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Short communication

Characterization of the binding epitope of ciprofloxacin bound to human serum albumin

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Abstract

Aqueous solutions of ciprofloxacin in phosphate buffer were measured by NMR under physiological conditions. The chemical shifts differ substantially compared to earlier investigations at low pH or in DMSO. Protein binding experiments using saturation transfer were optimized to measure proton resonances of ciprofloxacin that are in close proximity to human serum albumin. The relative intensities were mapped on the molecule to define the binding epitope. According to this methodology the cyclopropane ring and the chinolon ring constitute the binding epitope. Competition experiments with increasing amounts of salicylic acid did not change the saturation transfer to the ciprofloxacin protons indicating at least two different binding sites.

Keywords: Ciprofloxacin; NMR; Human serum albumin; Binding epitope

1. Introduction

Ciprofloxacin (Fig. 1) is a quinolone carboxylic acid that has Gram-negative and Gram-positive bactericidal activity and lacks cross reactivity with penicillins, cephalosporins and aminoglycosides. In the treatment of severe infections mixtures of drugs are applied complicating the pharmacokinetics of the antibiotic therapy. As a major factor the reversible non-covalent binding to human serum albumin (HSA) has to be tested. The protein binding has been studied by a number of techniques such as electrophoresis [1], chromatography [2], crystallography [3], mass spectrometry [4], ultrafiltration [5] and NMR [6]. HSA exhibits six binding sites three of which are high-affinity sites [7–9]. Due to inherent flexibility a high number of sites per drug molecule are typically found which complicates the definition of a simple binding epitope [7]. Nevertheless, solution NMR techniques have the advantage to maintain almost physiological conditions during analysis. A powerful way with atomic resolution is based on saturation transfer difference (STD) spectroscopy which allows to characterize the binding epitope of a drug molecule [10]. In this report we present data on the ciprofloxacin protons involved in binding to human serum albumin under physiological conditions.

2. Materials and methods

The NMR spectra were measured at 25 °C with a 700 MHz Bruker Avance spectrometer. A ciprofloxacin HCl stock solution of 50 mM in H_2O was prepared. Starting from this stock solution samples of ciprofloxacin and human serum albumin in sodium phosphate buffer (50 mM, pH 6.7, H_2O:D_2O = 90:10) were made. The ratio was varied to optimize saturation transfer experiments. STD experiments and STD factors were determined according to Mayer and Meyer [10]. A pulse sequence with a spinlock to suppress protein resonances, a saturation of 2 s and 128 scans were used. Water suppression was done by using the WATERGATE sequence. Proton chemical shifts in ppm were obtained with DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) as internal standard (0.00 ppm).

3. Results and discussion

In order to simulate physiological conditions an aqueous buffer system with a neutral pH was chosen for NMR measurements of ciprofloxacin.

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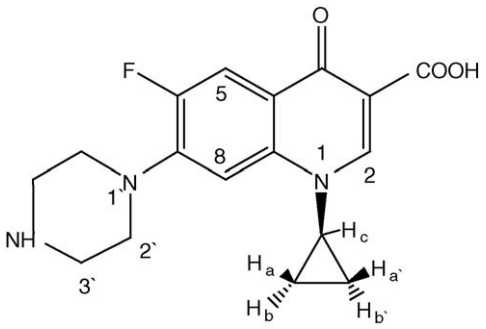

Fig. 1. Chemical formula of ciprofloxacin.

Because the solubility is decreased when compared to acidic conditions [11], ciprofloxacin has been typically analysed by NMR under non-physiological conditions (acidic D_2O, DMSO [12–14]). As a comparison the proton chemical shifts of this study and of Sakai et al. [15] are shown in Table 1. The proton spectrum of ciprofloxacin in phosphate buffer is shown in Fig. 2E, after addition of HSA the spectrum of Fig. 2D was recorded; only the aromatic part is shown in Fig. 2A.

For the saturation transfer experiments the ratio between ciprofloxacin and HSA was varied. A compromise between disturbing protein signals and intensity of STD signals was found for combinations such as 0.1 mM HSA and 1 mM ciprofloxacin (Fig. 2C, and only the aromatic part in Fig. 2B). It was verified that the proton resonances of ciprofloxacin did not shift in the concentration range between 0.1 and 2 mM. Titration experiments with increasing amounts of HSA did not affect the proton chemical shift of ciprofloxacin. The saturation time was optimized for the STD experiment (2 s). The resulting STD factors are listed in Table 2 for the ciprofloxacin protons. Normalized values with the STD factor of Ha/Ha′ set as 100% are shown in Fig. 3.

The values indicate that the north and eastern part of ciprofloxacin is in close proximity to HSA in the binding site (H2, H5 and the cyclopropyl protons Ha/Ha′). A 3D model is shown in Fig. 4. The crystal structure of ciprofloxacin alone showed that the cyclopropane ring is located almost perpendicular to the plane of the chinolon ring [16,17]. This fixed position of the cyclopropane ring could be the same in the HSA bound state because the Ha/Ha′ would then face the same side like H2. The lower values for the other cyclopropane protons (Hb/Hb′, Hc) might indicate the greater distance in this locked conformation. The STD factors for the protons of the piperazine ring are substantially lower indicating a minor contribution to binding.

Table 1
Proton chemical shifts of ciprofloxacin under different measurement conditions (in both cases with DSS as reference)

Protons	Phosphate buffer pH 6.8, 90% H_2O, 298 K (this study)	D_2O, pD 2.5, 310 K [15]
H2	8.54	8.63
H5	7.83	7.46
H8	7.57	7.52
Hc	3.62	nd
H2′/H6′	3.52	3.66/3.56
H3′/H5′	3.42	3.66/3.56
Hb/Hb′	1.29	1.47
Ha/Ha′	1.09	1.22

Table 2
STD factors of the ciprofloxacin protons

Protons	STD factor (A_{STD})
H2	8.7
H5	9.6
H8	3.1
Hc	6.7
H2′/H6′	2.5
H3′/H5′	3.3
Hb/Hb′	7.9
Ha/Ha′	10.7

A position of the cyclopropane ring pointing with the Ha/Ha′ protons to the eastern part of the molecule was substantiated in DMSO by an NOE between Ha/Ha′ and H2 [14]. Therefore, the binding groove of HSA would fit to a band of atoms starting from H5, extending to the carboxylate and ending with H2 and one face of the cyclopropane ring (Ha/Ha′). This would comply with binding to site II of HSA because the deprotonated acid function could serve as central part of the binding epitope. In conclusion, the binding epitope of ciprofloxacin bound to HSA seems to be located in the cyclopropane and chinolon part of the molecule.

This is in sharp contrast to the findings of Zlotos et al. [5]. A series of gyrase inhibitors was synthesized and binding to HSA was measured by ultrafiltration lacking direct atomic resolution. Using a structure–activity approach they concluded that the phenyl part of the chinolon ring and the piperazine ring take part in the interaction with HSA. The above mentioned methodological difference might explain this conflicting results. A recent development allowed the determination of ligand binding to HSA via electrospray ionization mass spectrometry [4]. High sensitivity and no need for labeled compounds are two advantages of this method. However, it is generally not able to deliver further structural information and in the case of HSA the entire protein could only be analyzed with insufficient resolution. Consequently, only the binding of ligands to a fragment of HSA was measured.

Titration of a 1 mM ciprofloxacin/0.2 mM HSA solution with salicylic acid up to 2 mM (saturating the available binding sites) did not change the STD factors for the ciprofloxacin protons. STD signals of the known HSA binder salicylic acid were clearly seen (Fig. 5A and B). Since salicylic acid binds to the high-affinity site I and this site is known to allow independent binding of two different compounds, ciprofloxacin might bind to the high-affinity site I or a different site such as II [7]. The latter high-affinity site binds carboxylic acids such as ibuprofen and diclofenac. Typically a hydrophobic part and a negatively charged group can be found which reflects

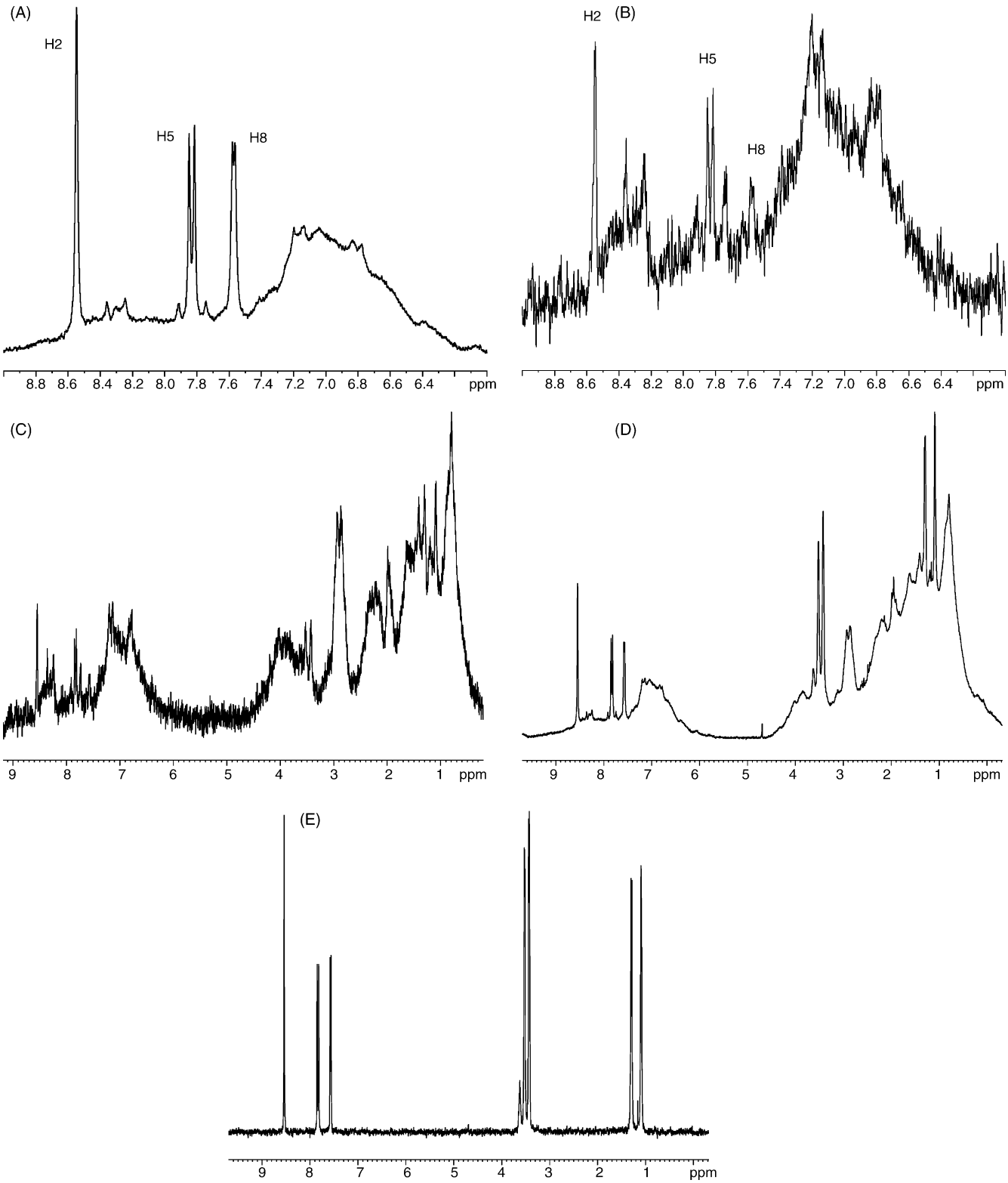

Fig. 2. (A) Aromatic part of the proton spectrum of ciprofloxacin 1 mM and 0.1 mM HSA. (B) Aromatic part of the proton STD spectrum of ciprofloxacin 1 mM and 0.1 mM HSA. (C) STD spectrum of ciprofloxacin 1 mM and 0.1 mM HSA. (D) Proton spectrum of ciprofloxacin 1 mM and 0.1 mM HSA. (E) Proton spectrum of ciprofloxacin 1 mM.

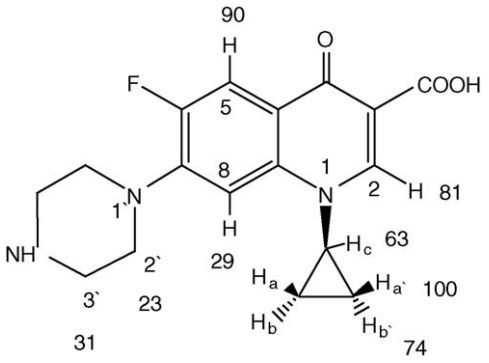

Fig. 3. Relative STD factors (in %) of ciprofloxacin bound to HSA.

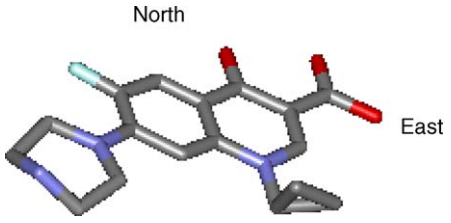

Fig. 4. 3D model of ciprofloxacin with indicated regions.

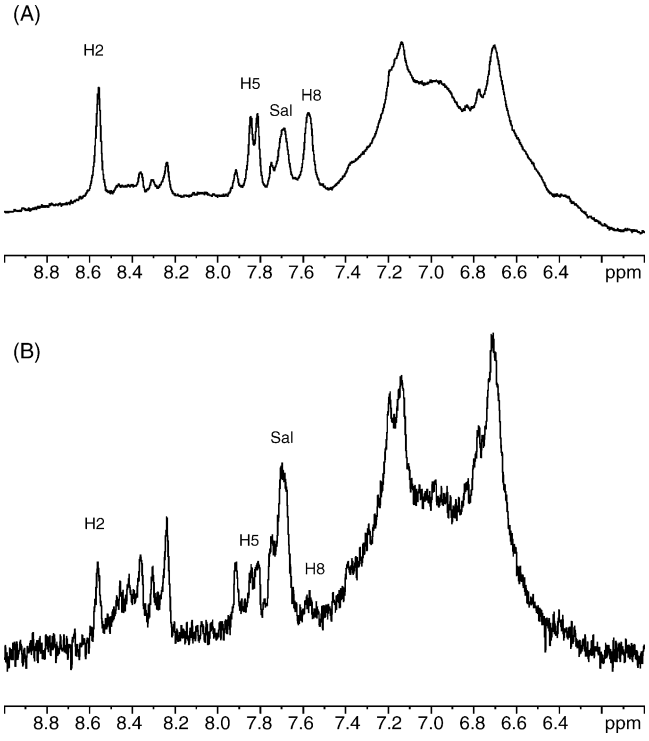

Fig. 5. (A) Aromatic part of the proton spectrum of ciprofloxacin (0.1 mM), salicylic acid (1 mM) and human serum albumin (0.2 mM). (B) Aromatic part of the proton STD spectrum of ciprofloxacin (0.1 mM), salicylic acid (1 mM) and human serum albumin (0.2 mM).

the situation for ciprofloxacin under physiological conditions. Near neutral pH in aqueous solution roughly 50% can be found in the mono-anionic form and 40% in the non-ionized form [18]. Mutagenesis of the relevant amino acids in HSA (Trp214 for site I) could further clarify which high-affinity site is the most important for the binding of ciprofloxacin to HSA.

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References

[1] H.S. Kim, J. Austin, D.S. Hage, Methods Mol. Biol. 276 (2004) 169–187.
[2] T. Ohkawa, R. Norikura, T. Yoshikawa, J. Pharm. Biomed. Anal. 31 (2003) 1167–1176.
[3] S. Curry, H. Mandelkow, P. Brick, N. Franks, Nat. Struct. Biol. 5 (1998) 827–835.
[4] K. Benkestock, P.-O. Edlund, J. Roeraade, Rapid Commun. Mass Spectrom. 19 (2005) 1637–1643.
[5] G. Zlotos, A. Bücker, J. Jürgens, U. Holzgrabe, Int. J. Pharm. 169 (1998) 229–238.
[6] C. Dalvit, M. Flocco, B.J. Stockman, M. Veronesi, Comb. Chem. High T. Scr. 5 (2002) 645–650.
[7] U. Kragh-Hansen, V.T.G. Chuang, M. Otagiri, Biol. Pharm. Bull. 25 (2002) 695–704.
[8] X.M. He, D.C. Carter, Nature 358 (1992) 209–215.
[9] F. Hervé, S. Urien, E. Albengres, J.C. Duché, J.P. Tillement, Clin. Pharmacokinet. 26 (1994) 44–58.
[10] M. Mayer, B. Meyer, Angew. Chem. Int. Ed. 38 (1999) 1784–1788.
[11] B.A. Firestone, M.A. Dickason, T. Tran, Int. J. Pharm. 164 (1998) 119–128.
[12] I.A. Parshikov, J.P. Freeman, J.O. Lay, R.D. Beger, A.J. Williams, J.B. Sutherland, FEMS Microbiol. Lett. 177 (1999) 131–135.
[13] G. Fardella, P. Barbetti, I. Chiappini, G. Grandolini, Int. J. Pharm. 121 (1995) 123–127.
[14] A. Zieba, A. Maslankiewicz, J. Sitkowski, Magn. Reson. Chem. 41 (2004) 903–904.
[15] M. Sakai, A. Hara, S. Anjo, M. Nakamura, J. Pharm. Biomed. Anal. 18 (1999) 1057–1067.
[16] I. Turel, P. Bukovec, M. Quiros, Int. J. Pharm. 152 (1997) 59–65.
[17] S.C. Wallis, L.R. Gahan, B.G. Charles, T.W. Hambley, P.A. Duckworth, J. Inorg. Biochem. 62 (1996) 1–16.
[18] D.-S. Lee, H.-J. Han, K. Kim, W.-B. Park, J.-K. Cho, J.-H. Kim, J. Pharm. Biomed. Anal. 12 (1994) 157–164.