ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
18 (1998) 847–858

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Determination of protein binding of gyrase inhibitors by means of continuous ultrafiltration

G. Zlotos [a], M. Oehlmann [b], P. Nickel [b], U. Holzgrabe [a,*]

[a] *Pharmaceutical Institute, University of Bonn, Kreuzbergweg 26, 53115 Bonn, Germany*
[b] *An der Immenburg 4, 53121 Bonn, Germany*

Received 15 May 1998; received in revised form 31 July 1998; accepted 16 August 1998

Abstract

In order to characterize the protein binding of a drug, it is necessary to have a method which is close to in vivo conditions and fast in the course of measurement. The continuous ultrafiltration fulfils both requirements for substances with a high extent of protein binding. In this study, 18 gyrase inhibitors in clinical practice, characterized by a lower extent of protein binding, were subjected to the titration procedure of the continuous ultrafiltration using bovine and human serum albumin (BSA, HSA), and human plasma. The results of the continuous ultrafiltration were found to be similar to those obtained by means of the 'classical' discontinuous ultrafiltration using plasma (correlation between continuous and discontinuous ultrafiltration $r^2 = 0.87$). In the cases of pipemidic acid, enoxacin and rufloxacin, the continuous method gave $\sim 20\%$ lower degrees of protein binding than the discontinuous procedure, which utilizes plasma having the full range of proteins. It is likely that these drugs bind mainly to other proteins in plasma than HSA. This finding proves that this fast method is worthwhile in the whole range of protein binding. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Gyrase inhibitors; Protein binding; Continuous ultrafiltration

1. Introduction

For a long time, the role of plasma protein binding of drugs is identified as a relevant factor in drug disposition and efficacy. Especially the concentration of a drug in tissues and at the locus of action, and the glomerular filtration can be limited by a high extent of protein binding [1]. In order to estimate the protein binding in human beings, it is necessary to have an in vitro method which is reliable, fast and close to in vivo conditions.

Almost all drugs bind more or less specifically to plasma proteins, acidic compounds mostly to plasma albumin, basic drugs to α_1-acid glycoprotein. The fraction of a drug bound to plasma is determined by its concentration, its affinity for the protein binding site and the number of binding sites. Most of the methods reported in the literature [2], utilizing, e.g. electrophoresis, gel chromatography, or ultracentrifugation, suffer

* Corresponding author. Tel.: +49-228-732845; fax +49-228-739038; e-mail: holzgrabe@uni-bonn.de.

0731-7085/98/$ - see front matter © 1998 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(98)00220-9

from the disadvantage that the extent of protein binding can only be determined at certain concentrations of the drug and the protein. Since the protein binding responds to the simple mass-action equation, the formation of the drug–protein complex depends on the concentration of both components. Thus, a method is needed which is able to characterize the protein binding of a drug over a larger range of concentration. The continuous ultrafiltration, firstly developed by Campion et al. [3] and improved by Kinawi et al. [4] and later by Nickel et al. [5,6] fulfils all aforementioned requirements for drugs which are characterized by a high extent of protein binding. The purpose of this study was to check whether this method is suitable for the determination of the extent of protein binding over the entire range. Since gyrase inhibitors cover the whole range of plasma protein binding 18 compounds in clinical practice or trials as antibacterials (Scheme 1) were subjected to the titration procedure of the continuous ultrafiltration using fresh plasma, and human and bovine serum albumin (HSA and BSA, respectively) as the representative protein fraction of plasma. The obtained results will be compared with values which were determined by 'classical' methods.

2. Experimental

2.1. Materials

Nalidixic acid (nal) was supplied from Sanofi Winthrop GmbH (München, Germany), norfloxacin (nor) from MSD Sharp and Dohme GmbH (München, Germany), oxolinic acid (oxo) and enoxacin (eno) from Gödecke AG (Freiburg, Germany), ciprofloxacin (cipro) and enrofloxacin (enro) from Bayer AG (Leverkusen, Germany), grepafloxacin (grepa) from Glaxo Wellcome (Hertfordshire, UK), trovafloxacin (trova) from Pfizer GmbH (Karlsruhe, Germany), fleroxacin (flero) from Hoffmann La-Roche AG (Grenzach-Wyhlen, Germany), pipemidic acid (pip), pefloxacin (peflo), and sparfloxacin (spar) from Rhone-Poulenc Rorer GmbH (Köln, Germany), ofloxacin (oflo) and levofloxacin (levo) from Hoechst AG (Frankfurt, Germany), difloxacin (diflo) from Abbott (Abbott Park, USA), rufloxacin (ruflo) from Mediolanum (Milano, Italy), and cinoxacin (cin) from Eli Lilly Deutschland GmbH (Giessen, Germany). Methylciprofloxacin (N-me) was synthesized according to a method reported by Jürgens et al. [7]. The plasma was a gift from the Institut für Exp. Hämatologie und Transfusionsmedizin, Blutbank, Universität Bonn, Germany. HSA and BSA were purchased from Serva (Heidelberg, Germany). Commercially available acetonitrile of HPLC grade and twice distilled methanol was used throughout. All other reagents were commercially available and of analytical grade.

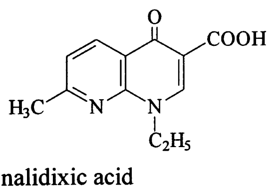
2.2. Discontinuous ultrafiltration

The determination of the extent of protein binding of grepafloxacin using the discontinuous ultrafiltration was performed with the micropartition system MPS-1 equipped with YMT membranes, exclusion limit 30 000 Da (Amicon, Lexington, MA) according to [8]. A stock solution of grepafloxacin was prepared by dissolution of 80 μmol of the drug in 0.1 ml 1 M NaOH and dilution to 2 ml with water. Aqueous solutions of grepafloxacin were prepared using the stock solution and added to a constant volume of human plasma (v/v 1:19) to obtain a drug concentration ranging from 0.005 to 0.5 μmol ml^{-1}. The mixture was incubated for 30 min at 37°C. An aliquot of 1 ml was ultrafiltrated using a micropartition system and was centrifuged at $2000 \times g$ for 20 min at 10°C. The total volume collected as filtrate was 25% of the starting volume. The concentration of the free gyrase inhibitor in the ultrafiltrate was determined by HPLC.

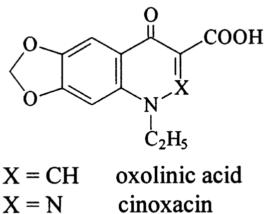
2.3. HPLC conditions

The mobile phase was composed of 220 ml acetonitrile and 780 ml buffer (0.1 M citric acid, 0.02 M NH_4ClO_4). To this was added 0.005 M TBAS, and the pH 2.5 was adjusted using 1 M NaOH. A RP18 column (Spherisorb ODS-2 endcapped, 5 μm, 250×4.6 mm, Grom, Herrenberg, Germany) was applied, the flow rate was 1.0 ml

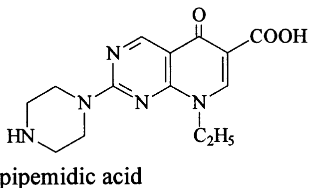
Gyrase inhibitors of the first and second generation



nalidixic acid

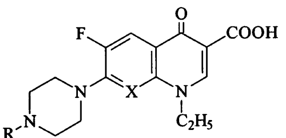


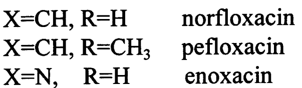
X = CH oxolinic acid
X = N cinoxacin

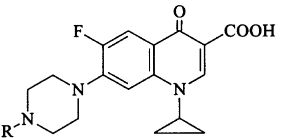


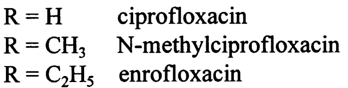
pipemidic acid

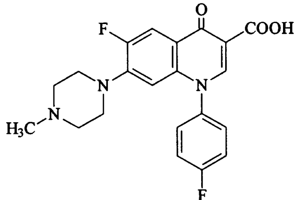
Gyrase inhibitors of the third generation



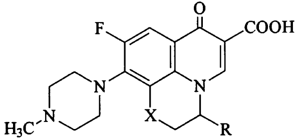


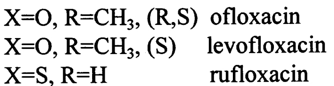


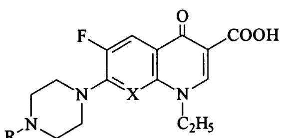




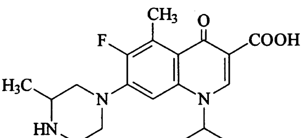
difloxacin



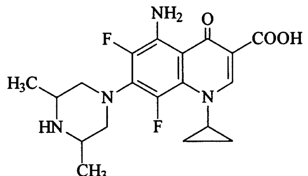




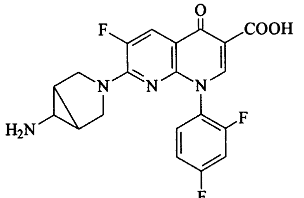
fleroxacin



grepafloxacin



sparfloxacin



trovafloxacin

Scheme 1. Structural formula of the gyrase inhibitor studied.

min^{-1}, and the measurements were performed at 37°C. The concentration of the analyte was calculated from the absorbance at the $\lambda_{max} = 280$ nm using an internal standard method (retention times: grepafloxacin 11.70 min, fleroxacin (int. standard) 5.40 min). Grepafloxacin shows an extent of protein binding of $28 \pm 3\%$ at the drug concentration in plasma of 0.01 µmol ml^{-1} and $29 \pm 5\%$, $27 \pm 4\%$, $25 \pm 4\%$, $25 \pm 4\%$ at drug concentrations of 0.005, 0.05, 0.1 and 0.5 µmol ml^{-1}, respectively (number of experiments at each concentration = 4).

2.4. Continuous ultrafiltration

A buffered drug solution is pumped through an ultrafiltration cell which contains a protein solution (buffer pH 7.4: phosphate buffer 0.03 M with 0.1 M NaCl; drug solution: 0.03–0.05 M in the buffer; protein concentration: 10–40 mg ml^{-1}). The cell for the ultrafiltration experiments was originally developed by Kinawi [9] and improved by Illy [10] and Oehlmann [11]. It has a volume of 1.5 ml. The top of the cell is closed with an ultrafiltration membrane (Type 14549 exclusion limit 20 000 Da, Sartorius, Göttingen, Germany). A buffer or buffered drug solution is pumped through the cell from the bottom to the top using an HPLC-pump (0.5 ml min^{-1} flow rate). Effective magnetic stirring is essential for good results. The eluate leaving the cell passes an HPLC UV detector. UV absorption of the eluate is measured in either maximum in a range 320–350 nm at intervals of 1 s.

Plotting the absorption values versus the time gives the elution curves as shown in Figs. 1 and 2. The curve at the left side is obtained when a drug solution is pumped through the cell (no protein in the cell). The curve at the right side is obtained when a drug solution is pumped through the cell containing a protein (HSA or BSA or plasma). After registration of the first (left) curve, the cell is rinsed by a pumping buffer through the cell until the UV absorption of the eluate decreases to zero. Subsequently, a protein solution is injected into the cell. The cell is again rinsed with buffer before the registration of the second (right) curve starts.

All of the steps of the process (changing the solutions, protein injection, pumping times, etc.) are computer controlled. Thus, the process runs fully automatically. Programs for the control of the process and for the calculation of the association constants are written in HP BASIC for WINDOWS version 6.32.

The size of the area between the two curves in Fig. 1 or Fig. 2 is proportional to the amount of drug bound to the protein in the ultrafiltration cell. From these two curves, a titration curve is calculated. By means of this titration curve, association constants are calculated, utilizing methods that are commonly used for the calculation of pK_a values from acid–base titration curves [12]. The results of these calculations are shown in Table 1.

3. Results

Fig. 1 shows the results of a continuous ultrafiltration experiment with oxolinic acid. This compound shows a relatively high degree ($75.6 \pm 2.2\%$) of binding to HSA. The area between the two curves is large. As can be seen from the standard deviation the reproducibility and reliability of the results are good. During the dead time, no UV absorption of the eluate can be detected; it is ~ 1.5 min. Variations of the dead time in the range of a few seconds (resulting in a shift of the curves along the X axis) will have no great influence on the size of the area between the two curves.

Fig. 2 shows the results of a continuous ultrafiltration experiment with *N*-methylciprofloxacin, a compound with a low degree (17%) of binding to HSA. Here again, the dead time is ~ 1.5 min. In this case, however, variations of the dead time in the range of a few seconds will have an important influence on the size of the area between the two curves. Small variations of the dead time are inevitable in spite of the facts that all steps of the ultrafiltration process (changing the position of the valves, protein injection, etc.) are computer controlled. Thus, reproducibility and reliability of the results of continuous ultrafiltration experiments will decrease with decreasing protein binding of the investigated drug. This fact is reflected

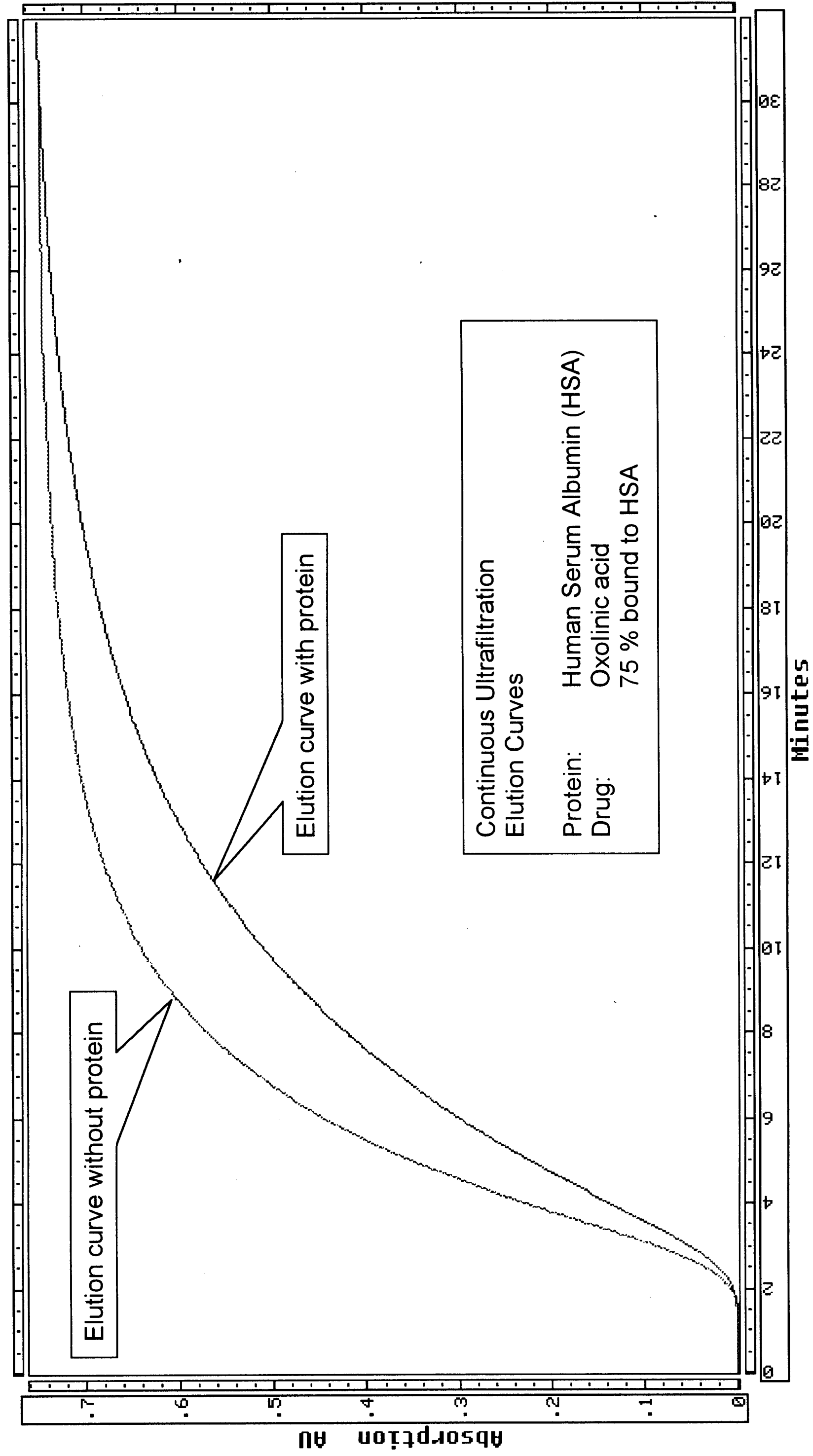


Fig. 1. Results of a continuous ultrafiltration experiment with oxolinic acid and HSA, original elution curves.

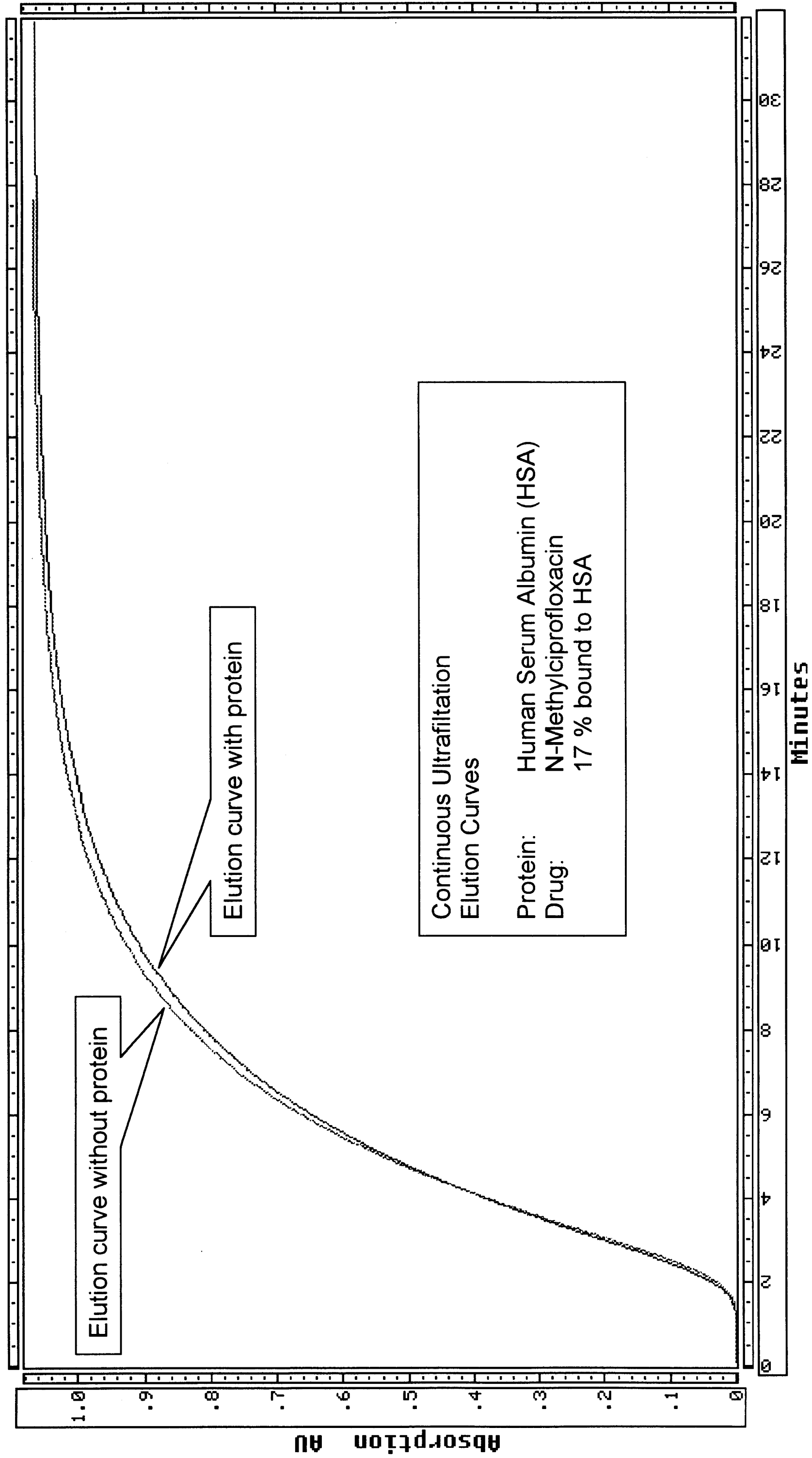


Fig. 2. Results of a continuous ultrafiltration experiment with *N*-methylciprofloxacin and HSA, original elution curves.

Table 1
Protein binding data of gyrase inhibitors with human serum albumin (HSA), bovine serum albumin (BSA) and human plasma[a]

Gyrase inhibitor	Protein binding determined by					Protein binding reported in the literature
	Continuous ultrafiltration				Discontinuous ultrafiltration	
	HAS		BSA		Human plasma	
	pK	% bound	pK	% bound	% bound	% bound
Nalidixic acid[b]	4.32; 3.88	94.6 ± 2.4	5.37; 3.67	99.3	93 ± 2	93–96 [14], 71–94 [15]
Oxolinic acid[b]	3.60; 3.31	75.6 ± 2.2	–	–	80 ± 2	23 [16]
Cinoxacin	3.46	65.2 ± 4.7	3.53	66.9	68 ± 3	65–71 [15]
Pipemidic acid	1.96	5.9 ± 3.3	–	–	19 ± 2	23–26 [15], 15–39 [17]
Norfloxacin	2.56	26.9 ± 15.4	–	–	24 ± 2	14 [18], 15 [19]
Pefloxacin	2.72	29.5 ± 8.6	3.43	61.8	27 ± 2	20–30 [17,18], 20 [20]
Enoxacin	2.13	9.3 ± 0.9	–	–	38 ± 4	32[18], 51 ± 10 [17]
Fleroxacin	2.56	25.2 ± 13.6	–	–	27 ± 2	23 [18,19], 27 [21], 47 [22]
Ciprofloxacin	2.27	15.7 ± 4.2	2.58	18.7	21 ± 2	20 [23], 22 ± 4 [24], 20–40 [18], 35 [19], 40 [17]
N-methylcipro.	2.39	17.3 ± 2.8	–	–	28 ± 4	28 [8]
Enrofloxacin	2.76	34.6 ± 13.8	–	–	40 ± 2	36–45 [25]
Grepafloxacin	2.48	18.0 ± 5.3	–	–	28 ± 3	50 [26]
Sparfloxacin	2.55	20.4 ± 2.5	2.42	13.5	20 ± 2	40 [27], 44 [17]
Ofloxacin	2.51	21.5 ± 12.0	3.35	56.8	22 ± 4	8–30 [19], 10 [28]. 25 [17,18]
Levofloxacin	2.47	23.3 ± 11.9	3.03	38.9	22 ± 3	47–50 [29]
Rufloxacin	2.83	37.4 ± 5.6	–	–	58 ± 2	55–64 [30], 60 [17]
Difloxacin	2.59	25.6 ± 1.4	–	–	34 ± 2	42 [17]
Trovafloxacin	3.24	55.0 ± 5.4	–	–	62 ± 4	72 [31]

[a] All binding data in the table are calculated for a drug/protein ratio = 1/100 (the number of experiments was at least 3 in each case).

[b] The calculation of the binding constants leads to the assumption that at least two different protein-binding sites exist for these compounds; pK = log (K), where K is the association constant.

by the standard variations for the percentage of bound drug shown in Table 1. They are low for highly bound drugs and high for weakly bound drugs. Therefore, the continuous ultrafiltration will yield good results mainly for compounds with a high degree (≥ 50%) of protein binding.

After optimization of the entire procedure, the extent of plasma protein binding was measured using bovine and human serum albumin and human plasma. The application of human plasma turned out to create problems because the membranes tend to be blocked by some ingredients of low molecular weight of the plasma (Fig. 3). In some cases, the membrane was destroyed in the course of the filtration procedure. Thus, the plasma protein binding of only a few representative gyrase inhibitors could be determined (Table 2). With the exception of ofloxacin, the data found were in accordance with the data obtained from the discontinuous ultrafiltration.

From the continuous ultrafiltration data obtained with HSA and BSA, the number of binding sites and the association constants can be calculated. Furthermore, it is possible to calculate the percentage of bound drug for any ratio drug/protein. The binding data shown in Table 1 correspond to a ratio drug/protein = 1/100. This ratio corresponds to a total drug concentration in the serum of patients of 10 μM, which is approximately the therapeutic blood level of the gyrase inhibitors. At this low concentration, only one binding site of the protein will be occupied even in

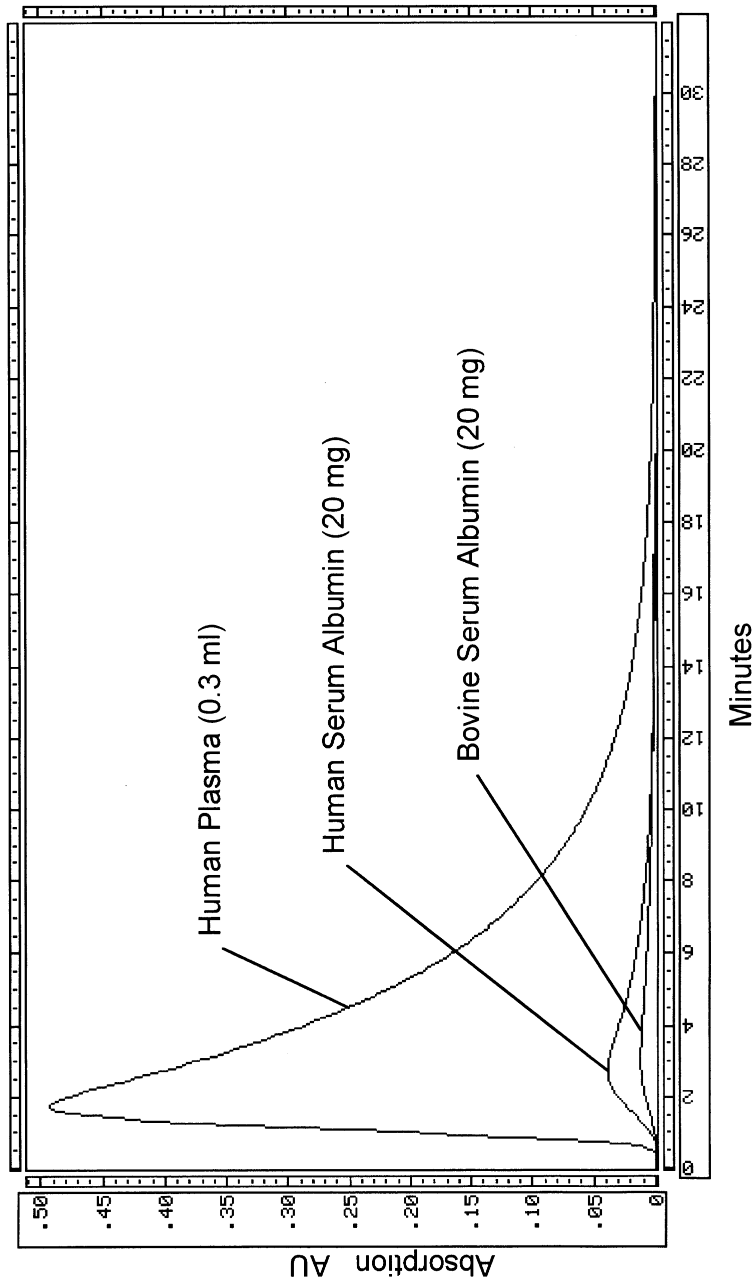


Fig. 3. Elution curves for HSA, BSA and human plasma at 280 nm.

Table 2
Protein binding data of gyrase inhibitors obtained with human plasma by means of continuous and discontinuous [8] ultrafiltration[a]

Gyrase inhibitor	% Bound to protein (continuous ultrafiltration)	% Bound to protein (discontinuous ultrafiltration)
Nalidixic acid	93.6	93
Cinoxacin	68.6	77
Ofloxacin	38.2	25
Sparfloxacin	20.1	23
Ciprofloxacin	27.4	23

[a] Drug/protein ratio = 0.1.

those cases of highly binding drugs for which more than one binding site can be assumed.

4. Discussion

BSA was included in the study in order to check whether the cheaper BSA gave results comparable to investigations with HSA. However, the comparison between the protein binding data obtained from BSA and HSA exhibited the difference between those two sorts of serum albumin (Table 1): The values obtained from bovine material were found to be higher, equal or lower than the data measured for HSA. Thus, it can be stated that BSA is not suitable to characterize the protein binding behaviour of a drug in human beings.

In a previous study, the extent of protein binding of gyrase inhibitors was determined by means of a discontinuous ultrafiltration using plasma protein in order to reveal the total fraction of the bound drug [8]. As can be seen from Table 2, the results of this former study are in accordance with the findings obtained here by means of the titration procedure, although the plasma created some problems during filtration. Thus, it is possible to compare the fraction of a drug bound to plasma with the fraction bound to albumin. The comparison of both methods is displayed in Fig. 4. In almost all cases, the columns show a similar height indicating that the gyrase inhibitors mainly bind to the albumin in plasma. The correlation between the amount of protein bound to plasma and albumin can be seen from the regression equation:

$$n = 18,\ r^2 = 0.87;\ s = 0.11;\ \text{and}\ F = 103. \quad (1)$$

Where n, number of drugs; F, the number of explained to unexplained variance; and r^2, the regression coefficient. The number in brackets is the standard deviation of the regression coefficient. The slope of almost 1 indicates the similarity of the data. However, the rather large Y-intercept gives evidence for some difference between plasma and albumin. The extent bound to plasma is mostly higher than the extent bound to albumin. This is easy to understand because plasma contains a lot of other proteins, e.g. α_1-acid glycoprotein [13], which are capable of binding the drug. Nevertheless, a homologous series of gyrase inhibitors, such as ciprofloxacin, *N*-methylciprofloxacin and enrofloxacin, shows a similar tendency of protein binding.

The diagram (Fig. 5) displaying the correlation between HSA and plasma bound fractions of the drugs clearly shows that rufloxacin, enoxacin and pipemidic acid (and to a certain extent trovafloxacin) bind in a substantially higher extent to the plasma than to the HSA. Taking additionally into account that, as shown in a previous study [8], heteroatoms in or at position 8 of the quinolone skeleton were found to enhance the plasma protein binding, this finding might reveal that this variation in the quinolone structure diminishes the affinity to the HSA and, at the same time, increases the affinity to other proteins in the plasma.

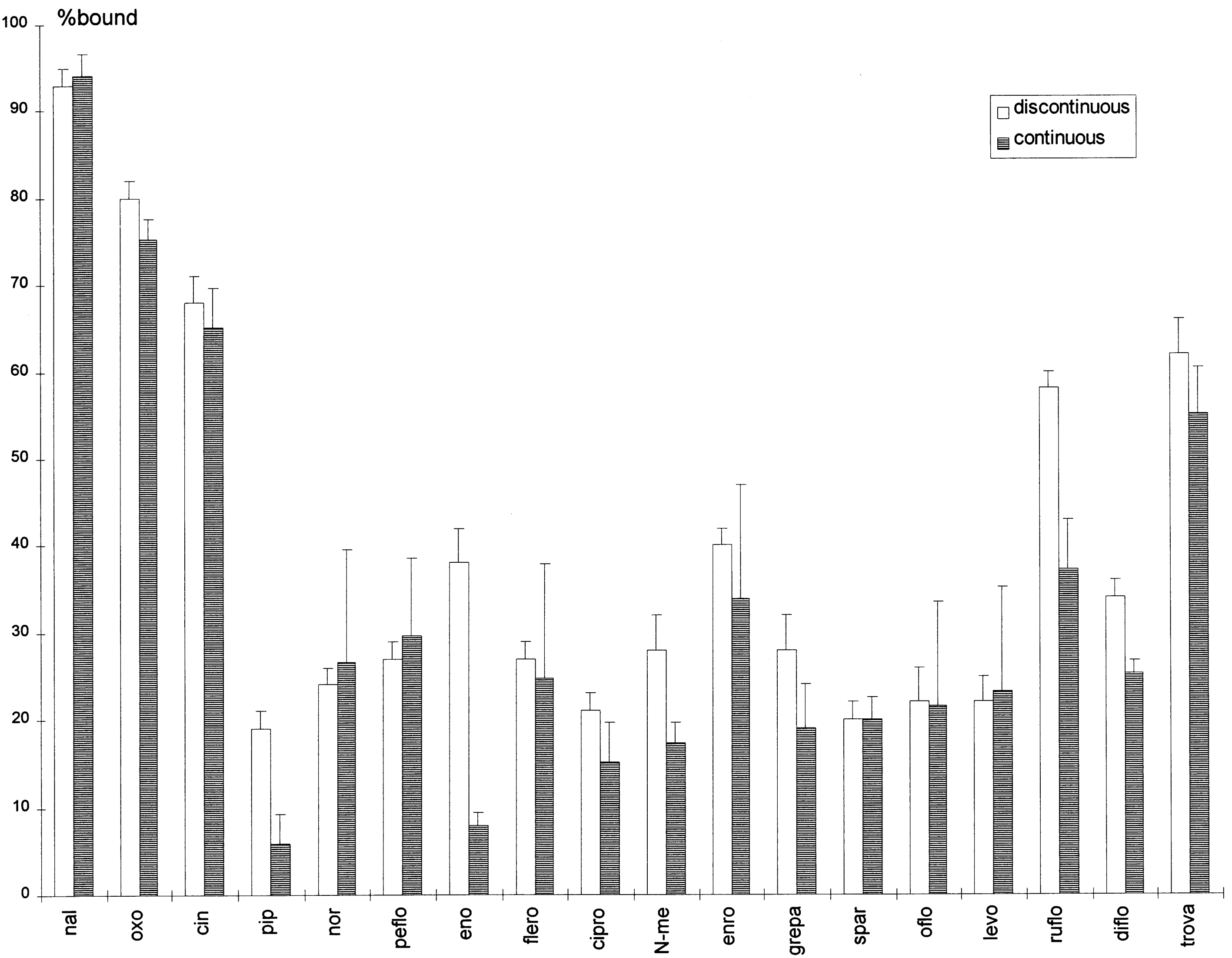


Fig. 4. Comparison between the extent of protein binding for gyrase inhibitors obtained with HSA by means of continuous ultrafiltration, and revealed from human plasma by means of discontinuous ultrafiltration (drug/protein ratio = 0.01).

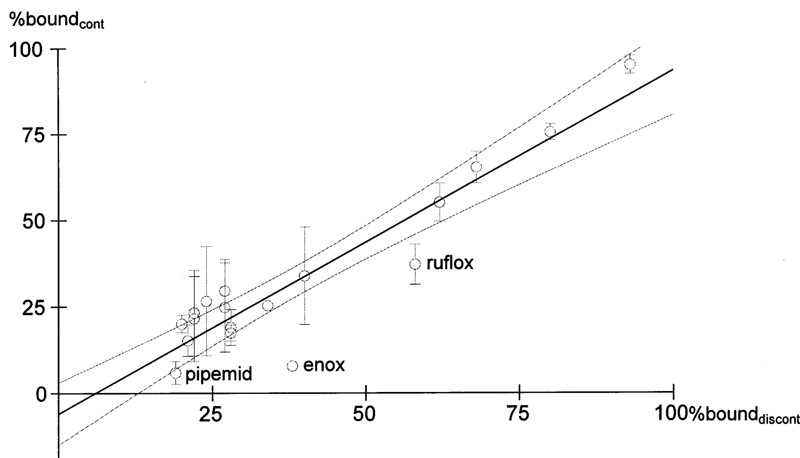


Fig. 5. Correlation between the extent of plasma and HSA binding of the gyrase inhibitors with standard deviation bars and 95% confidence limits.

5. Conclusion

The results presented here clearly show that it is difficult to estimate the total extent of plasma protein binding of a drug from HSA binding values, because the structure-binding properties relationships may slightly differ. However, the technique of the continuous ultrafiltration, which can be described as a simple titration, is suitable to determine the extent of protein binding fast and reliably. In addition, the method is closer to in vivo conditions than the 'classical' methods mostly used. The application of plasma to this method is under development.

Acknowledgements

G. Zlotos acknowledges a fellowship grant of the Cusanuswerk der Deutschen Bischöfe. In addition, thanks are due to Annegret Bücker for careful technical assistance.

References

[1] L.Z. Benet, D.L. Kroetz, L.B. Sheiner, in: J.G. Hardman, L.E. Limbird, P.B. Molinoff, R.W. Ruddon, A. Goodman Gilman (Eds.), Goodman and Gilman's, The Pharmacological Basis of Therapeutics, 9th ed., McGraw-Hill, New York, 1996, pp. 3–27.
[2] J. Oravcova, B. Bohs, W. Lindner, J. Chromatogr. B 677 (1996) 1–28.
[3] D.S. Campion, R. Olsen, J. Pharm. Sci. 63 (1974) 249–252.
[4] A. Kinawi, C. Teller, Arzneim. Forsch. 29 (1979) 1495–1499.
[5] P. Nickel, R. Schlerf, Pharm. Ztg. 128 (1983) 1234–1238.
[6] M. Oehimann, P. Nickel, Pharm. i. u. Z. 25 (1996) 202.
[7] J. Jürgens, H. Schedletzky, P. Heisig, J.K. Seydel, B. Wiedemann, U. Holzgrabe, Arch. Pharm. Pharm. Med. Chem. 329 (1996) 179–190.
[8] G. Zlotos, A. Bücker, M. Kinzig-Schippers, F. Sörgel, U. Holzgrabe, J. Pharm. Sci. 87 (1998) 215–220.
[9] A. Kinawi, Arch. Pharm. (Weinheim) 323 (1990) 523–524.
[10] H. Illy, Dissertation, Bonn, 1991.
[11] M. Oehimann, Dissertation, Bonn, 1996.
[12] A. Albert, E.P. Serjeant, The determination of ionisation constants, Methuen, London, 1971.
[13] R. Olsen, D.D. Christ, Ann. Rep. Med. Chem. 31 (1996) 327–336.
[14] T. Izumi, T. Kitagawa, Chem. Pharm. Bull. 37 (1989) 742–745.
[15] E. Okezaki, T. Terasaki, M. Nakamura, O. Nagata, H. Kato, A. Tsuji, J. Pharm. Sci. 78 (1989) 504–507.
[16] H. Jarboe, B. R. Toth, K.E. Shoemaker, K.J. Greenlees, K.M. Kleinow, Xenobiotica 23 (1993) 961–972.
[17] F. Sörgel, M. Kinzig, Am. J. Med. 94 (1994) 56–69.
[18] W. Cullmann, A.M. Geddes, E. Wiedekamm, H. Urwyler, A. Braunsteiner, Int. J. Antimicrob. Agents 2 (1993) 203–230.
[19] C.H. Nightingale, Am. J. Med. 94 (1994) 38–43.
[20] G. Montay, Y. Goueffon, F. Roquet, Antimicrob. Agents Chemother. 25 (1984) 463–472.
[21] N. Manek, J.M. Andrews, R. Wise, Antimicrob. Agents Chemother. 30 (1986) 330–332.

[22] E. Brunt, J. Limberg, H. Derendorf, J. Pharm. Biomed. Anal. 8 (1990) 67–71.
[23] R. Wise, J.M. Andrews, L.J. Edwards, Antimicrob. Agents Chemother. 23 (1983) 559–564.
[24] B. Joos, B. Ledergerber, M. Flepp, J.-D. Bettex, R. Luthy, W.J. Siegenthaler, Antimicrob. Agents Chemother. 27 (1985) 353–356.
[25] L. Kaartinen, M. Salonen, L. Alli, S. Pyorala, J. Vet. Pharmacol. Ther. 18 (1995) 357–362.
[26] A. Dalhoff, Pharmacokinetics of Selected Antibacterial Agents in Antibiotics and Chemotherapy, Karger, Basel, vol. 49, 1998, p. 85.
[27] M.A. Cooper, J.M. Andrews, J.P. Ashby, R.S. Matthews, R. Wise, J. Antimicrob. Chemother. 26 (1990) 667–676.
[28] Arzneimittelinformationen, Pharm. Ztg. 18 (1987) 1098.
[29] R. Davis, H.M. Bryson, Drugs 47 (1994) 677–700.
[30] R. Wise, J.M. Andrews, R.S. Matthews, M. Wolstenholme, J. Antimicrob. Chemother. 29 (1992) 649–660.
[31] Brochure of Pfizer GmbH.