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Characterization of binding properties of ephedrine derivatives to human alpha-1-acid glycoprotein

Sebastian Schmidt, Markus Zehe, Ulrike Holzgrabe *

Institute for Pharmacy and Food Chemistry, Am Hubland, Wuerzburg D-97074, Germany

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ABSTRACT

Most drugs, especially those with acidic or neutral moieties, are bound to the plasma protein albumin, whereas basic drugs are preferentially bound to human alpha-1-acid glycoprotein (AGP). The protein binding of the long-established drugs ephedrine and pseudoephedrine, which are used in the treatment of hypotension and colds, has so far only been studied with albumin. Since in a previous study a stereoselective binding of ephedrine and pseudoephedrine to serum but not to albumin was observed, the aim of this study was to check whether the enantioselective binding behavior of ephedrine and pseudoephedrine, in addition to the derivatives methylephedrine and norephedrine, is due to AGP and to investigate the influence of their different substituents and steric arrangement. Discontinuous ultrafiltration was used for the determination of protein binding. Characterization of ligand-protein interactions of the drugs was obtained by saturation transfer difference nuclear magnetic resonance spectroscopy. Docking experiments were performed to analyze possible ligand-protein interactions. The more basic the ephedrine derivative is, the higher is the affinity to AGP. There was no significant difference in the binding properties between the individual enantiomers and the diastereomers of ephedrine and pseudoephedrine.

AGP	alpha-1-acid glycoprotein
BGE	background electrolyte
CE	capillary electrophoresis
DUF	discontinuous ultrafiltration
E	ephedrine
HDAS-β-CD	heptakis-(2,3-di-O-acetyl-6-sulfo-)-β-cyclodextrin
K_a respectively pK_a	acidity constant
K_{aff} respectively pK_{aff}	affinity constant
ME	methylephedrine
NE	norephedrine
PE	pseudoephedrine
STD-NMR	saturation transfer difference NMR

1. Introduction

Many different factors influence the drug therapy, among them bioavailability, metabolism, and solubility. Above all, binding to proteins such as albumin, has huge influence on the success of the respective drug therapy. If a drug is strongly bound, the effective dosage is substantially reduced and hence, the drug cannot develop its full effect. Albumin is the protein with the largest proportion among all plasma proteins and performs the most important functions, such as transport of endogenous and exogenous substances through the body. It binds mainly acidic and neutral substances at two binding sites (Sudlow et al., 1975). Diseases can increase or decrease protein concentration, which affects the extent of protein binding (Tillement et al., 1978). Besides albumin, there are other plasma proteins which only make up a small proportion of the total quantity such as lipoproteins or alpha-1-acid glycoprotein (AGP) also known as orosomucoid, with AGP being only 3% of the plasma proteins. However, it is the second most important transport protein for drugs besides albumin. AGP is a highly glycosylated acute phase protein with a molecular mass of 42 kDa and widely used as an inflammation marker in laboratory diagnostics. Its carbohydrate content is very high at 45% and responsible for the immunomodulatory activities of the protein (Fournier et al., 2000). There are two variants of AGP, variant A and F1*S, which occur in a 1:2 to 1:3 ratio in humans (Yuasa et al., 1993). AGP transports mainly basic substances but lipophilic ones as well. This is significant as most drugs have basic structural elements (Charifson and Walters, 2014). The binding

* Corresponding author.
E-mail address: ulrike.holzgrabe@uni-wuerzburg.de (U. Holzgrabe).

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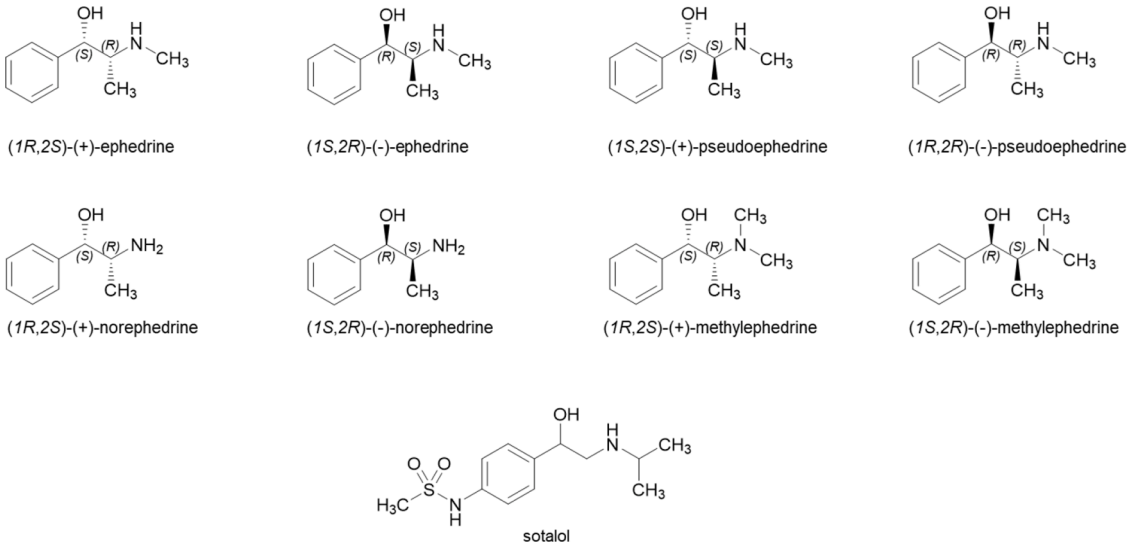

Fig. 1. Structural formulas of studied drugs

behavior of drugs to AGP was often described as binding to a single dominant high-affinity binding site with several low-affinity binding sites or two main binding sites with the same affinity and these two binding sites differ primarily in their affinity for basic and acidic drugs (Berezhkovskiy, 2007). In 2008, Schönfeld et al. characterized the non-glycosylated crystal structure of AGP with one main binding site, which differs depending on the variant (Schönfeld et al., 2008). In a review by Israili and Dayton the binding of many different drugs to AGP was evaluated (Israili and Dayton, 2001). The basic drugs ephedrine (E) and pseudoephedrine (PE), which are used to treat hypotension and colds, have not been characterized. Nevertheless, there have been studies on protein binding of E and PE with albumin (Guo et al., 2003; Huang et al., 2011; Till and Benet, 1979; Yang and Hage, 1994).

Volpp and Holzgrabe showed that the binding of E and PE is increased in human serum compared to the isolated albumin and indicating that these drugs may bind to another plasma protein (Volpp and Holzgrabe, 2019). In addition, differences in protein binding of the individual enantiomers and diastereomers were found in human serum, which suggest a stereoselectivity of the binding process. Due to the basic structure of E and PE, binding to AGP seems obvious, which is why binding experiments with E and PE as well as the derivatives norephedrine (NE) and methylephedrine (ME) were performed. For the determination of the extent of the binding of ephedrine derivatives to AGP discontinuous ultrafiltration (DUF) was used and for structural studies saturation transfer difference NMR (STD-NMR) was chosen. To the best of our knowledge, STD-NMR has only been used by Becker and Cruz (Becker and Larive, 2008; Cruz and Larive, 2012) to investigate protein binding of drugs to AGP due to the challenging nature of a polar binding site and the strong glycosylation. One of the drugs examined was the β-receptor antagonist propranolol, which has some structural similarity to the ephedrine derivatives. Stereoselective differences in binding behavior were also found for both propranolol enantiomers, which is why enantioselective binding seems possible for the ephedrine derivatives and their enantiomers. Overall, the aim of this study was to investigate the binding properties of different ephedrine derivatives and their respective enantiomers (cf. Fig. 1) to AGP and to study the influence of structural differences and stereoselective binding.

2. Materials and methods

2.1. Chemicals

(+)-Ephedrine hemihydrate, (−)-pseudoephedrine, (+)-pseudoephedrine, (+)-norephedrine, (−)-norephedrine, sotalol hydrochloride and α_1-Acid Glycoprotein from human plasma were purchased from Sigma Aldrich (Basel, Switzerland), while (−)-ephedrine from Caelo (Hilden, Germany). (+)-Methylephedrine and (−)-methylephedrine were obtained from Fluka (Buchs, Switzerland). The buffer salts sodium dihydrogenphosphate (anhydrous), disodium hydrogenphosphate, tripotassium phosphate, sodium azide and sodium chloride were purchased from Sigma Aldrich (Basel, Switzerland), phosphoric acid 85% and sodium hydroxide from VWR Chemicals (Darmstadt, Germany), hepktakis-(2,3-di-O-acetyl-6-sulfo-)-β-cyclodextrin (HDAS-β-CD) from Regis Technologies (Morton Grove, Texas, US), 0.1 M hydrochloric acid solution from Bern Kraft (Duisburg, Germany) and deuterated water from Deutero GmbH (Kastellaun, Germany), while Millipore water was obtained from an in-house water purification system from Merck Millipore (Darmstadt, Germany).

2.2. Instruments

pH measurements were performed with a pH meter from Metrohm (Filderstadt, Germany). The samples were centrifuged with a centrifuge 5702 and incubated in a thermomixer, both from Eppendorf (Hamburg, Germany). The CE system used was a P/ACE MDQ system from Beckman Coulter (Brea, California, US) with a photodiode array detector.

2.3. Discontinuous ultrafiltration

As incubation medium a 30 mM sodium dihydrogen/disodium hydrogen phosphate buffer, with 100 mM sodium chloride and a pH of 7.40 was used. The pH was adjusted with 0.1 M sodium hydroxide solution. The ionic strength and pH of the buffer correspond to the physiological conditions of the blood (Oehlmann, 1996). 180 μM AGP, 360 μM racemic mix of each drug derivative and 225 μM sotalol stock solution were prepared by dissolving the required amount in incubation

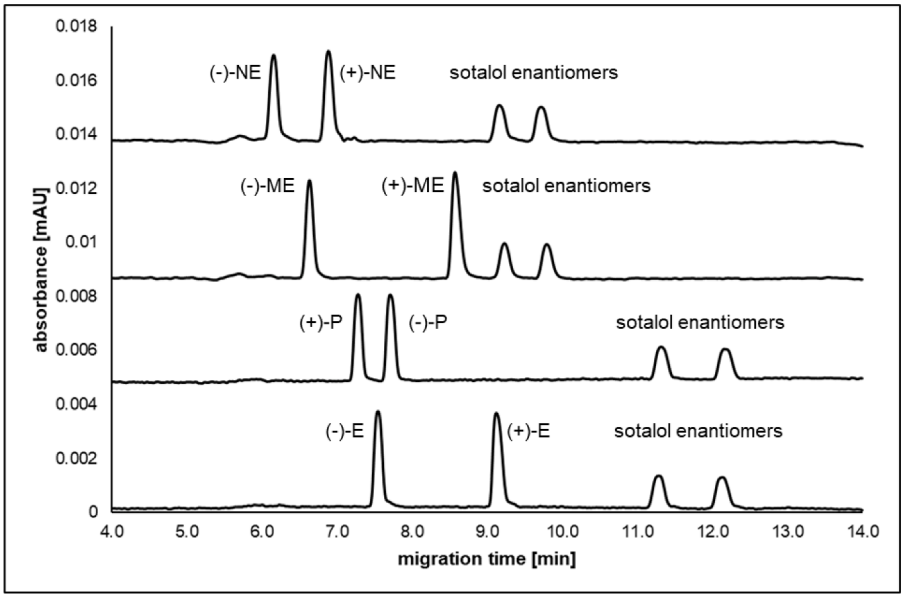

Fig. 2. Electropherogramm of the drugs used at a concentration of 100 μM and 50 μM internal standard; method parameters: 20.0 kV, 100 mM sodium dihyrogenphosphate/phosphoric acid buffer pH value 3.0, 3 mM HDAS-β-CD, total capillary length 52 cm, effective capillary length 40 cm

buffer. Five drug-protein ratios were incubated and covered the range from 0.8 to 1.5. The protein concentration was held constant at 80 μM while the determined drug concentration differed in the range from 50 - 120 μM. Drug and protein stock solutions were mixed with the respective amount of incubation buffer in 1.5 ml Eppendorf tubes and incubated at 37 °C for 30 min. After equilibration was reached the internal standard sotalol was added in a final concentration of 50 μM. The solution was transferred directly into the Amicon® Ultra 0.5 ml ultracentrifugation unit with a molecular cutoff of 3 kDa (Merck, Darmstadt, Germany) and centrifuged for 5 min at 4400 rpg. After centrifugation, the solution was filled into vials and assessed by means of capillary electrophoresis (CE). This was performed in triplicate per drug-protein ratio.

2.4. Capillary electrophoresis

A fused silica capillary from BGB Analytik Vertrieb (Rheinfelden, Germany) with an internal diameter of 50 μM, a total length of 52 cm and an effective length of 40 cm was chosen for the separation. The background electrolyte (BGE) consisted of a 100 mM sodium dihydrogenphosphate/phosphoric acid (85%, w/w) buffer, adjusted to a pH of 3.0 with 1.0 M sodium hydroxide solution, and 3 mM HDAS-βCD. Samples were injected at a pressure of 10.0 psi for 5.0 s and separated at room temperature in the cationic injection mode, using a constant voltage of 20.0 kV for 20.0 min. The capillary was conditioned first with 1.0 M sodium hydroxide solution, second with 2.0 M hydrochloric acid and third with Millipore water at a pressure of 30.0 psi, each for 10.0 min. Subsequently, the capillary was rinsed with BGE for 2 min and a voltage of 20.0 kV for 20 min was applied. Before each sample injection, the capillary was rinsed 2.0 min with 0.1 M hydrochloric acid, 2.0 min Millipore water and 5.0 min with BGE at a pressure of 20.0 psi. Analytes were detected at a wavelength of 194 nm. Data evaluation was performed with 32 Karat Software 8.0 from Sciex (Darmstadt, Germany).

2.5. Saturation transfer difference NMR

The measurements were carried out on a Bruker III Avance spectrometer operating at 400.13 MHz equipped with a PABBI inverse probe (Karlsruhe, Germany). The pulse frequency stddiffesgp.3 was used, coupled with an excitation sculpting pulse frequency for water signal suppression at 4.703 ppm. The scan count was 8 scans with 16 dummy scans and a loop counter of 64. The spectral width was set to 15.98 ppm and the transmitter offset to 4.70 ppm. The measurement temperature was 300 K. The solvent used was a deuterated 30 mM phosphate buffer containing 25 mM sodium chloride. For preparation, tripotassium phosphate was weighed, dissolved in deuterium oxide, and adjusted to a pD of 7.40 with deuterated hydrochloric acid. For stability to microbial attack 0.02% sodium azide was added. 2.0 mM from each enantiomer and 40 μM protein stock solutions were prepared by dissolving the required amounts in the buffer**.** The ligand-protein solutions were prepared by mixing the stock solutions with the respective amount of buffer to obtain a ligand-protein ratio of 20:1. Protein concentration was held constant at 15 μM. Each sample was measured at four different saturation times from 1 to 4 s with a constant relaxation delay of 4 s. Data were integrated and evaluated with TopSpin 4.0.9 from Bruker (Karlsruhe, Germany).

2.6. Molecular docking

Docking studies with AutoDock (Morris et al., 1998) were executed on the crystal structure of the human AGP A variant in complex with amitriptyline (PDB code 3APV, chain A (Nishi et al., 2011)). Using MOE (Molecular Operating Environment; Chemical Computing Group ULC, Montreal, QC, Canada) ligand atoms as well as water molecules and other small molecules were extracted, terminal residues were kept in the charged form. Polar hydrogens were added with the program PROTONATE of the AMBER software package (Case et al., 2005, 2008). Kollmann charges and solvation parameters were assigned to generate the PDBQS-files required for the calculation of the docking grids by AutoGrid (60×60×60 grid points, spacing 0.375 Å; centered on binding site placing the grid center near atom OE2 of Glu-92). Ligand building, addition of protons and energy minimization (gradient: 0.001 kcal/mol/Å; MMFF94x forcefield) were performed with MOE. The docking was carried out using the Lamarckian Genetic Algorithm (10×10^6 energy evaluations, population size of 350 individuals, max. 50.000 generations, 300 iterations in Solis and Wets local search, 50 GA runs) which resulted in a very accurate reproduction of the experimentally observed binding mode of amitriptyline (98% top poses with

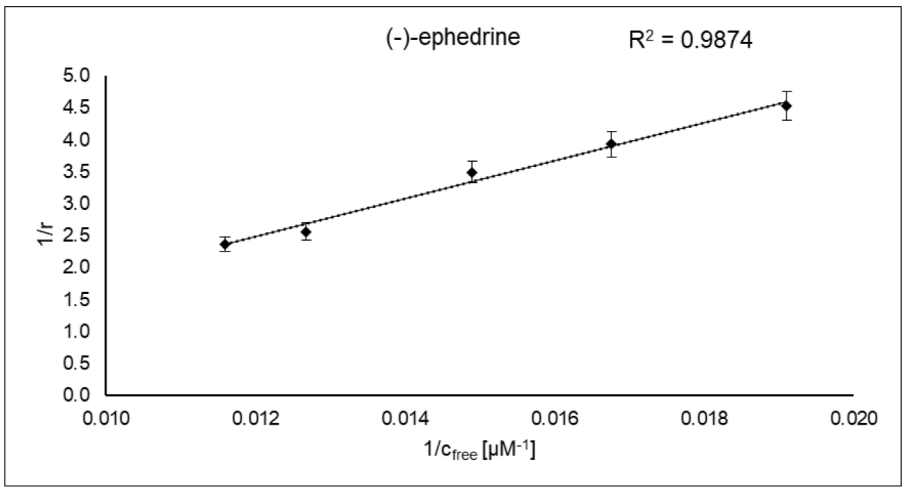

Fig. 3. Klotz plot of (−)-ephedrine; reciprocal application of r against c_{free} with r equal to the quotient of c_{bound} by $c_{protein}$, error bars indicate the relative standard deviation

RMS-deviations below 2 Å).

3. Results and discussion

3.1. Discontinuous ultrafiltration and capillary electrophoresis analysis

The procedure of DUF is characterized by mixing drug and protein solution. After incubation, the formed drug-protein complex is centrifuged off while the free drug remains in the centrifugate. The free drug concentration was determined by means of CE. HDAS-β-CD was chosen as the chiral selector because good separations of ephedrine derivatives and their enantiomers have already been shown in the past (Wedig et al., 2002). In addition, a good separation of the sotalol enantiomers had to be achieved because only the racemic sotalol is commercially available. Sotalol hydrochloride was used as internal standard because it is reported not to bind to APG (Belpaire et al., 1982; Israili and Dayton, 2001). To avoid possible binding of sotalol to AGP, the internal standard was added just before centrifugation. As can be seen in Fig. 2, a separation of all ephedrine derivatives as well as the sotalol enantiomers was accomplished within 15 min.

The greater the excess of the drug concentration the more the binding sites of the protein are occupied. The binding of a ligand to a protein is based on an equilibrium reaction following.

$$\text{ligand} + \text{protein} \rightleftharpoons \text{ligand} - \text{protein complex} \quad (1)$$

This reaction can be described by the affinity constant K_{aff}, which is the applied law of mass action to Eq. (1) (Du et al., 2016). To characterize the affinity constant K_{aff} and the binding behavior, several different drug-protein ratios must be incubated, and the free drug concentration measured. Either the protein concentration or the drug concentration is kept constant. In healthy humans, the plasma concentration of AGP is between 10 and 20 µM (Bteich, 2019). Since the drug excess would have been too large and no significant change in free concentration would have been detected it was not possible to use a physiological concentration of AGP in the DUF experiments. Therefore, a AGP concentration of 80 µM was chosen. Lowering the drug concentration was no option, as drug concentrations would have fallen below the limit of quantification. The LOQs of the drug used can be found in Table S1. LC-MS is also used in combination with ultrafiltration (Fabresse et al., 2020), as the better sensitivity allows work in physiological conditions. However, CE was chosen because of its advantages in the separation of enantiomers, as the stereoselectivity of protein binding was to be investigated. In this study an approach was chosen to work in a drug-protein ratio of 0.8–1.5 resulting in a constant protein concentration of 80 µM. The fraction bound to the protein (f_{bound}) is calculated according to Eq. (2) and Eq. (3) by subtracting the concentration of unbound drug (c_{free}) determined by CE in the sample from the total concentration of drug (c_{total}) used considering the concentration of the internal standard and dividing the resulting bound concentration (c_{bound}) of drug by c_{total}.

$$c_{bound} = c_{total} - c_{free} \quad (2)$$

$$f_{bound} = \frac{c_{bound}}{c_{total}} \quad (3)$$

K_{aff} constant can be determined using graphical approaches, e.g. the Klotz or Scatchard plot (Klotz and Hunston, 1971; Scatchard, 1949). The amount of drug which is bound per mole of protein can be expressed by r which corresponds to the quotient of the bound drug concentration to the total protein concentration ($c_{protein}$).

$$r = \frac{c_{bound}}{c_{protein}} \quad (4)$$

The Klotz plot makes use of the relationship between r and the free drug concentration as a double-reciprocal approach. If there is only one binding site, a linear graph is gained; if there are several binding sites, a curve is obtained whose evaluation is much more complex. Fig. 3 shows the linear Klotz plot of (−)-ephedrine as an example indicating the presence of one main binding site.

K_{aff} is the quotient of the intercept with the slope of the determined regression line while n, number of binding sites, is the reciprocal value of the intercept (Klotz and Hunston, 1971). The enantiomers of E, PE, and ME all showed a linearized Klotz Plot, while both NE enantiomers could not be graphically analyzed. The diastereomers E and PE including their respective enantiomers showed no significant difference in their binding behavior. ME showed low protein binding with the dextrorotatory enantiomer showing even lower binding compared to E and PE. Hardly any binding was found for both NE enantiomers. As was mentioned earlier, one main binding site is present when a linearized Klotz plot is obtained. For all derivatives except NE this is the case. An overview of the obtained plots is given in Figure S1. To confirm the result, K_{aff} for each ligand-protein ratio and the resulting total K_{aff} as average of each individual K_{aff} was calculated by Eq. (5) with the generated data and transformed into the pK_{aff}, which is the negative decimal logarithm of K_{aff}, for better usability. In the presence of only one main binding site and selective binding, pK_{aff} must be constant over the different ratios (Volpp and Holzgrabe, 2019).

Table 1
Comparison of pK_{aff} calculated according to Eq. (5) as the average value of the respective enantiomer, dissociation constant K_D as reciprocal value of K_{aff}, the determined protein binding by means of DUF and its graphical evaluation by means of Klotz plot with n being equal to the number of binding sites

				Klotz plot	
	pK_{aff}	K_D	% bound	pK_{aff}	n
(+)-E	3.62 ± 0.02	240 µM	25.0 ± 0.9	3.29	1.82
(−)-E	3.65 ± 0.03	221 µM	26.5 ± 1.6	3.56	0.91
(+)-PE	3.71 ± 0.03	176 µM	29.1 ± 1.4	3.46	1.45
(−)-PE	3.68 ± 0.04	209 µM	27.6 ± 2.0	3.79	0.46
(+)-ME	3.34 ± 0.03	429 µM	14.9 ± 1.0	3.63	0.68
(−)-ME	3.42 ± 0.03	383 µM	17.3 ± 0.9	3.43	1.16
(+)-NE	2.96 ± 0.14	956 µM	6.8 ± 1.9	graphical evaluation	
(−)-NE	3.02 ± 0.11	1087 µM	7.7 ± 1.7	was not possible	

$$K_{aff} = \frac{f_{bound}}{1 - f_{bound}} \times \frac{1}{c_{protein}} \quad (5)$$

Figure S2 shows this was the case for the enantiomers of E, PE, and ME, while the enantiomers of NE showed a larger scatter. The results obtained with both methods are compared in Table 1. The ultrafiltration units were tested for non-specific binding of the drugs. No significant adsorption on the ultrafiltration device was observed. The minimal deviations were considered in the results presented. The values of the pK_{aff} differ significantly but are of the same order of magnitude. Both approaches support the notion, that there is only one main binding site. The protein binding of all tested drugs was less than 30%, which is why it can be assumed that the binding of AGP is not clinically relevant.

3.2. Saturation transfer difference-NMR

STD-NMR is a well-established method in screening for possible binding of small molecules to respective targets, e.g. proteins (Krishnan, 2005). The principle of STD NMR is based on the nuclear overhauser effect. When a ligand binds to a protein, a proton of the protein can interact with a proton of the ligand and transfer energy in form of saturation. Saturating the protein's protons with the aid of a specific pulse, this saturation is transferred to the proton of the ligand in case of binding. A completely saturated proton does not show a signal in the spectrum any longer. Since the saturation is not completely transferred to the proton of the ligand, an attenuated signal is obtained. By subtracting on-resonance-spectrum (with saturation pulse) from off-resonance-spectrum (without saturation pulse) a difference spectrum is obtained. Different signals indicate ligand protons that are in proximity to protons of the protein and thus were saturated by saturation transfer through the protein. For each protein, the appropriate saturation pulse must be found, depending on the chemical structure. Ideally, the saturation pulse should not interfere with the signals of the ligand. Three different saturation pulses were applied with 6.22 ppm, 1.97 ppm and −1.00 ppm. Only the saturation pulse at −1.00 ppm did not interfere, so it was chosen as the saturation pulse for the STD measurements. The corresponding NMR spectra are shown in Figure S3. The drug excess should be at least equal to 0.5 to 2 times of the dissociation constant K_D to ensure adequate saturation (Mayer, 2001). K_D values were calculated as reciprocal values from the previously determined K_{aff} values from the DUF measurements and are shown in Table 1. Fig. 4 shows the ^1H spectra of (−)-E and AGP. The CH-N signal at 3.50 ppm of the ephedrine derivatives overlapped with the signals from AGP and was therefore not suitable for evaluation. The H_{benzyl} signal at 5.00 ppm was canceled out by the water suppression and could thus not be detected. The aromatic protons are overlapping, thus individual analysis was not possible. The exchangeable protons of the hydroxy group and the amine group were not seen due to a deuterium exchange by the buffer used. An overlay between an off-spectrum (black) and a difference spectrum (red) of a mixture of (+)-E with AGP is displayed in Fig. 5.

The STD factor is calculated according to Eq. (6) with the intensity of each individual proton.

$$\text{STD factor} = \frac{\text{intensity}_{off-res.} - \text{intensity}_{on-res.}}{\text{intensity}_{off-res.}} \times 100 \quad (6)$$

For epitope mapping, all STD factors are normalized to the strongest one (Viegas et al., 2011). The STD factor intensity depends on the saturation time, rebinding processes, drug and protein concentration and binding kinetics, respectively (Walpole et al., 2019). With longer saturation times, more intensive signals are obtained. The measurements were performed at saturation times of 1, 2, 3 and 4 s. Plotting STD factor against the saturation time, revealed an adsorption isotherm, which can be described by Eq. (7). STD factor (t_{sat}) is the factor at a certain saturation time, k_{sat} the saturation rate constant, t_{sat} the saturation time and STD $factor_{max}$ the maximum STD factor intensity.

$$\text{STD factor}(t_{sat}) = \text{STD factor}_{max} \times \left(1 - e^{-k_{sat} \times t_{sat}}\right) \quad (7)$$

To account for ligand and protein concentration (c_{ligand} respectively $c_{protein}$), the STD amplification factor (STD AF) is used instead of the STD factor. It is calculated according to Eq. (8).

$$\text{STD AF} = \text{STD factor} \times \frac{c_{ligand}}{c_{protein}} \quad (8)$$

As can be seen in Fig. 6, at a saturation time starting from 3 s, almost complete saturation is reached for protons H_{phenyl}, N-CH_3, and C-CH_3. In the rising part of the adsorption isotherm, the STD factor is still relatively independent of the saturation time. Therefore, a saturation time of 2 s was chosen for epitope mapping.

In contrast to the DUF it was possible to use a physiological concentration of 15 µM AGP. Becker et al. showed that the influence of nonspecific interactions by increasing the ligand excess does not discriminate between the individual enantiomers (Becker and Larive, 2008). The higher the ligand excess, the more the STD factors of some protons of the individual enantiomers converged. To investigate this effect, different ligand excesses between 10:1 to 40:1 were tested. The results are shown in Table S2. No influence of the ligand concentration on the STD factor could be detected. A drug concentration of 300 µM representing an excess of 20:1 showed good results for all investigated drugs and was therefore used. Results are summarized in Table 2. In all ephedrine derivatives, the aromatic protons H_{phenyl} and C-CH_3 from the sidechain are the most affected ones. The alkyl substituents attached to the amine show weaker effects. In E and ME, the substituent of the dextrorotatory enantiomer showed a more intensive STD-factor while in PE there is no significant difference between the enantiomers.

3.3. Molecular docking

Kaliszan et al. showed a proposal for the main binding site of AGP (Kaliszan et al., 1995). According to their findings, the high affinity binding pocket for basic substances is funnel shaped, with a negatively charged anionic region at the inner end and lipophilic regions at the edge and outer end. At the anionic area, mainly the positively charged basic nitrogen interacts, while at the lipophilic regions, mainly aliphatic and aromatic hydrocarbon interactions take place. The funnel shaped pocket is also a steric restriction and thus enantiomers can bind differently. In 2008, Schönfeld et al. succeeded in characterizing the crystal structure of the non-glycosylated protein. Variant F1*S has a third domain in the binding pocket compared to variant A and thus a larger opening which facilitates the entry of ligands and is a steric restriction. However, both variants have the anionic region postulated by Kaliszan. Previous experiments (Nishi et al., 2011) showed that variant A is mainly responsible for binding basic substances, which is why the docking experiments were carried out with this variant. The results of E and PE can be seen in Fig. 7, the results of ME and NE can be found in Figure S4. Despite structural differences, the same interactions were

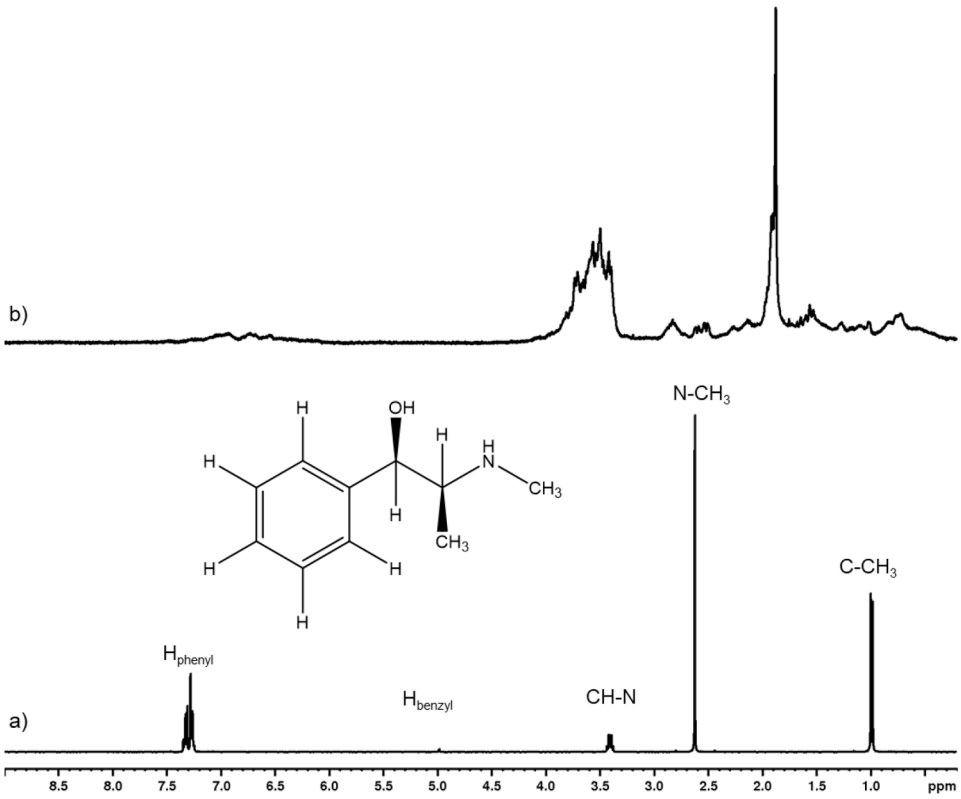

Fig. 4. NMR spectra being a) a ^{1}H-spectrum of AGP b) a ^{1}H-Spectrum of (−)-ephedrine; both spectra were recorded with water signal suppression

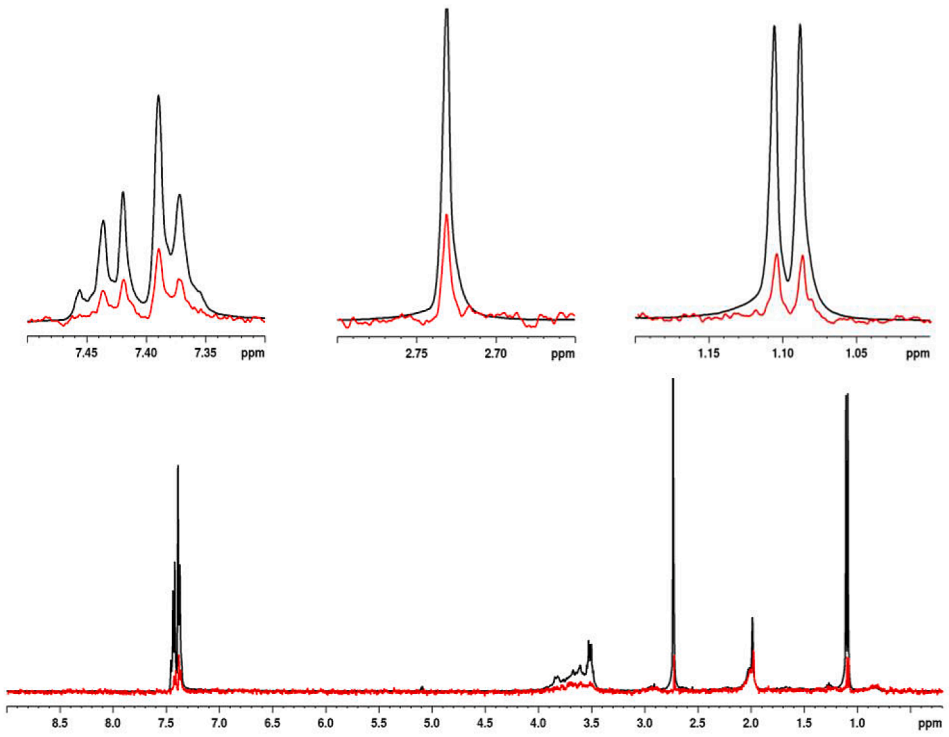

Fig. 5. Overlay of the off-resonance spectrum (black) and the difference spectrum (red) of (−)-ephedrine with a saturation pulse of 400 Hz. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

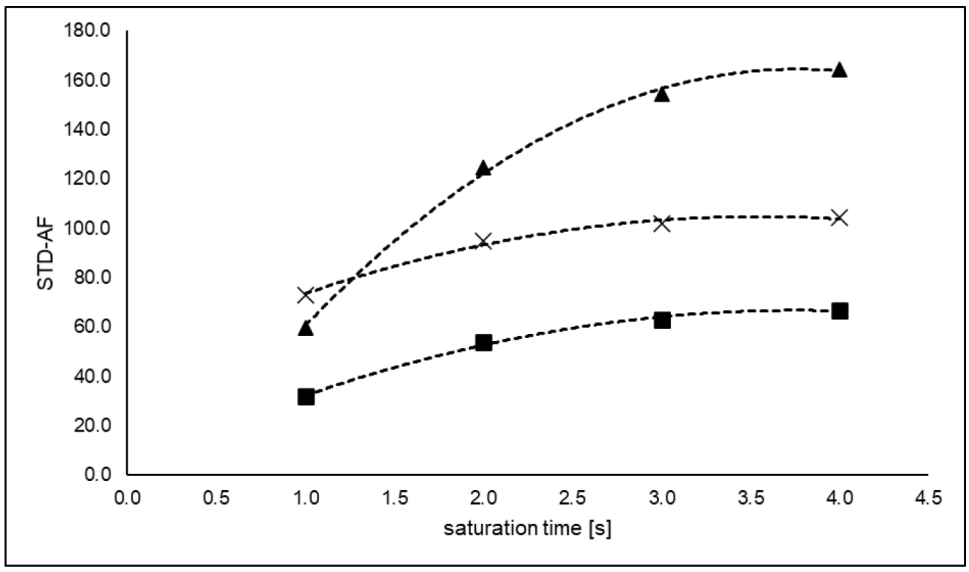

Fig. 6. Adsorption isotherm of the protons of (−)-E, application of the STD amplification factor = STD-AF at the saturation times 1, 2, 3 and 4 s with STD AF being the obtained STD factor multiplicated with the ligand excess, ▲= H_{phenyl}, ■ = C-CH_3, *X* = N-CH_3

Table 2
STD epitope maps for the used ephedrine derivatives at a concentration of 300 μM per ligand and a AGP concentration of 15 μ M (values are expressed as a percentage in comparison to the most intense normalized STD factor signal)

	E		PE		ME		NE	
	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
H_{phenyl}	93.6%	100.0%	100.0%	100.0%	100.0%	93.7%	100.0%	93.8%
C-CH_3	100.0%	79.5%	85.2%	84.1%	96.8%	100.0%	86.1%	100.0%
N-CH_3	79.2%	40.0%	60.8%	58.8%	72.9%	52.8%	n.a.	n.a.

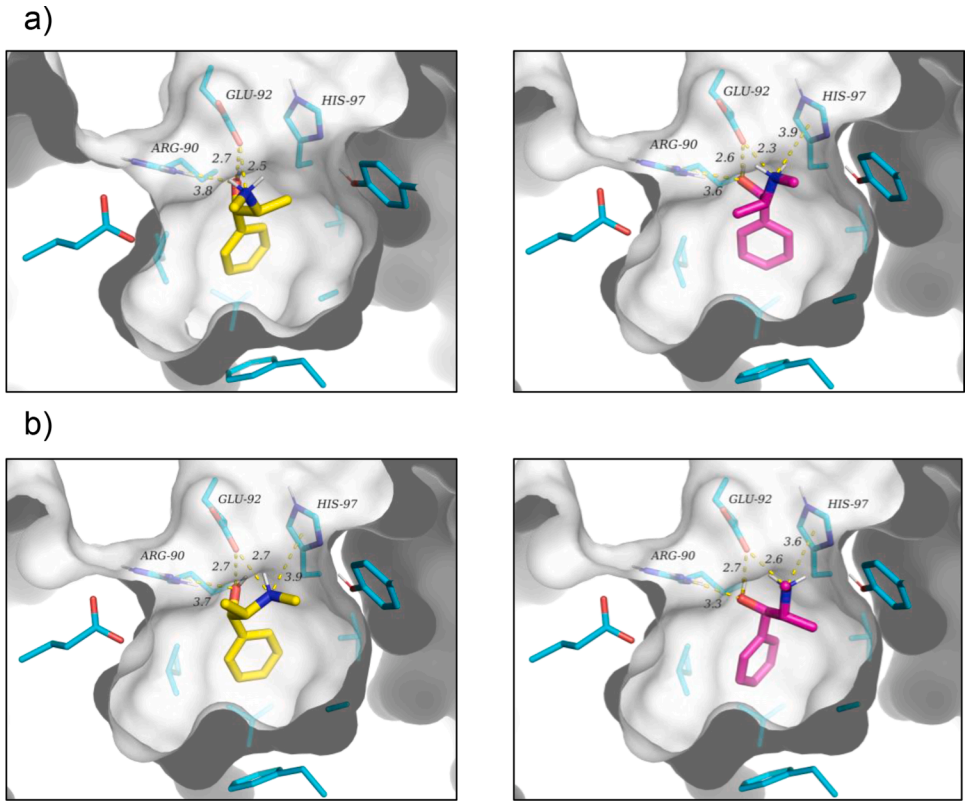

Fig. 7. Docking results of a) ephedrine and b) pseudoephedrine with variant A of AGP; yellow = (+)-enantiomer; pink = (−)-enantiomer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
pK_a values of the measured drug substances (Dong et al., 2015; Shalaeva et al., 2008) and ratio of charged to uncharged species at pH 7.40

drug	pK_a	ratio of charged to uncharged species at pH 7.40
pseudoephedrine	9.74	218:1
ephedrine	9.64	173:1
methylephedrine	9.40	100:1
norephedrine	9.12	52:1

found on all docked ligands. The phenyl ring shows van der Waals and π-π interactions in the hydrophobic pocket. The amine function forms a salt bridge to Glu92 and a cation π- interaction to His97. The hydroxyl group is stuck between Arg90 and Glu92 and forms hydrogen bonds to both sidechains. The respective enantiomers are present in the binding pocket in a different orientation (cf. Fig. 7) but show the same interactions. Based on the docking results, there is no difference in the binding process between the enantiomers of each ligand.

Nevertheless, stereochemistry and molecule size can have an influence on binding. As already mentioned, the opening of the binding pocket has steric restrictions, which is why some drugs can penetrate more easily than others. In addition, a substituent on the basic nitrogen increases the size of the molecule and thus makes penetration into the binding pocket more difficult. However, the tested ligands measured showed no significant differences between their enantiomers and penetrated the binding pocket without restriction.

The influence of a substituent of the basic nitrogen is less decisive for the molecular size than for the degree of protonation and the solvation of the amine, which has a significantly influence on the binding. Negatively charged and uncharged xenobiotics bind primarily to albumin, while positively charged drugs bind preferred to AGP. Therefore, binding to AGP should be favored significantly by increased protonation. All investigated ephedrine derivatives are weak bases (cf. Table 3 for exact pK_a values and ratio of charged to uncharged species at physiological pH value).

The basicity of the drug substances decreases in the following order, considering their respective substitution: PE > E > ME > NE. NE and ME are least charged at physiological pH of 7.40 and thus should have a lowered protein binding to AGP in comparison to E and PE. The pK_a value difference between the diastereomers E and PE is 0.1 and thus they differ only slightly in their protonation and solvation. Still PE is more charged than E at physiological pH. Comparing the results of the DUF with basicity of the drugs shows the same pattern. While PE and E show similar protein binding with PE being the more stongly bound ligand, the protein binding decreases with declining basicity. ME thus has a lower binding affinity than PE and E, and NE the lowest. An alkyl substituent of the basic nitrogen thus only influences the protein binding not via its change in molecular size but via the change in basicity.

4. Conclusions

The binding behavior of various ephedrine derivatives to AGP was successfully determined by means of DUF and STD-NMR and supported by docking experiments. The decrease in protein binding correlates with the decrease in protonation at physiological pH value. The more basic the ligand is, the higher is its affinity to AGP. The docking experiments showed three main interactions, van der Waals and π-π-interactions of the phenyl ring, a cation interaction of the amine and hydrogen bonding of the hydroxy group. The DUF, STD-NMR and docking experiments showed no significant differences in the binding behavior between the respective enantiomers. The binding of the ephedrine derivatives to AGP is therefore not enantioselective.

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Sebastian Schmidt: Investigation, Methodology, Writing – original draft, Visualization, Validation. **Markus Zehe:** Investigation, Methodology, Software, Visualization. **Ulrike Holzgrabe:** Conceptualization, Resources, Writing – review & editing, Supervision.

Conflict of Interests

None of the authors of this paper does have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Data availability

Data will be made available on request.

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Supplementary materials

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