 ng/ml for DHA and 3.0 ng/ml for ARST. LLOQ using 300 µl of plasma treated with PP were 8.0 ng/ml for both DHA and ARST. Proposed range for calibration curves are for DHA; LLOQ – 2500 ng/ml and for ARST; LLOQ – 1000 ng/ml, which would correlate with therapeutic plasma levels of assayed compounds.

Since LLOQ for both LLE and PP are very closed, the own sensitivity of MS detector can be considered as a main determinat for practical applicability of the method. However, decrease of pH and usage of precipitation agent is forbidden when some percentage of haemolysis can be expected in plasma samples (bad prepared blood samples and or samples from patient infected by malaria parasite), unless vast signal deterioration is observed. Using organic solvent free SPE described in the article[53] can partly solve the problem with tiny percentage of haemolysis in plasma samples.

In order to prevent responses reduction of AR caused by haemolysis and to enable assay of AR in rat`s embryo, addition of iron chelator is useless. The leading mechanisms of

degradation is probably haem alkylation. Process of alkylation is extremely accelerated by precipitation of proteins and pH decrease. Issue of pH decrease within process of extraction with organic solvent is a problem particularly for isolation of ARST, this can be solved by usage of ion-pair extraction. On the other hand, it is also questionable whether isolation of ARST is so important, taking into account it`s extremely short half-life. Additionally, it also worth thinking if portion of ARST in rat`s embryo cannot be counted from portion of DHA (it`s metabolite) assayed.

Considering the fact that the highest affinity to haem has QHS, it`s usage as an I.S. is strictly inappropriate. Usage of another I.S. should be an object of further investigation.

Recovery, hence reduction of responses assayed compounds will be probably extremely dependent on portion of hemoglobin and also myoglobin. Therefore, it must be performed further investigation if reproducibility of this LC-MS method for investigation in rat`s embryo using LLE would encounter required parameters. If not, other analytical approach should be taken into account, i.e. radioanalysis.

Considering a high affinity of AR derivatives to haem and probably also to erythrocytes, it can be object of debate if their plasma levels (which is the only levels mentioned in all articles) are predicative enough about their concentration in blood stream.

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