Universidade de Lisboa

Faculdade de Farmácia

Quantification of Chloroquine by LC-MS and the Macular Degeneration Disease

David Miguel Cunha Pereira

Mestrado Integrado em Ciências Farmacêuticas

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Abstract

Age-related macular degeneration is a visual disease that involves a progressive loss of central vision which its consequences are dramatic for the patient's quality of life. Age is a major risk factor for macular degeneration. Other risk factors include, ethnicity, genetics, smoking and drugs. Chloroquine appears to be the most toxic drug to the retina, which might be explained by the extensive accumulation and very long retention of chloroquine bound to melanin. Chloroquine is used for malarial prevention and treatment, as well to various inflammatory diseases such as rheumatoid arthritis and lupus erythematosus. Chloroquine toxicity in age-related macular degeneration is of serious ophthalmologic concern because it is not treatable. Nonetheless, it has been demonstrated that central vision can be preserved if damage is recognized before there are changes in the retinal pigment epithelium. With proper screening, bull's-eye retinopathy, no longer should be seen. The focus of this study is the development of a precise and sensible method of quantification of chloroquine, by using liquid chromatography–mass spectrometry, to determine the amount of chloroquine that binds to melanin when using a tissue sample and to relate the melanin-chloroquine binding to macular degeneration or age-related macular degeneration, from the premise that the extensive binding of chloroquine to the melanin of the eye contributes to a quicker evolution of the macular degeneration disease. A method of melanosome extraction and melanin-cloroquine binding was accomplished. It was also developed a liquid chromatography–mass spectrometry method. HPLC was done in an Agilent 1260 Infinity instrument with a Poroshell 120 SB-C18 column (2.1 x 50 mm, 2.7 µm). Mass spectrometry used an Agilent 6495 Triple Quadrupole Mass Spectrometer. The results showed the suitability of the method, which was improved with a stability study of chloroquine, included on this work. The results showed that more than 50% of the chloroquine binds to melanin, independently of the concentration of the melanin used. In the future, a more sensible method should be developed with other columns of HPLC. The present work joins the studies that associate the relation between chloroquine therapeutic and its influence on retinopathy, and therefore on AMD, making its consequences appear sooner or greater.

Keywords: AMD, Chloroquine, Eye, Melanin, LC-MS.

Resumo

A degenerescência macular da idade (DMI) é uma doença oftálmica que envolve a perda progressiva de visão central e cujas consequências são dramáticas para a qualidade de vida do doente. A idade é um importante fator de risco para a degenerescência macular. Outros fatores de risco incluem: etnia, genética, tabagismo e medicamentos. A cloroquina parece ser o fármaco mais tóxico para a retina, dada a sua extensa acumulação e retenção longa quando ligada à melanina. A cloroquina é utilizada na prevenção e tratamento da malária, sendo também utilizada na terapêutica de doenças inflamatórias, como a artrite reumatoide e o lúpus eritematoso. A toxicidade da cloroquina na degenerescência macular da idade é uma preocupação oftalmológica séria dada a ausência de tratamento. No entanto, demonstrou-se que o centro da visão pode ser preservado se o dano for reconhecido antes de ocorrerem alterações no epitélio do pigmento da retina. Com rastreio apropriado, a retinopatia do "olho de boi" deixará de se expressar. Este trabalho tem como objetivo o desenvolvimento de um método preciso e sensível de quantificação de cloroquina, usando cromatografia líquida-espectrometria de massa, para determinar a quantidade de cloroquina que se liga à melanina, usando uma amostra de tecido, e relacionar a associação da cloroquina à melanina com a degenerescência macular ou degenerescência macular da idade. Foi realizado um método de extração melanossomal e de ligação melanina-cloroquina. Foi, igualmente, desenvolvido um método de LC-MS. O HPLC foi executado num aparelho *Agilent 1260 Infinity* com uma coluna *Poroshell 120 SB-C18* (2,1 x 50 mm, 2,7 µm). A espectrometria de massa foi realizada com um espectrómetro *Agilent 6495 Triple Quadrupole*. Os resultados mostraram a adequação do método, sendo que este foi melhorado com um estudo de estabilidade da cloroquina, incluído neste trabalho. Os resultados mostraram que mais de 50% da cloroquina se liga à melanina, independentemente da concentração de melanina utilizada. No futuro, deverá ser desenvolvido um método mais sensível com o uso de outras colunas de HPLC. O presente trabalho junta-se aos estudos que associam a relação entre a terapêutica de cloroquina e sua influência na retinopatia e, portanto, na AMD, fazendo com que suas consequências aparecem mais precocemente.

Palavras-chave: Cloroquina, DMI, LC-MS, Melanina, Olho.

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List of acronyms

Acronyms	
ACN	Acetonitrile
AMD	Age-related macular degeneration
CV	Coefficient of variation
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
EMA	European Medicines Agency
FA	Formic Acid
HPLC	High-performance liquid chromatography
IS	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
LC-MS	Liquid chromatography–mass spectrometry
MES	2-(N-morpholino)ethanesulfonic acid
MWCO	Molecular weight cut-off
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
QC	Quality Control
RED	Rapid equilibrium dialysis
RPE	Retinal pigment epithelium
SD	Standard deviation
UEF	University of Eastern Finland

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1. Introduction and objectives

1.1. Introduction

Macular degeneration, also known as age-related macular degeneration (AMD), has its genesis on the deterioration of the central portion of the retina, the inside back layer of the eye that turns light into electrical signals and then sends these electrical signals through the optic nerve to the brain, where they are translated into the images we see. The retina's central portion and its most sensitive part, known as the macula, which is made up of millions of light-sensing cells that provide sharp, is responsible for focusing central vision in the eye, and it controls our ability to read, drive a car, recognize faces or colours, and see objects in fine detail. (1) When the macula is damaged, the center of your field of view may appear blurry, distorted, or dark. (1,2)

This disorder of the macula is characterized by the presence of at least intermediate-size drusen (63 μm or larger in diameter), retinal pigment epithelium (RPE) abnormalities such as hypopigmentation or hyperpigmentation, reticular pseudodrusen and occurrence of any of the next features: geographic atrophy of RPE, choroidal neovascularization (exudative or wet), polypoidal choroidal vasculopathy, or retinal angiomatous proliferation. (3–5)

As seen above, RPE alterations plays a major importance in AMD. RPE or pars pigmentosa, present on the outermost layer of the retina, is composed of a monolayer of polygonal cells. The RPE together with the capillary wall constitutes the blood–retinal barrier. RPE has three main functions: phagocytosis and degradation of the continuously shedding tips of photoreceptor cells, vitamin A transport and storage, and active ion transport. The RPE cells of pigmented animals contain melanin in melanosomes or melanin granules. (6)

AMD by itself does not lead to complete blindness. However, the loss of central vision in AMD can interfere with simple everyday activities, which makes it an incapacitating disease. (2) Anyhow is a leading cause of severe, irreversible vision impairment in developed countries. (3)

The classification of AMD, by the Age-Related Eye Disease Study, is based on the size of the drusens, RPE abnormalities and the presence of another retinal features, e.g. choroidal neovascularization, polypoidal choroidal vasculopathy and others. There are four types of AMD, which includes "No AMD", "Early AMD", "Intermediate AMD" and "Advanced AMD". (3)

Prevalence data about age-related macular degeneration show that AMD is responsible for an estimated 46% of cases of severe visual loss in persons over 40 years

of age in the United Sates and that 1.75 million people aged 40 years or older in the United States were estimated to have either neovascular AMD or geographic atrophy in at least one eye. (3–5,7) In Portugal, the prevalence of AMD is estimated to be approximately 300 000 cases and its estimated incidence it is about 45 000 new cases every year. (8)

Age is a major risk factor for AMD. The disease is most likely to occur after the age of sixty, but it can occur earlier. Other main risk factors for AMD include, ethnicity (AMD is more common among Caucasians than among African-Americans or Hispanics/Latinos) (9) and genetics. Modifiable risk factors include smoking, hypertension, cardiovascular disease, antioxidants levels, diet and drugs. (2,5,10)

Beaver Dam Eye Study reported two times the incidence of late macular degeneration in patients who used aspirin at least twice weekly for ten years compared with those who used no aspirin. (3,11) Other studies have shown a potential protective effect of aspirin against the development of AMD. (3,12)

Medical and experimental literature contains a very large volume of data regarding the reputed retinal toxicology of drugs. Chloroquine and phenothiazines are often used as examples of drugs associated with retinal toxicity, (6) in which chloroquine appears to be the most toxic drug to the retina. (13) The association of retinopathy with chloroquine therapy was only discovered in 1959, because the symptoms of retinopathy did not decrease on cessation of chloroquine treatment. (13–15) This might be explained by the extensive accumulation and very long retention of chloroquine bound to melanin. (13,14,16)

Melanin is a polyanionic polymer derived from the amino acid tyrosine mainly via enzymatic and spontaneous reactions. (13,17) It is synthesized in melanosomes, which contains cells that are called melanocytes. After the earliest stages of maturation, melanosomes have an organized, fibrillar matrix where the synthesized melanin will bind. Melanin is synthesized if there is active tyrosinase in the melanosome. When the melanosome is full, the tyrosinase activity ceases, ending the melanin synthesis. (17,18)

All the functions of melanin are not entirely known. In the eye, the absorption of light is thought to be the most important function. Melanin absorbs near infrared light, visible light, and ultraviolet (UV) radiation. In the iris it controls the visible and UV light entering the eye and protects the eye of their harmful effects. In the RPE melanin protects the neural retina from getting exposed to too much light, minimizing reflection and improving image quality. (18)

Melanin acts as an antioxidant and protects the eye against free radicals. (10,17) This may help protect against diseases such as uveal melanoma and AMD. On the other hand, constant exposure of melanocytes to oxidizing agents (free radicals, light) in the

aging eye may reduce the antioxidant effect of melanin and melanin may even act as a pro-oxidant. (17,18)

Melanin is known to bind many chemicals, protecting the eye from their harmful effect. Among these are free radical forming heavy metal ions like iron and copper, harmful organic compounds and drugs. By binding to chemicals melanin can also act as reservoir. Depending on the nature of the chemical, this effect can be harmful, if it causes the chemical to accumulate in melanocytes. (17–19) The nature of the drug influences the different mechanisms that contribute to drug-melanin binding. There is a relationship between the physicochemical properties of a drug and its binding to melanin. All basic and lipophilic drugs can be expected to bind to melanin. This is expected, since the melanin polymer is polyanionic. (13,18)

The exact mechanism of melanin binding to drugs remains undetermined. Despite this, there is evidence to suggest that the association of most drugs with melanin is a reversible process, since little or no evidence was obtained for covalent binding of drugs to melanin. (6) Electrostatic interactions are thought to be the main contributing mechanism of binding. Other mechanisms include van der Waals forces and hydrophobic interactions. (13,18) Chloroquine seems to be an exception, electrostatic forces are not the main mechanism of its binding. Non-electrostatic interactions are important in melanin-chloroquine binding and the binding of chloroquine may even be partly irreversible. The van der Waals forces were suggested to be the main contribution of interaction in the binding of chloroquine. (13)

Chloroquine is a small molecule that possess an average molecular mass of 319.87 g/mol. Its chemical formula is $C_{18}H_{26}ClN_3$. Chloroquine's IUPAC name is 7-chloro-N-[5-(diethylamino)pentan-2-yl]quinolin-4-amine. (20,21)

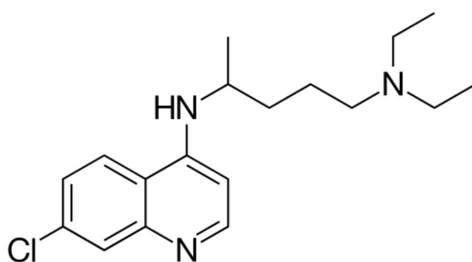

Figure 1: Chloroquine. **(22)**

Chloroquine was originally used for malarial prevention and treatment. (21,23–27) Malarial disease is one of the most widespread threats to health in the more than 100 countries of the tropical and subtropical regions of the world with approximately 300–500 million clinical cases reported annually, out of which around 2–3 million people die

due to complications. (26,28,29) Later, its anti-inflammatory property was disclosed, thus its use was expanded to treat various inflammatory diseases such as rheumatoid arthritis and lupus erythematosus. (15,21,23,24)

Although chloroquine has been used for a long time, its mechanism of action is not well understood. Chloroquine is well absorbed when administered orally. (23,30) The bioavailability is nearly 80%. It has a high volume of distribution and its protein binding capacity is approximately 55%. Chloroquine is metabolized by the cytochrome P450 (CYP) system in which CYP2C8, CYP3A4 and CYP2D6 are the major enzymes. Chloroquine is de-ethylated to desethylchloroquine, its major active metabolite. (23,30) The elimination half-life of chloroquine ranges from ten days to two months. The most serious adverse effect is maculopathy, such as macular degeneration. (14,23,30–33)

The incidence of chloroquine retinopathy varies considerably but appears to be related to the total dose of the drug and the duration of therapy. (13) Chronic high-dose anti-inflammatory therapy of chloroquine produces a dose-dependent retinopathy. (6,15)

The most common symptoms of chloroquine retinopathy are reading difficulties (words or letters missing), photophobia, blurred distance vision, missing or blackout areas in the central or peripheral visual field, and light flashes and streaks. The earliest abnormalities in fundus appearance are mild pigment stippling or mottling of the macula and loss of foveal reflex. In many cases, even when permanent damage has occurred, the effects remain restricted to the macula. The macula, on the other hand, may vary in appearance from an extremely subtle pigment disturbance to a complete loss of pigment. (13) The stages in between are described as having a “bull’s eye” appearance; (13,15,31,32,34) this is characterized by granular hyperpigmentation of the macula surrounded by a clear zone of depigmentation which is, in turn, encircled by another ring of pigment. Histopathological investigations of the eye of man have indicated that the primary lesions occur in the retinal pigment epithelium, which becomes extremely enlarged with an increase in the content of the pigment granules. (13)

High experimental doses have acute effects on the metabolism of retinal cells, but it is not clear how these short-term metabolic effects relate to the slow and chronic damage that characterizes the clinical state of toxicity. Binding to melanin in the RPE may serve to concentrate the agents and contribute to, or prolong, their toxic effects. It has been verified that inner and outer retina are damaged by chloroquine exposure in animal studies. In clinical practice, the primary damage is in the photoreceptors, and as the outer nuclear layer degenerates, there is secondarily disruption of the RPE. (15)

Chloroquine toxicity in AMD is of serious ophthalmologic concern because it is not treatable. Nonetheless, it has been demonstrated that central vision can be preserved if damage is recognized before there are changes in the retinal pigment

epithelium (RPE). With proper screening, bull’s-eye retinopathy, as classically described, no longer should be seen. (15)

1.2. Objectives

This thesis aims to develop a precise and highly sensible method of quantification of chloroquine, by LC-MS, to determine the amount of chloroquine that binds to melanin, by using a tissue sample, and to relate the melanin-chloroquine binding to AMD.

2. Materials and methods

2.1. Chemicals and reagents

Chloroquine diphosphate salt, pamaquine naphthoate (internal standard), dimethyl sulfoxide (≥99.7%), methanol, acetonitrile (ACN), formic acid (FA) and inhibitors (PMSF and protease inhibitor cocktail) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (2.7 mM KCl, 2.7 mM KH_2PO_4, 8 mM Na_2HPO_4, 138 mM NaCl, pH 7.4), Tris-HCl buffer (50 mM Tris-HCl, 150 mM KCl, pH 7.4), hypotonic buffer (10 mM Tris-HCl, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.4) and MES buffer (25 mM MES, 5 mM NaCl, 115 mM KCl, 1.3 mM $MgSO_4$, pH 7.40) were obtained from Thermofisher (Invitrogen, NY, USA). Milli-Q Water was obtained by reverse osmosis Millipore, provided by the UEF.

2.2. Melanosome extraction

Porcine eyes were obtained from a local slaughterhouse and kept in PBS on ice during the transportation. Extraocular material was rejected, and the anterior part of the eye was removed with a scalpel. The vitreous and neural retina were removed with tweezers and PBS was added into the eyecup. After 5 min incubation at room temperature, the RPE was gently removed with a small paintbrush and collected. Cells were pelleted with 6238 g centrifugation for 5 min and stored at -20°C until further processing. (35)

Melanosomes were isolated using OptiPrep1 (Axis Shield, Oslo, Norway) density gradient centrifugation. The RPE tissue was slowly defrosted on ice and suspended into hypotonic buffer containing inhibitors. Cells were disrupted with nitrogen cavitation (Parr 4639, Parr Instrument Co., Moline, IL, USA) at 450 psi with 15 min equilibration time. Whole cell lysate was centrifuged at 3 000 g for 5 min and the supernatant (crude lysosomal fraction) was removed and pelleted at 20 000 g for 20 min. Crude melanosomal pellet was re-suspended into buffer (pH 7.40) and layered on top of discontinuous OptiPrep1 gradient. Gradient tubes were centrifuged at 135 000 g for 1 h at +4°C (Sorvall™ WX Ultra Centrifuge, TH-641 Swinging Bucket Rotor, Thermo Fisher Scientific Inc., Waltham, MA USA). The melanosomal pellet was recovered and re-suspended into buffer (pH 7.40) and re-centrifuged in another OptiPrep1 gradient. After the second gradient centrifugation, melanosomes were re-suspended into MES buffer and residues of OptiPrep1 were removed by two 10 000 g centrifugations. The total

protein concentration of the purified melanosomal fraction was measured with the Bradford method (Bio-Rad Protein reagent, Bio-Rad, Hercules, CA, USA). (35)

2.3. Melanin-chloroquine binding

Melanossomal pellet previously obtained (2 mg/ml) was pre-warmed in a shaker (+37°C) and then diluted, with PBS, to obtain three melanin concentrations (0.2 mg/mL, 0.5 mg/ml and 1 mg/mL). A stock solution of chloroquine (1 mg/mL) was diluted, in PBS, to obtain two working solutions, one for the melanin-choroquine binding test (1100 ng/mL) and the other to the quality control of the assay (550 ng/mL). (36)

Melanin suspensions were sonicated and added to the RED device (Thermo Scientific™ RED, 8K MWCO) in the sample chamber. Pre-warmed PBS (+37°C) was added to the buffer chamber. RED device possesses one equilibrium dialysis membrane and two chambers. The sample chamber, where the melanin-chloroquine binding is accomplished, and the buffer chamber, where the free chloroquine will be suspended on buffer. The chloroquine solution (1100 ng/mL) was added to the sample chamber and after 6 h of incubation, three samples were taken from the buffer chamber. (36)

Quality control test, with no melanin, was done by adding the chloroquine solution (550 ng/ml) to the sample chamber. Pre-warmed PBS (+37°C) was added to the buffer chamber. At 0h it was taken three samples from the sample chamber. RED device was covered and put on a shaker (+37°C for 6 h). After 6 h of incubation three samples were taken from the sample chamber and the buffer chamber. (36)

2.4. Liquid chromatography–mass spectrometry (LC-MS)

2.4.1. Chromatography

High-performance liquid chromatography (HPLC) was performed in an Agilent 1260 Infinity consisting of a micro degasser, binary pump SL in low delay volume configuration (damper and mixer bypassed), high performance autosampler SL, and column thermostat SL (Agilent Technologies, Waldbronn, Germany). A Poroshell 120 SB-C18 column (2.1 x 50 mm, 2.7 µm) was used. The column was maintained at 40ºC and the autosampler tray at ambient temperature. Injection volume was 2 µL. Flow rate was 0.5 mL/min and a gradient elution was used with water (eluent A) and methanol (eluent B). The gradient was as follows: 0 to 2.0 min: 0%→2% B, 2.0 to 7.0 min:

2%→100% B, 7.0 to 7.1 min: 100%→2% B, 7.1 to 9.0 min: 2% B. The total running time from injection to injection was 9 min.

2.4.2. Mass spectrometry

Mass spectrometry analysis was performed in an Agilent 6495 Triple Quadrupole Mass Spectrometer with electrospray ion source (Agilent Technologies, Palo Alto, CA, USA). Nitrogen was used as a drying, nebulizer, and collision gas. The following ion source conditions were employed: positive ion mode, drying gas temperature of 200 ºC, drying gas flow of 16 L/min and nebulizer pressure of 25 psi.

2.4.3. Preparation of standard solutions

A PBS chloroquine stock solution was used to prepare working standards with different concentration levels, ranging from 1 – 500 ng/mL. These standards solutions were prepared in PBS or 1% formic acid in 80% acetonitrile. To each standard solution was added a solution of the internal standard, pamaquine, in PBS. The concentration of pamaquine in each standard solution was 100 ng/mL.

Polypropylene 2 mL tubes and pipette tips were used to prepare the standard solutions while vial glass was used to perform the LC-MS experiment.

To demonstrate the method suitability, precision, accuracy and calibration curve were calculated according to the "Guideline on bioanalytical method validation", by European Medicine Agency (EMA). (37) Within the precision and accuracy, it was used 3 standard solutions (5 ng/mL, 50 ng/mL and 500 ng/mL), which were the quality control (QC) samples of the method suitability. They were prepared by diluting the PBS chloroquine stock solution with PBS or 1% formic acid in 80% acetonitrile. The standard solutions were injected 5 times in a row. Precision is expressed as the coefficient of variation (CV) and it is calculated according to the formula ahead:

$$CV = \left(\frac{SD}{\bar{X}}\right) \times 100\% \quad (1)$$

Accuracy is reported as percent of the nominal value and it is calculated accordingly with the formula below:

$$Accuracy = \left(\frac{Determined\ Value}{True\ Value}\right) \times 100\% \quad (2)$$

A calibration curve with 9 concentration levels (1, 2.5, 5, 10, 25, 50, 100, 250 and 500 ng/mL) was used. A blank sample, without chloroquine and pamaquine, and a zero sample, with pamaquine and without chloroquine, both in PBS or 1% formic acid in 80% acetonitrile, were prepared. The blank and zero samples were not considered to calculate the calibration curve parameters. A linear regression adjustment was used to obtain the calibration curve and to achieve the best linear fit it was done a $1/x^2$ weighing factor.

2.4.4. Preparation of samples

The internal standard was added to the samples obtained from the melanin-chloroquine binding test, in a final concentration of 100 ng/mL. Polypropylene 2 mL tubes and pipette tips were used to prepare the samples while vial glass was used to perform the LC-MS experiment.

2.4.5. Stability Studies

To comprehend the behaviour of the binding of chloroquine to surfaces in different solvents and materials it had to done stability studies of this molecule.

A chloroquine stock solution, in PBS, was diluted to prepare solutions with different solvents that had a final concentration of 50 ng/ml of chloroquine. The solvents were water, PBS, 50% methanol, acetonitrile, 80% methanol and 1% formic acid in 80% acetonitrile. The solutions were prepared in polypropylene tubes or glass tubes, the vials used to perform the LC-MS were made of the same material as the one used to prepare the solutions. Polypropylene pipette tips were used to prepare the solutions. The LC-MS method used was the same described above.

2.4.6. Data Analysis

Software MassHunter Qualitative Analysis B.06.00, from Agilent, was used to analyse the data.

3. Results

A LC-MS method was used to separate, identify and quantify chloroquine.

3.1. Optimization of chloroquine and pamaquine detection

Initially, an optimization was needed to obtain a reliable and trustworthy method able to quantify the real amount of chloroquine in the samples.

Standard sample solutions of chloroquine and pamaquine (IS) were directly infused into the mass spectrometer to optimize the transitions and operating conditions. The obtained spectra of chloroquine and pamaquine are presented in figure 2 and 3, respectively. Chloroquine's $[M+H]^+$ showed a 320 m/z (figure 4) while pamaquine's $[M+H]^+$ showed a 316 m/z (figure 6). The predominant $[M+H]^+$ ion for chloroquine and IS were used as the precursor ion to obtain the product ions spectra.

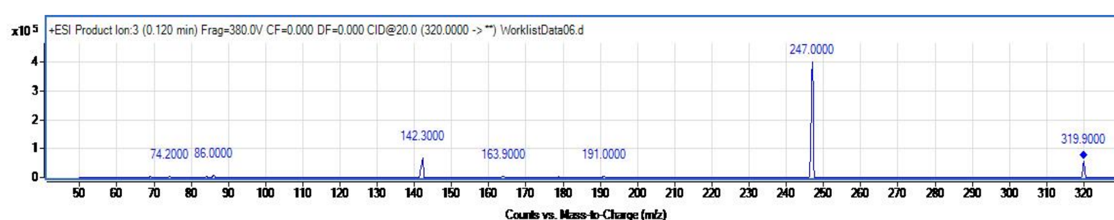

Figure 2: Electrospray ionization of chloroquine.

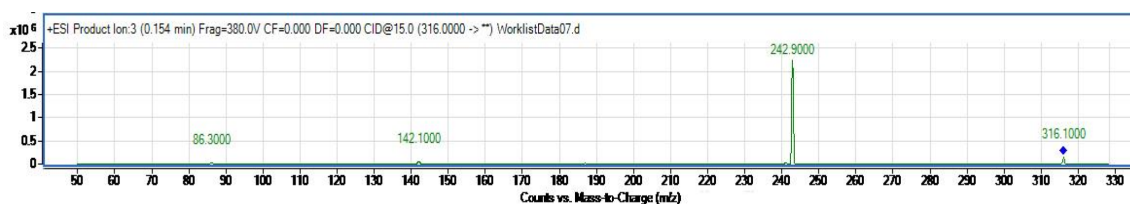

Figure 3: Electrospray ionization of pamaquine.

Chloroquine and pamaquine transitions, with collision energy and polarity, are summarized on table 1. Chloroquine precursor ion gave the following major product ions, 247 m/z (figure 5), 142 m/z and 86 m/z. IS precursor ion gave as major product ions, 243 m/z (figure 7), 187 m/z and 86 m/z.

The optimal mass transitions were 320→247 m/z (figure 4 to figure 5) to chloroquine and 316→243 m/z (figure 6 to figure 7) to pamaquine. This optimal mass transitions are also described by Wang et al (2016), Dongre et al (2009) and Singhal et al (2007). (21,38,39)

Table 1: LC-MS ions, with high response, of chloroquine and pamaquine.

	Precursor Ion (m/z)	Product Ions (m/z)	Collision Energy (V)	Polarity
Chloroquine	320	247	20	Positive
		142		
		86		
Pamaquine	316	243	15	
		187		
		86		

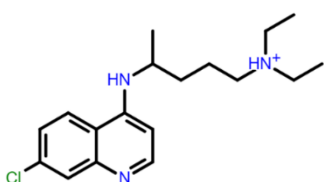

Figure 4: Chloroquine's Precursor Ion 320 m/z.

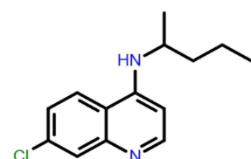

Figure 5: Chloroquine's Product Ion 247 m/z.

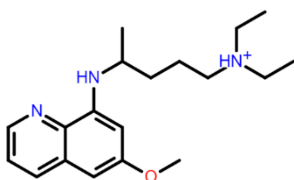

Figure 6: Pamaquine's Precusor Ion 316 m/z.

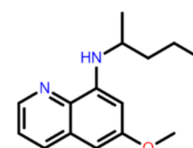

Figure 7: Pamaquine's Product Ion 243 m/z.

3.2. Optimization of quantification method

The selected HPLC column showed a suitable chromatographic elution and symmetric peak. The mobile phase provided a good peak shape and signal intensity.

Two calibration curves were prepared with different concentration ranges, in which the working solutions were prepared in 50% MeOH, since it appeared to be the best solvent to use. One calibration curve had a concentration interval between 0.01 to 1 ng/mL (figure 8).

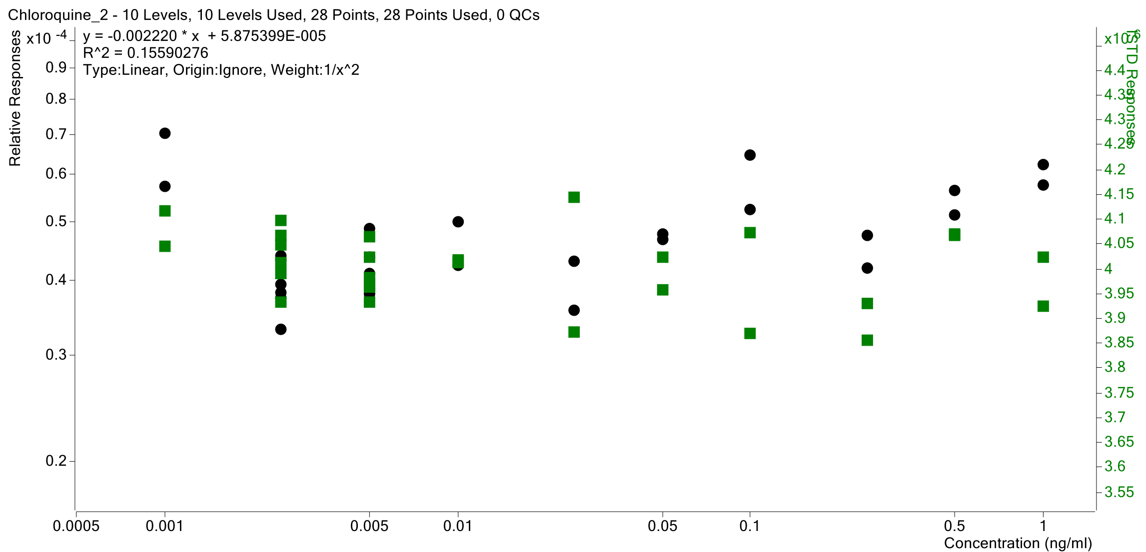

Figure 8: Experimental calibration curve (0.001ng/mL to 1ng/mL).

The other calibration curve had chloroquine concentration with a range of 1 to 1000 ng/mL (figure 9).

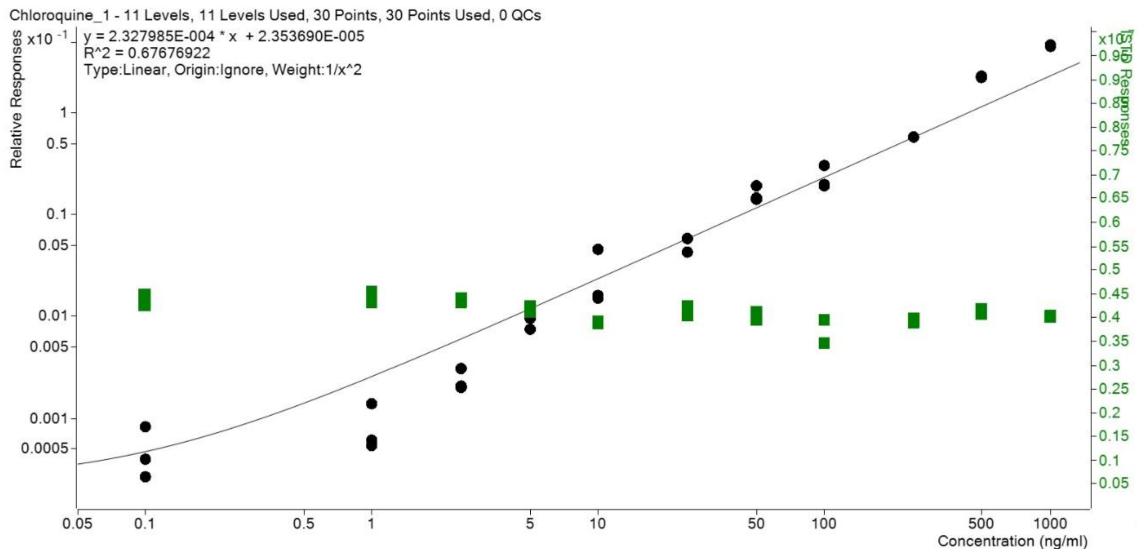

Figure 9: Experimental calibration curve (0.1ng/mL to 1000ng/mL).

Green dots, in figure 8 and 9, represent the IS concentration. The coefficient of variation of the IS was calculated and it was 2.8% for the calibration curve with lower concentration and 10,1% for the calibration curve with higher concentration.

Due to the poor-quality results, which can be seen by the low coefficient of determination, r^2, stability studies of chloroquine were performed.

3.3. Stability Studies

Table 2: Cloroquine's stability study in glass and plastic.

Material	Time (h)	Solvents					
		Water	PBS	50% Methanol	ACN	80% Methanol	1% FA in 80% ACN
Glass	0	26 800	172 223	49 640	73 071	18 980	194 930
	48	5 003	169 485	20 111	9 213	7 119	193 497
Plastic (Polypropylene)	**Time (h)**	Water	PBS	50% Methanol	ACN	80% Methanol	1% FA in 80% ACN
	0	128 821	179 968	168 937	148 237	192 226	200 705
	48	114 583	166 570	165 293	123 581	189 405	193 060

Chloroquine quantification, in the stability study done in LC-MS, was determined by the peak area of the chromatogram. When the solutions were prepared and analysed in glass, the values of response of chloroquine, in all most all solvents, have shown a very low response in comparison with the results obtained from the solutions prepared in plastic, only PBS and 1% formic acid and 80% acetonitrile show similar results between glass and plastic. The same applies with the time of analysis.

With this new information it was done a new calibration curve and the sample quantification, both in PBS or 1% formic acid in 80% acetonitrile, prepared with polypropylene 2 mL tubes and pipette tips, while the LC-MS experiment was done in vials of glass.

3.4. Calibration curve

The difference between the previous calibration curve and the new one relies on the use of new solvents to dilute the stock solution and the use of polypropylene tubes to prepare the dilutions.

The calibration curve which showed better results was the one that had the solutions prepared in PBS.

The calibration curve is linear over the concentration range of 10 ng/mL to 500 ng/mL of chloroquine in PBS and the coefficient of determination was 0.99. The linear regression equation of the calibration curve was $y = 0.161x - 0.117$, where y is the relative peak area of the analyte to the IS and x is the relative concentration of the analyte. Calibration curve can be seen on figure 10.

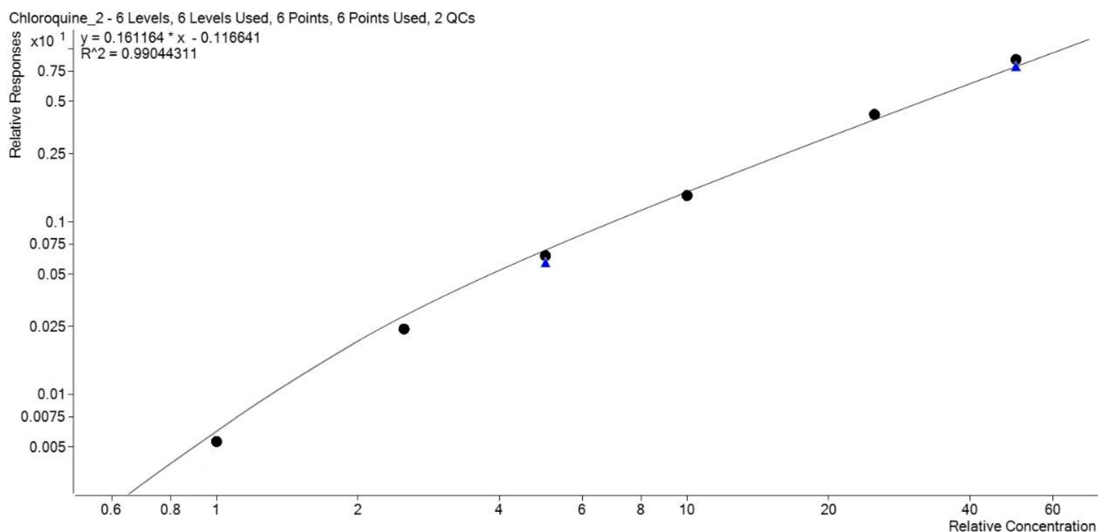

Figure 10: Calibration curve of chloroquine, ranging from 10ng/mL to 500ng/mL.

Blue triangles represent quality control standards of 50 ng/mL and 500 ng/mL of chloroquine in PBS.

From figure 11 we can be seen that the chromatogram of the standard solution 500 ng/mL that shows a slight tail.

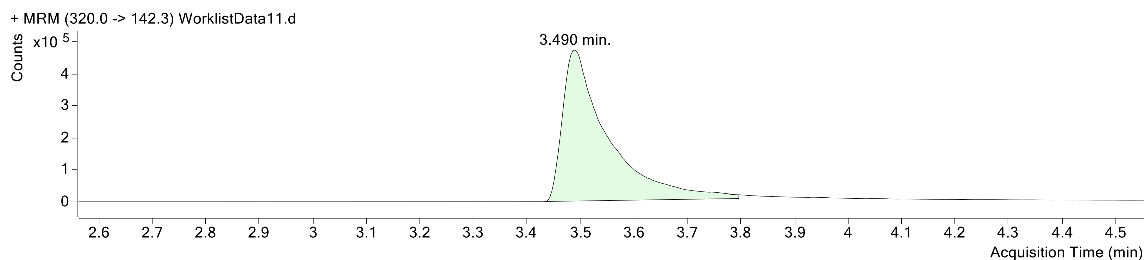

Figure 11: LC-MS chromatogram of 500ng/mL of chloroquine.

3.5. Precision and accuracy

Quality control standards of 50 ng/mL and 500 ng/mL prepared for precision evaluation, presented a CV% of 5.94% and 3.90%, respectively. As in accuracy, the 50 ng/mL quality control standard had 94.03% and the 500 ng/mL quality control had 103.61%. Values for calculation of precision and accuracy are presented on table 3.

Table 3: Chloroquine LC-MS method CV % and accuracy.

True Value (QC)	Determinated Value	CV%	Accuracy	Accuracy [Mean]
50 ng/mL	43.73 ng/mL	5.94%	87.46%	94.03%
	46.76 ng/mL		93.50%	
	50.56 ng/mL		101.12%	
500 ng/mL	501.46 ng/mL	3.90%	100.29%	103.61%
	546.51 ng/mL		109.30%	
	506.21 ng/mL		101.24%	

3.6. Bound chloroquine quantification

The melanin-chloroquine control binding study was done to know how much chloroquine would bind to the RED device, since it is made of plastic, and if there was a true equilibrium of chloroquine concentration between the two chambers.

Samples of chloroquine from melanin-chloroquine control binding study were analysed in the described LC-MS method. The results obtained are shown in Table 4.

Table 4: Melanin-chloroquine binding control.

Melanin concentration (0 mg/mL)	Time	Sample	Chloroquine concentration (ng/mL)	Chloroquine concentration (ng/mL) [Average]	Chloroquine theoretical concentration (ng/mL)
Sample chamber	0 h	1	478.34	507.60	550
		2	510.66		
		3	533.80		
Sample chamber	6 h	1	151,77	138.52	225
		2	132,11		
		3	131,67		
Buffer chamber		1	128,82	134.77	225
		2	132,89		
		3	142,61		

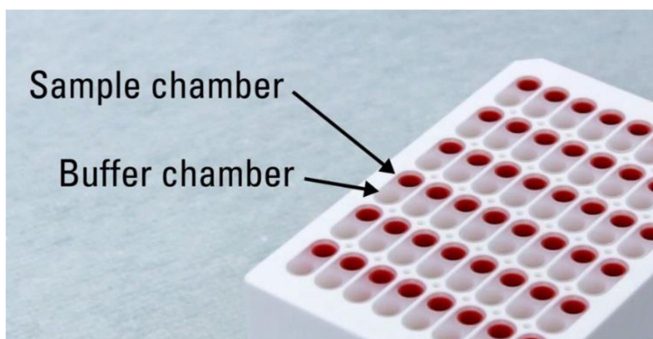

Figure 12: RED device (Thermo Scientific™ RED, 8K MWCO) with differentiation between sample and buffer chamber. **(40)**

Figure 13: RED device in detail. **(41)**

At 0 h, 42.4 ng/mL of chloroquine was bound to the RED device. After 6 h of incubation, 61.48 ng/mL and 65.23 ng/mL of chloroquine was bound to the RED device in the sample chamber and in the buffer chamber, respectively, as seen on table 4.

Samples of chloroquine from melanin-chloroquine binding study were analysed in the described LC-MS method. The results obtained from the LC-MS method gave the amount of free chloroquine on the RED device after 6 h of incubation. In table 5 it is presented the concentration of chloroquine that was bound to melanin and to the RED device. The calculation of the bound chloroquine concentration was accomplished by subtracting the concentration of free chloroquine to 100 ng/mL of chloroquine, the concentration of chloroquine used on the melanin-chloroquine binding study. From the three different concentrations of melanin used, in the 0.2 mg/mL of melanin was achieved a concentration of 92.42 ng/mL of bound chloroquine, in the 0.5 mg/mL of melanin the amount of chloroquine that was bound was 92.74 ng/mL and in the 1 mg/mL of melanin, 92.69 ng/mL of chloroquine was not free, as presented on table 5.

Table 5: Bound chloroquine quantification in samples.

Melanin concentration (6h)	Sample	Bound chloroquine concentration (ng/mL)	Bound chloroquine concentration (ng/mL) [Mean]
0,2 mg/mL	1	92.02	92.42
	2	92.48	
	3	92.75	
0,5 mg/mL	1	92.74	92.74
	2	92.72	
	3	92.76	
1 mg/mL	1	92.71	92.69
	2	92.70	
	3	92.66	

4. Discussion

The two first calibration curves obtained were meant to have a sensitive and wide range of chloroquine concentration that would be able to integrate the chloroquine values obtained from the chloroquine-melanin binding test. This two calibration curves revealed a low coefficient of determination indicating its lack of suitability. A possible explanation for this problem is the binding of chloroquine to the equipment/column. (42–44) Despite this, the precision of the IS, which directly correlates with the work precision of the person who did the solutions, it is below 15%, ensuring its work precision. (37)

Studies of binding and stability of chloroquine were done and previous literature was examined. (42–46) With this, it was clear that chloroquine tends to bind preferably to glass and over time binds greatly to plastic. Acknowledging this, the method of preparation of the standard solutions and samples was improved. On one hand, the dilutions of the standards and samples were done in plastic, since the chloroquine would have a short time in contact with this material. On the other hand, the LC-MS vials were made of glass, because the LC-MS procedure takes a few hours to analyse all samples and the amount of free chloroquine would not variate. Also, with the binding and stability studies of chloroquine, the solvents that shown the highest response of chloroquine, were PBS and 1% formic acid in 80% acetonitrile, reason why they were both used to prepare the standards for the calibration curve. Anyhow, the calibration curve that showed the best results was the one with PBS.

With a coefficient of determination of 0.99, the calibration curve obtained showed its reliability and linearity (figure 10). Precision, accuracy and quality controls respect EMA's Guideline on bioanalytical method validation parameters, proving instrument and method suitability (table 3).

In figure 11, it is possible to see a slight tail of the chromatogram, which can be explained by the biding of chloroquine to the instrument. While most of the drug leaves the column, small portions of chloroquine that are attached to the column are released with the flow and slowly leave the column, appearing in the chromatogram as tail of the main curve.

From the result analysis of the melanin-chloroquine binding control test it is relevant to point the amount of chloroquine obtained at 6h on the two chambers of the RED device and the differences between the theoretical concentration and the concentrations obtained. The concentration of chloroquine on the two chambers of the RED device, at 6h, is almost the same, ensuring that chloroquine flows freely between the two chambers and that the sampling is suitable. Chloroquine binds positively to the RED device and over time, since at 0h, there's a decrease of, approximately, 8% from

the theoretical concentration and at 6h, there is a bigger decrease of the chloroquine concentration, approximately 39%. With this finding it is knowledgeable that the results obtained from the melanin-chloroquine binding test are inflated due to the binding of chloroquine to the RED device.

Despite it is known the amount of chloroquine that binds to the RED device after 6h of incubation, it is not possible to use this information to extrapolate the real amount of bound chloroquine to melanin in the melanin-chloroquine binding test. The concentration of chloroquine used on these two tests was not the same and we do not know if chloroquine has equal affinity to plastic and melanin. Regardless, it can be theorized that the amount of chloroquine used on the two tests was the same and that the affinity of chloroquine to plastic and melanin was also the same, with this, the corrected results of the melanin-chloroquine binding study are, in the 0.2 mg/mL of melanin, 56.38 ng/mL of bound chloroquine to melanin, in the 0.5 mg/mL of melanin, 56.57 ng/mL and in the 1 mg/mL of melanin, 56.54 ng/mL.

The results from the melanin-chloroquine binding study, with subtraction of the percentage of chloroquine that bound to the RED device, makes it clear that chloroquine binds heavily to melanin, as reported, (13,14,16) and without discrimination of the concentration of melanin used.

5. Conclusion

The aim of the scientific work of this thesis was the development of a method that could quantify small amounts of chloroquine, precisely and accurately. To achieve this premise, stability and binding studies were done and granted essential information to optimise the method of quantification. From the information extracted from those studies, a sensible and reliable method was accomplished. In the end, the results from the melanin-chloroquine binding test were concordant with previous studies that used blood/plasma samples or tissues samples and show that chloroquine binds greatly and unequivocally to melanin.

In the future, a more sensible method should be developed. At this time, other columns of HPLC need to be tested, where, the walls' column need to be permeable to chloroquine. Also, the next melanin-chloroquine binding control and the melanin-chloroquine binding test should have the same amount of chloroquine concentration on them.

The evidence of the relation between chloroquine therapeutic and ophthalmic diseases is undeniable. Some studies defend the connection between the melanin-chloroquine binding and its influence on retinopathy, and therefore on AMD, making its consequences appear sooner or greater.

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