Original Article

Method Optimization for Acetylcholinesterase Modulators-Albumin Interactions

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Summary

Plasma proteins such as human serum albumin (HSA) and alpha 1-acid glycoprotein (AGP) play an important role in the pharmacokinetic and pharmacodynamic properties of all endogenous or exogenous substances presented in the blood vessels. Their abundance in human blood not only helps to maintain hemostasis, but also makes them ideal candidates as transporters or possible depot reservoirs. Numerous methods e.g. equilibrium dialysis, ultrafiltration, ultracentrifugation, high-performance affinity chromatography were introduced to determine potential interactions with plasma proteins and possible quantification.

In this work method was developed for the determination of drug-human serum albumin interactions and applied to obtain pharmacokinetic profile of AChE modulators prepared at the Faculty of Military Health Sciences. For method validation commercially used compounds HI-6 and obidoxime were chosen. HI-6 did not bind at all while obidoxime showed 7% binding potency.

Key words: human serum albumin; alpha 1-acid glycoprotein; plasma proteins; acetylcholinesterase reactivators; drug-protein interaction

Introduction

In the blood we can find around 70–75 g/l of proteins that makes them significant players in the functionality of our system. The focus on plasma proteins has been mainly because of their relatively easy isolation from blood and plenty of available blood donors. Naturally, it was not long before the structure of certain proteins was decoded and many interesting things were learned from it.

Majority of plasma proteins are synthetised in the liver. Nevertheless, some exceptions occur e.g. cells of the immune system produce γ-globulins, which also belong to the protein family [1][2][3].

Plasma proteins in general are synthetised on polyribosome bound to the cell membrane and are part of the main secretion cell pathway (endoplasmic membrane Golgi apparatus plasma membrane cell exit).

Nearly all plasma proteins are part of the glycoprotein family with oligosaccharide chains
bound either by N- or O- linkage. However, albumin is an important exception as it does not contain any saccharide residues. Moreover, many plasma proteins show polymorphic characteristics and each protein also has different half-life e.g. for albumin half-life is approximately twenty days [4].

Certain proteins (e.g. AGP) are inflammatory markers. Their concentration in blood increases as the part of immunological reaction during acute inflammation or as a secondary reaction on tissues damage [5].

Several methods were developed to quantify interactions that can occur between plasma proteins and therapeutics, and subsequently improve dosing and safety of numerous medications. Furthermore, we cannot ignore interactions that two or more substances can have on each other while bonded to human serum albumin. It has been reported in the literature that interactions proved to be life threatening (e.g. warfarin, digoxin, lithium) [6, 7]. As experience shows it is of outer most importance for novel therapeutics to undergo protein binding tests not only for better understanding of pharmacokinetics and thorough determination of therapeutic doses, but also to eliminate any potential threat to the patient.

Albumin (Fig. 1) is one of the main plasma proteins (45 g/l) with molecular weight around 69 000 Da (585 amino acids). Approximately 40% of albumin is located in the blood plasma while the rest is in extracellular compartments. Albumin is synthetised in the liver at the rate of 12 g per day in the form of a preprotein. Subsequent post-translational modification is needed to produce functional protein [9]. Under normal conditions albumin has a life span around 27 days [10]. Naturally, this can be affected by illness such as liver disease (albumin synthesis is decreased) or nephritic syndrome and protein-losing enteropathy (increased loss of albumin) [11].

HSA is a heart-shaped molecule with approximate dimensions of 80x80x80x30 Å with 67% of its conformation formed by α-helices [12] interestingly no β-helices are present. With the help of x-ray diffraction it has been identified that the protein has three helical domains (I – III). Each domain is composed of two sub-domains (A and B). The A and B sub-domains consists of six and four α-helices respectively, both domains are connected with flexible loops. The whole conformation is stabilised by 34 disulfide bonds with one cysteine group remaining vacant, 34Cys [13].

Sudlow et. al. 1975 [14]are considered pioneers in the discovery of HSA binding sites.
They determined two major binding sites towards majority of drugs show the highest affinity. They are since then called site I, site II or Sudlow site I, Sudlow site II. In addition to these two sites, other less potent binding sites have been discovered for several drugs: thyroxine/propofol, halothane/ibuprofen, indomethacin/azapropazone etc. It is important to understand that substances can bind to several sites with different affinity[15, 16].

The aim of this work was to find and adapt the most suitable method for drug-protein interactions. Numerous experimental methods are used worldwide either for commercial use or laboratory needs, each with understandable pros or cons. Our main focus was to adapt the most appropriate method for AChE modulators and test it at commercially used AChE reactivators, HI-6 and obidoxime (Fig.2).

**Figure 2.** Structures of tested compounds.

**METHOD DESCRIPTION**

**Method and Materials**

Drug-protein interactions have been studied by numerous types of validated experimental in vitro methods before any new compound is administered to a patient. It is necessary to acquire as much knowledge about the behavior of the new compound as possible. Equilibrium dialysis (ED), ultrafiltration, ultracentrifugation, spectroscopic methods, affinity chromatography and electrophoretic methods are the most commonly used techniques [17]. Ultrafiltration is a simple, cost-effective and fairly quick method. Moreover, analysis of the filtrate can be done by HPLC methods that are well established at the Faculty of Military Health Sciences.

**Chemicals**

Albumin, phosphate buffer saline and acetonitril super gradient grade G Chromosolve® were purchased from Sigma-Aldrich (Prague branch, Czech Republic). AChE modulators were synthesised at the Faculty of Military Health Sciences. Water was obtained by Millipore system via reverse osmosis.

**Apparatus**

The HPLC system consisted of gradient pump LC-10ADVP Shimadzu (Prague branch, Czech Republic), 7125 injection valve – 10 µl loop (Rheodyne, Cotati, USA), Coulchem II detector with analytical cell model 5011, and CSW...
Chromatography Station 1.5 software (DataApex, Praha, Czech Republic).

For ultracentrifugation Centrifree® Ultrafiltration Devices (Millipore, Ireland BV, Tullagreen, Carrigtwohill, Country Cork, Ireland) was used. Membrane present is Ultracel regenerated cellulose with surface area 0.92 cm² designed to retain 99.9 % of serum protein.

Sample Preparation

HPLC method for tested compounds was validated. Even though, each compound is structurally different the same mobile phase worked for both.

Concentration of HSA is known to be 45 g/l, which was adapted in our method. Samples were prepared by pipetting 900 μl of albumin solution (HSA and phosphate buffer saline) and 100 μl of phosphate buffer saline with tested substance into vials. The real concentration of HSA corresponds with the average concentration typical for human blood. The final concentration of AChE modulators corresponded with the maximum concentration acquired after application of therapeutic dose. Therapeutic doses for HI-6 and obidoxime were taken from previous distribution studies 15.26 µg/ml and 23.62 µg/ml respectively [17]. Each sample was incubated for at least 90 minutes at 37°C and continually shaken. The ideal incubation time was determined on obidoxime. Tested time intervals were 30, 60, 90, and 120 minutes. Obidoxime when incubated for 30 minutes did not bind, at the 60 minutes interval analysis showed 3% binding, and the 90 minutes interval binding affinity has risen to 7%, which was same for the last time interval.

After the incubation the next step was to transfer samples into Centrifree® Ultrafiltration Devices and according to manufactures specifications centrifuged at 1,000-2,000 x g for 90 minutes at 37°C. The middle value (1,500 g) was chosen for these experiments.

HPLC Method

For analysis previously validated and published reverse phase HPLC method with electrochemical detection was used [18]. The analytical column used for measurements of AChE modulators was LiChrospher® 60, 250 x 4.6 (5μm) containing a guard column 4 x 4 (RP – select B) (Merck. Damstadt, Germany). The mobile phase composition was 20:80 (v/v) acetonitrile and aqueous component containing 3mM octane sulfonic acid (sodium salt) and 1mM tetramethylammonium chloride. The pH level was adjusted by H₃PO₄ to suit HI-6 (pH 2.4) and obidoxime (pH 2.1) [18] [19]. The flow rate of the mobile phase 1.0ml/min is optimal for this kind of measurement. Electrochemical detector measurement cells had potentials of +350 mV/+650 mV. Potential of the guard cell was +1000 mV. All chromatograms were obtained at conditioned room temperature (23°C).

Samples and blanks were prepared and analysed in triplicates. Calibration and measurements were analysed on the same day to obtain accurate results. The calibration curve for obidoxime and HI-6 had linear relationship R²>0.9979 (Fig. 3). Calibration samples were in the range of 0-20 μg/ml.

![Figure 3. Calibration curve of HI-6.](image-url)
RESULTS AND DISCUSSION

Before any measurements took place, appropriate method parameters had to be determined and exactly specified. Constant conditions are as follows; incubation temperature (37°C), incubation time (90 minutes), pH calibration (albumin had tendencies to slightly acidify the solution), and ultracentrifugation parameters (37°C, 90 minutes, 1,500 g). HPLC method turned out to be a variable parameter because every compound has a unique structure meaning different detector settings had to be used each time and of course mobile phase had to be adjusted also. However, this is of no relevance since calibration was conducted before every measurement as described above.

Two sets of three samples were prepared for tested compound. First set of three vials had 900 μl of albumin solution and 100 μl of phosphate buffer saline with tested substance in specified concentration, while the second set was designed as a control (900 μl of phosphate buffer saline and 100 μl of phosphate buffer saline with tested substance in the same concentration). HI-6 and obidoxime concentrations used were taken from previous pharmacokinetic experiments by Karasova et. al. 2010 [18].

After ultracentrifugation of all samples HPLC analysis of all filtrates were carried out. Below (Fig.4) is an example of acquired chromatogram for HI-6. The top line represents sample with albumin, middle line is a control, and bottom line represents pure phosphate buffer saline to prove no contamination was present.

From above chromatogram it is apparent that HI-6 did not demonstrate any affinity towards HSA. AUC and its corresponding concentration of the top (10.92±0.18 μl/ml) and middle peak (10.97±0.12 μl/ml) was exactly the same.

Interactions of therapeutics with plasma proteins are determined for every novel drug with the ambition of being registered and administered to potential patients. The importance to understand this idea is underlined by regulatory authorities requesting relevant data as part of the registration process. AChE modulators are specific in terms of their primary use, but it is without doubt that more detailed understanding of their interactions is needed.

Described method is relatively simple to conduct and flexible enough to be applicable to broad spectrum of substance. Naturally, more sophisticated methods using HPLC and HSA bound to silica gel columns are available but described method mimics more precisely the blood vessel environment.

Previous work has proven that pharmacokinetic profile of AChE modulators corresponds closely to
A set of structurally unique compounds was tested each with difference in elimination. The hypothesis for this phenomenon is that possible interaction with albumin can alter elimination times based on the structure of tested AChE modulators. Once AChE modulators-albumin interactions are determined and compared with their pharmacokinetic profile interesting results will be surely obtained [20].

CONCLUSION

A method to determine the binding potency of AChE modulators is presented. A combination of ultrafiltration and reverse phase HPLC analysis with electrochemical detection was tested on two compounds, HI-6 and obidoxime. Understandably more modulators will have to be measured before any conclusion can be made. However, even preliminary results are mentioned in this manuscript. Two structurally similar compounds HI-6 and obidoxime have apparently different affinity towards HSA.

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