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Measurement of drug–protein binding by immobilized human serum albumin-HPLC and comparison with ultrafiltration

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Abstract

An HPLC method employing CHIRAL-I (150 mm × 3 mm), 5 μm column from Chrom. Tech., immobilized with human serum albumin (HSA), was used to determine in vitro protein binding of several compounds. Experimentally obtained plasma protein data exhibited good correlation with the reported values. The method was compared with the conventional ultra filtration technique and both yielded similar results. Proprietary compounds that could not be analyzed by ultra filtration due to high non-specific binding to filter membrane were successfully analyzed by HSA-HPLC method. On the other hand, two proprietary compounds did not elute from HSA column due to strong binding, but were successfully analyzed by ultra filtration. This proves that both the techniques have their own merits and demerits and should be exploited judiciously as per the requirement. The plasma protein binding studies conducted on four gyrase inhibitors in rat and human plasma exhibited no interspecies difference via ultra filtration method. Further, it was also observed that the protein binding obtained for the four gyrase inhibitors by HSA-HPLC method was not only similar to that obtained by ultra filtration in human plasma but was also in accordance with ex vivo and in vitro protein binding obtained for rat plasma after ultra filtration because these compounds predominantly bind to HSA The binding of several compounds to α1-acid glycoprotein (AGP), another important plasma protein, was also examined using AGP immobilized column. However, the data could not be relied upon since some anti-bacterials and non-steroidal anti-inflammatory drugs (NSAIDS), which are known to predominantly bind to HSA, were also found to bind to AGP.
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Keywords: Plasma protein binding; Immobilized human serum albumin-HPLC; Ultra filtration

1. Introduction

Plasma protein binding of a drug is an important factor that influences its pharmacokinetic parameters [1–8] (i.e., distribution, clearance and elimination half-life) and pharmacodynamic parameters (i.e., efficacy and toxicity [9]). A drug may bind to several components/macromolecules, i.e., albumin, α1-acid glycoprotein (AGP), lipoproteins, immunoglobins (IgG) and erythrocytes within the blood. The formation of *a drug–protein complex* is termed as *drug–protein binding*. Most drugs bind to proteins in a reversible manner by means of weak chemical bonds such as ionic, van der Waals, hydrogen and hydrophobic bonds with the hydroxyl, carboxyl or other reversible sites available in the amino acids that constitute the protein chain. The major contribution to drug binding in the plasma is made by albumin [10], which is synthesized in the liver and constitutes about half of total plasma proteins. The molecular weight of albumin ranges between 65,000 and 69,000 Da. Acidic drugs are known to bind tightly [10] to human serum albumin (HSA), which has two ligand-specific binding sites [11,12] namely, site-I and site-II. The ligand selectivity is comparatively broader for these two sites, allowing a range of drug molecules to bind at these sites. This broad selectivity is considered to be a consequence of the significant allosteric effects in HSA [13] and drug molecules can also interact nonspecifically with HSA. HSA is responsible for maintaining the osmotic pressure of the blood and is a carrier of many molecules [14] such as: free fatty acids, bilirubin and various hormones (such as cortisone, aldosterone, thyroxin, etc.).

α1-Acid glycoprotein (AGP) is a relatively low molecular weight (approximately 40,000 Da) protein. Its concentration in plasma is about 40–100 mg/100 ml and primarily binds to basic (such as lidocaine, propranolol, imipramine and quinidine) and

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Table 1
Reported protein binding of some antibacterial [16,36], anti-retroviral [18] and NSAIDS [36]

Compound	Category	% PPB
Ciprofloxacin		
Norfloxacin		20
Gatifloxacin		20
Ofloxacin	Antibacterial	30
Gentamicin		3
Moxifloxacin		39–52
Levofloxacin		24–38
Nelfinavir		99
Lopinavir		99
Sequinavir	Antiretroviral	98
Ritonavir		98
Amprenavir		98
Indinavir		60
Rofecoxib		87
Celecoxib		97
Valdecoxib		98
Piroxicam		99
Meloxicam		99
Fenoprofen		>99
Ibuprofen	Antiinflammatory	99
Indomethacin		92–99
Meclofenamic acid		99.8
Naproxen		99–99.5
Oxyphenbutazone		97–98
Phenylbutazone		98–99
Sulindac sulfide (active metabolite)		93–98

neutral drugs [10] in addition to some acidic drugs [15]. In general, gyrase inhibitors [16] and NSAIDS (non-steroidal anti-inflammatory drugs [17] bind to albumin and anti-retroviral compounds [18] bind to AGP. Table 1 exhibits the protein binding of some antibacterial, antiviral and NSAIDS.

Binding of drugs to lipoproteins, red blood cell and other membranes is not a true binding reaction but is similar to dissolving of the drugs in the lipids of the membrane. Very lipophilic drugs partition preferentially into the membrane lipids rather than the plasma water. Some drugs bind strongly to particular tissue components such as DNA (e.g., some anticancer drugs) and melanin-rich tissues (e.g., chloroquine and amiodarone).

Free drug concentration in plasma is responsible for the observed pharmacological effect or therapeutic response [19–21]. The drug bound to plasma protein is not available for distribution, hepatic metabolism and renal elimination. The drug/protein complex does not permeate phospholipid bilayers, including capillary membranes, glomerular membranes in the nephrons and the blood brain barrier [1–8]. Bound drugs are also less available to the enzymes involved in first-pass metabolism. The driving force for drug excretion in kidney is the free drug concentration in the plasma. The glomerular capillaries permit the passage of most of the drug molecules but restrict the passage of plasma proteins and drug–protein complex and therefore, only free or unbound drug is filtered. After the metabolic and excretory processes have cleared much of the free drug, the reversible *drug–protein complex* serves as a depot to replenish the concentration in vivo [20]. Drugs with high protein binding tend to have a greater elimination half-life compared to those with low binding. The prolonged pharmacological activity resulting from these factors may be desirable, or may promote the emergence of undesirable side effects. Therefore, estimation of the extent of drug–protein binding is crucial for the clinical drug development. Ideally, determination of both total and unbound plasma drug concentrations is necessary to obtain an understanding of drug available for pharmacological effect.

Models to predict binding affinities to human serum albumin (HSA) should be very useful in the pharmaceutical industry to speed up the design of new compounds, with favourable pharmacokinetics [20,22,23].

In the past, several techniques have been explored for quantitative determination of drug–protein binding in vitro. Among those, equilibrium dialysis, gel-filtration, ultra-filtration and ultra-centrifugation [16,24–28] have been conventionally and most commonly used. These conventional methods suffer from long analysis time. Different in vitro methods yield different results, i.e., equilibrium dialysis indicated 23% plasma protein binding for fleroxacin whereas, ultra filtration indicated 47% plasma protein binding [16]. A lot of inter laboratory variation in the plasma protein binding data obtained via the same technique leading to a broad range, i.e., 20–40% for ciprofloxacin, 8–30% for ofloxacin, norfloxacin and 30–50% for enoxacin [16] has been reported. Although ultra filtration method is believed to be comparable to in vivo processes such as ultra filtration of drug in kidney [16], still it suffers from the limitation of non-specific binding of the drug to the membrane and leakage of drug from membrane.

In comparison to the conventional methods, chromatography based methods [24,29–33] employing columns immobilized with plasma proteins have gained popularity over the years because of their simplicity, specificity and speed. The earliest work in the area of human serum immobilized chromatography was carried out by Wainer and his group [34–37]. Henceforth, different strategies for the development of columns immobilized with HSA have been reported [38–44]. In one method, the epoxy groups of this copolymer (glycidyl methacrylate and ethylene dimethacrylate) were used directly for the immobilization of HSA through its amine residues (i.e., the epoxy method); in other approaches, these epoxy groups were converted to diols for later use in the carbonyldiimidazole, disuccinimidyl carbonate, and Schiff base methods [38]. Stable and selective chiral stationary phases were also prepared by covalent binding of HSA to silica particles via reactive-polymers. Poly(acryloyl chloride), poly(methacryloyl chloride) and poly(vinyl chloroformate) derivatives were compared. Human serum albumin (HSA) has been reported to be successfully bonded to silica with *N*-hydroxysuccinimide [39] and s-triazine as activator [44]. Stationary phases obtained by immobilization of HSA on [C8] and [C18] reversed-phases and on poly(1-vinylimidazole)-coated silica have also been reported [41,43].

The present investigation reports an HPLC-based (HSA) method for drug–protein binding study of UV active compounds. A comparison of the method with ultra filtration has also been made.

2. Experimental

2.1. Chemicals and reagents

Working standards of fluconazole, gatifloxacin, norfloxacin, ofloxacin, ciprofloxacin, venlafaxine, carbamazepine, citalopram, rofecoxib, celecoxib, propanolol, metformin, paroxetin, omeprazole, nelfinavir, sequinavir and ritonavir (purity above 95.0%) as exhibited in Fig. 1,were provided by Zydus Research Center, Cadila Healthcare Ltd. *iso*-Propanol Omnisolv® was purchased from Merck, KgaA and Darmstadt, Germany. Potassium phosphate was procured from Merck, Germany. HPLC Type II Water from Millipore's Milli-Q System was used throughout the study.

2.2. Chromatographic conditions

Chromatographic separation was performed on a Class VP chromatograph LC 2010 from Shimadzu Corporation, Kyoto, Japan. The data acquisition was carried out by Class VP6.01 version data system from Shimadzu Corporation Kyoto Japan. Sample (10.0 μl) was injected into an immobilized CHIRAL-I (150 mm length, 3 mm inner diameter and 5 μm particle size) column from Chrom. Tech. maintained at 25 °C with an isocratic mobile phase (7% *iso*-propanol in 20 mM potassium phosphate buffer, pH 7.0) flowing through it at a flow rate of 0.8 ml/min. All analytes were detected at a wavelength of 220 nm. For AGP-HPLC, CHIRAL-AGP (150 × 3) mm, 5 μm column from Chrom. Tech. was used.

2.3. Plasma protein binding experiment by ultra filtration

The stock solution (1.0 mg/ml) of test compounds was prepared by dissolving appropriate amount of working standard of compounds in water:methanol (50:50, v/v) mixture. Twenty-five microliters of the above solution was added to a glass test tube and evaporated to dryness under a stream of nitrogen in the thermostatically controlled water-bath maintained at 55 °C for

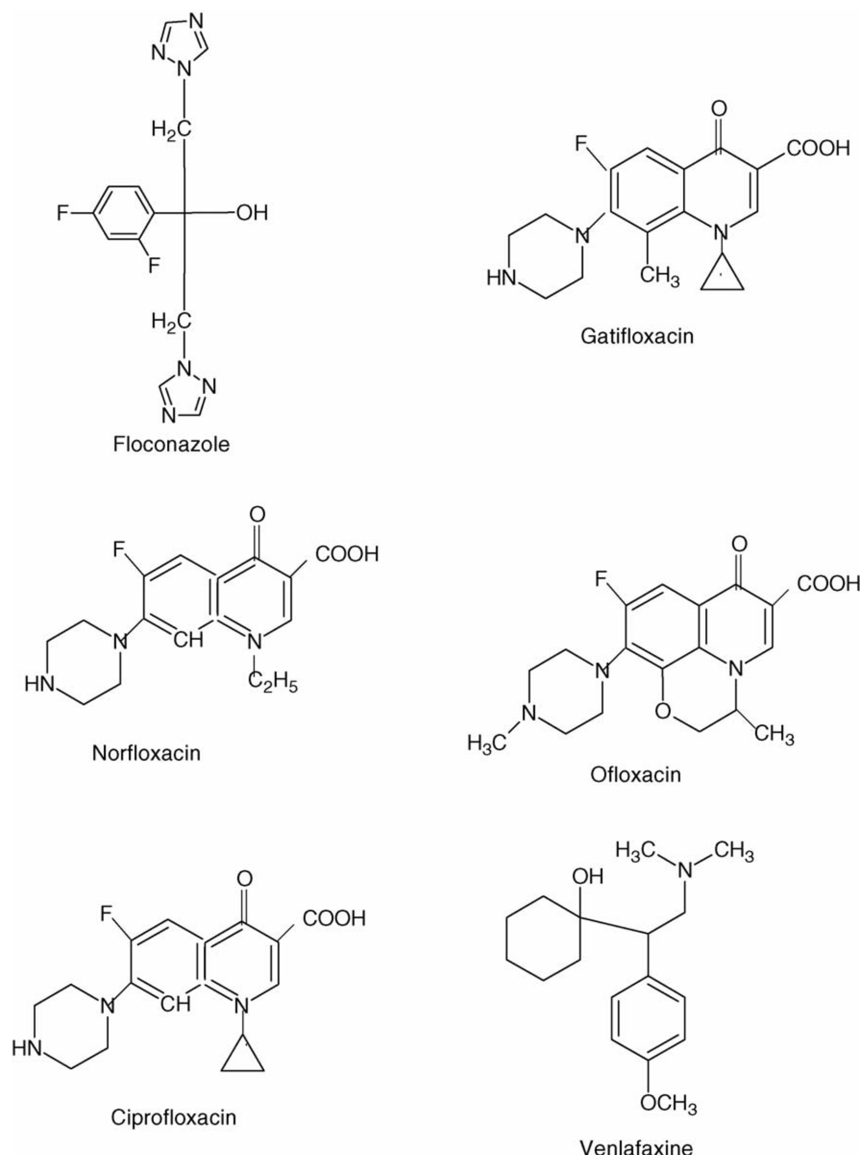

Fig. 1. Structural formula of compounds studied.

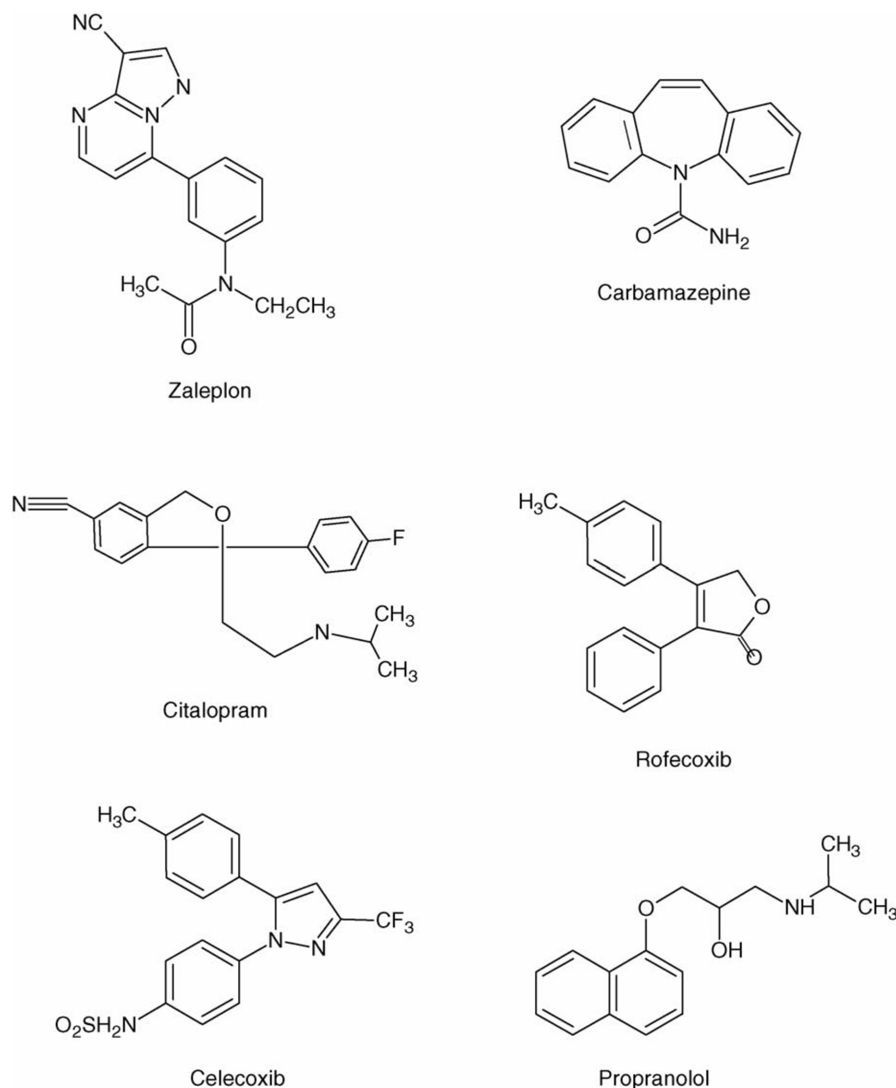

Fig. 1. (*Continued*).

about 20 min. Drug free plasma (2.5 ml) was then added to it and vortexed for 45 s. The mixture was incubated at 37 °C for 30 min. Thereafter, 1 ml aliquot of the sample was loaded into the sample reservoir of centricon filter device with a filter pore size of 30 kDa and subjected to ultra filtration with centrifugation for 45 min at 2465 × *g*. The filtrate was analyzed for the drug content by HPLC.

In order to estimate the adsorption of the drug to the filter membrane 1 ml of solution (10 μg/ml) of each compound studied was subjected to ultra filtration by centrifuging for 45 min at 2465 × *g* in a Centricon filter device with a filter pore size of 30 kDa. The concentration of the filtrate was analyzed by HPLC. The adsorption to the membrane was <5% in each case.

The filtrates were analyzed by HPLC with a mobile phase consisting of 0.05% trifluroacetic acid and acetonitrile in the ratio 75:25 (v/v) was continuously passed through the analytical column at a flow rate of 1.0 ml/min. About 100 μl of samples was kept in auto sampler maintained at 15 °C and injected into equilibrated column. The separation was achieved on Inertsil ODS-3V, (250 mm length, 4.6 mm inner i.d. and 5 μm particle size) analytical column maintained at 30 °C with detection at 220 nm. The plasma protein binding by ultra filtration was performed in duplicates for all the compounds studied.

2.4. *Ex vivo estimation of plasma protein binding in rat plasma*

Male rats 8–10 weeks of age; were used for the study. Each group consisted of four animals. All animals were fasted for 18 h prior to the administration of the drug. Food was supplied after 4 h of drug administration and there was free access to water through out the study. Each group of animals received a single oral dose of 30 mg/kg of different compounds. A homogenous suspension of four gyrase inhibitors was prepared separately in a vehicle comprising of 0.5% (w/v) carboxy methyl cellulose (CMC) in water and polyethylene glycol (PEG) 400 (90:10, v/v). The animals were sacrificed at C_{max} by incising the juglar vein and blood was withdrawn from the retro-orbital plexus of a rat into heparinized eppendorf tubes. Samples were kept on ice until centrifugation. Plasma was separated by centrifugation at

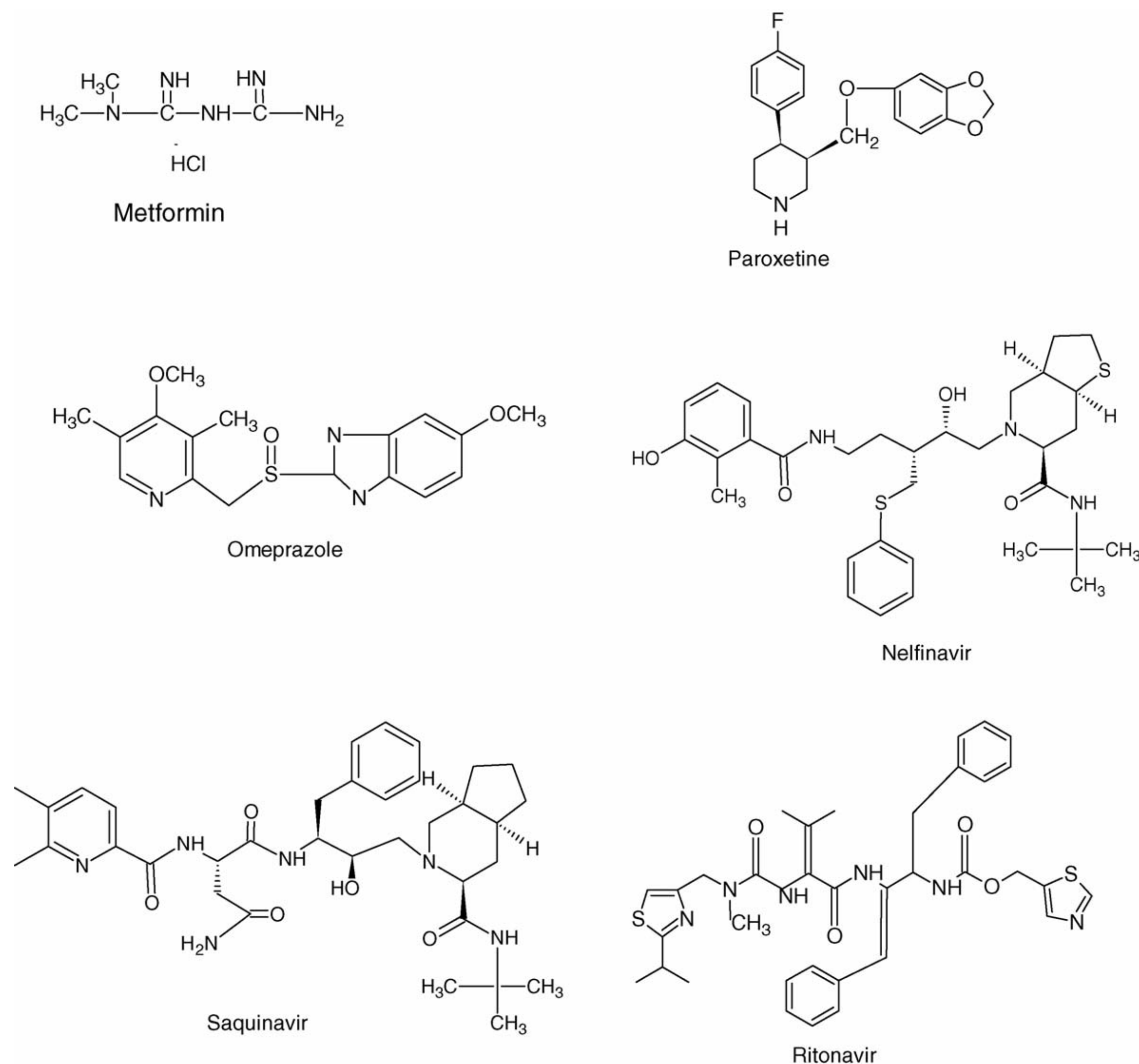

Fig. 1. (*Continued*).

approximately 3220 × *g* for 5 min at 25 ± 5 °C and immediately analyzed. The pending samples were stored in the deep freezer at −70 ± 5 °C until analyzed. The study adheres to "Principles of Laboratory Animal Care" and is approved by the animal care committee IAEC/CPSEA—Institutional Animal Ethics Committee/Committee for the Purpose of Control and Supervision of Experiments on Animals. Plasma samples obtained from animals were divided into two parts. One part was subjected to the estimation of total drug concentration at C_{max} after liquid–liquid extraction. The other part was subjected to ultra filtration for the estimation of plasma protein binding as per Section 2.2.

3. Results and discussions

Around 14 different drugs with plasma protein binding ranging from 12 to 95% were analyzed by immobilized human serum albumin-HPLC. The compounds with low plasma protein binding eluted at a lower retention time and exhibited sharp peak shapes as compared with the highly protein bound compounds. Representative chromatograms are shown in Fig. 2. There was no significant advantage in terms of column efficiency and retention time when the temperature was increased from ambient (25 °C) to body temperature (37 °C) and therefore all HPLC analysis was carried out under ambient conditions only. This might have some effect on the comparison of the HPLC and ultrafiltration results (which were done at 37 °C), since most drug–protein binding increases in strength as the temperature is lowered over this range. The retention factor (k') for a compound was calculated as [35,24]:

$$\frac{t_R - t_M}{t_M} \tag{1}$$

where t_R is the retention time of the compound and t_M is the retention time of the unretained marker compound (water in this case). The value of (k') was further substituted in Eq. (2) in order to obtain the percentage protein binding [35,24]:

$$\%\,\text{plasma protein binding} = 100\left(\frac{k'}{k'+1}\right). \tag{2}$$

Fig. 2. Representative chromatograms of some compounds analysed on HSA column.

The protein binding of the compounds obtained by immobilized human serum albumin-HPLC is shown in Table 2. A plot of % plasma protein binding observed by immobilized HSA-HPLC versus reported plasma protein binding (Fig. 3) exhibited a good agreement, i.e., correlation (r^2) of 0.986 between the two values, indicating the suitability of the method.

The plasma protein binding of five known compounds and five proprietary compounds (**ABCDE**) was determined (Table 3) by immobilized HSA-HPLC as well as ultra filtration and a comparison of the two techniques was made. It was found that for most of the compounds, % plasma protein binding obtained by both the techniques was in close agreement (Table 3) except for compounds **CDE**. This supports the earlier reports [45–47] that the binding properties of immobilized HSA are similar to those observed for HSA in solution. However, immobilized human

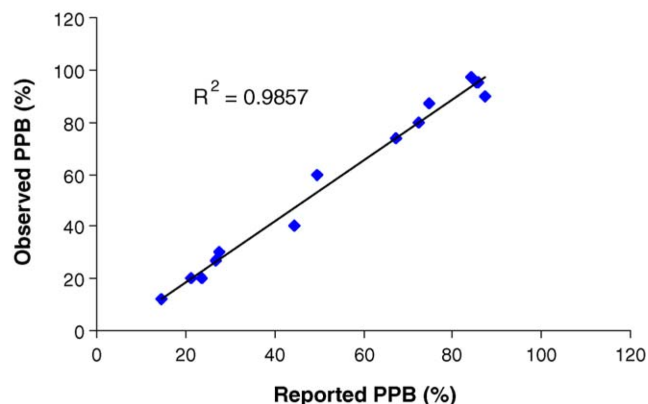

Fig. 3. Comparison of protein binding of some compounds by immobilized HSA column HPLC with the literature values.

Table 2
Protein binding of some compounds by immobilized HSA-HPLC

Compound	Retention time (min)	Retention factor (k')	$k'/k'+1$	Observed %PPB	Reported %PPB
Fluconazole	3.51	0.17	0.15	14.53	12.0
Gatifloxacin	3.80	0.27	0.21	21.05	20.0
Norfloxacin	3.93	0.31	0.24	23.66	20.0
Venlaflaxin	4.10	0.37	0.27	26.83	27.0
Ofloxacin	4.15	0.38	0.28	27.71	30.0
Ciprofloxacin	5.40	0.80	0.44	44.44	40.0
Zaleplon	5.93	0.98	0.49	49.41	60.0
Carbamazepine	9.12	2.04	0.67	67.11	74.0
Citalopram	10.90	2.63	0.72	72.48	80.0
Rofecoxib	11.80	2.93	0.75	74.58	87.0
Celecoxib	18.96	5.32	0.84	84.18	97.0
Metformin	23.40	6.80	0.87	87.18	90.0
Paroxetin	20.30	5.77	0.85	85.22	95.0
Omeprazole	21.30	6.10	0.86	85.92	95.0

Table 3
Comparison of protein binding of some compounds by ultra filtration and immobilized HSA-HPLC

Compound	% Protein binding (±S.D.)		
	Ultrafiltration	HSA-HPLC	Reported value
Gatifloxacin	17.5 8(1.32)	21.05 (0.03)	20.0
Norfloxacin	18.3 3 (0.98)	23.66 (0.05)	20.0
Ciprofloxacin	38.96 (0.89)	44.44 (0.02)	20–40
Ofloxacin	26.82 (0.65)	27.71 (0.04)	30.0
Rofecoxib	96.92 (0.87)	74.58 (0.05)	98
Celecoxib	97.69 (1.32)	84.18 (0.6)	12
Compound A	35.34(1.52)	38.23 (0.02)	NA
Compound B	32.14 (0.95)	30.26(0.04)	NA
Compound C	100.00 (0.00)	25.67 (0.01)	NA
Compound D	96.22 (1.21)	NA	NA
Compound E	92.63 (0.99)	NA	NA

NA: not applicable.

serum albumin-HPLC offered better 'standard deviation (S.D.)' as compared to ultra filtration. The degree of protein binding is also governed by lipophilicity to some extent and in general the protein binding increases with increasing lipophilicity [16]. In accordance with this generalization, it was observed that the gyrase inhibitors with low partition coefficient (norfloxacin: −9.5, ciprofloxacin: −1.1 and ofloxacin: −4.7) exhibit lower protein binding whereas, the cox-2 inhibitors with high lipophilicity (log p; celecoxib: 3.68, rofecoxib: 1.7) show higher protein binding The ultra filtration method indicated 100% plasma protein binding for compound **C**, whereas the HSA-HPLC method indicated a binding of 25%. The discrepancy arose due to high non-specific binding (60%) of the compound **C** on to the membrane filter of the ultra filtration tube. Compounds **D** and **E** did not elute from the HSA column under the chromatographic conditions mentioned in Section 2.2, because of very strong binding to HSA, and had to be eluted by increasing the *iso*-propanol concentration to 45%. Earlier workers [48] have also used high organic concentrations to elute strongly bound compounds from HSA immobilized column damaging the column. On the other hand, the ultra filtration method worked successfully for the compounds **D** and **E** yielding high plasma protein binding values of 96.22 ± 1.21 and 92.63 ± 0.99, respectively. The conventional ultra filtration method is believed to be comparable to in vivo processes such as ultra filtration of drug in kidney [16]. However, it is also reported [24] to be time consuming, requiring additional analytical step for estimating the actual drug concentration, and the test compounds are fraught with the dangers of non-specific binding of drugs on to the membrane. It should be borne in mind, ultra filtration provides the total estimate of drug–protein binding with all the plasma proteins whereas HSA-HPLC method estimates binding only to albumin and this difference is sufficient to cause discrepancy in the plasma protein binding data obtained from two the techniques. In spite of being a quick means of in vitro plasma protein binding estimation, HSA-HPLC could underestimate the plasma

Table 4
Comparison of ex vivo and in vitro protein binding of gyrase inhibitors

Compound	Protein binding (%) (±S.D.)				
	Ultrafiltration			HSA-HPLC	Literature value (human plasma)
	Ex vivo	In vitro			
	Rat plasma	Rat plasma	Human plasma		
Gatifloxacin	16.53 (0.98)	18.6 (0.99)	17.5 (1.32)	21.05 (0.03)	20.0
Norfloxacin	17.88 (1.32)	17.98 (0.73)	18.3 (0.98)	23.66 (0.05)	20.0
Ciprofloxacin	34.65 (0.62)	35.61(0.85)	38.9 (0.89)	44.44 (0.02)	20–40
Ofloxacin	24.65 (0.95)	23.48 (1.78)	26.82(0.65)	27.71 (0.04)	30.0

Table 5
Protein binding of some compounds by ultrafiltration and immobilized AGP-HPLC

Compound	Retention time (min)	Retention factor (k')	$k'/k'+1$	Observed %AGP	Reported %PPB
Fluconazole	3.40	0.27	0.21	21.11	12.0
Nelfinavir[a]	17.10	5.38	0.84	84.33	99
Sequinavir[a]	22.50	7.40	0.88	88.09	98
Ritonavir[a]	24.60	8.18	0.89	89.11	98
Norfloxacin	6.94	1.59	0.61	61.39	20.0
Linezolid	3.34	0.24	0.20	19.67	30.0
Ofloxacin	5.85	1.18	0.54	54.17	30.0
Levofloxacin	5.86	1.19	0.54	54.28	24–38
Gatifloxacin	2.84	0.06	0.06	5.64	20.0
Ciprofloxacin	7.06	1.63	0.62	62.03	20–40
Carbamazepine	11.84	3.42	0.77	77.38	800
Citalopram	6.99	1.61	0.62	61.70	80.0
Rofecoxib	6.43	1.40	0.58	58.35	87
Celecoxib	7.57	1.83	0.65	64.61	97
Paroxicam	15.57	4.81	0.83	82.79	98.5
Paroxetine	30.78	10.49	0.91	91.30	95
Propranolol[a]	9.22	2.44	0.71	70.95	90–96

[a] Specifically bind to AGP.

protein binding binding of those compounds that are bound to other plasma proteins. Therefore, both the techniques have their own advantages and limitations, which should be exploited judiciously.

The plasma protein binding for four antibacterial compounds was obtained ex vivo in rat model and Table 4 depicts that the protein binding obtained ex vivo was in agreement with in vitro protein binding in rat plasma, confirming the validity of the use of in vitro ultra filtration technique for plasma protein binding determination. Further, it was also observed that the rat plasma protein binding values were very close to those obtained for human plasma indicating interspecies similarity in protein plasma protein binding of these compounds. The HSA binding obtained by HSA-HPLC was also in accordance with the plasma protein binding obtained by ultra filtration in rat and human plasma. These antibacterial compounds predominantly bind to albumin [16] and therefore no discrepancy was observed between the HSA-HPLC and conventional ultra filtration plasma protein binding data.

Every compound binds in a certain extent to all the plasma proteins. The percentage protein binding obtained by the ultra filtration method measures all the specific and non-specific binding to all of the plasma components. On the other hand the HPLC method measures both the specific and non-specific binding to one particular protein. Comparison of the binding of all components, i.e., ultra filtration data with that of only one protein, i.e., HSA-HPLC presents a false picture. Therefore, the in vitro plasma protein binding of several compounds was also estimated (Table 5, Fig. 4) using AGP (α1-acid glycoprotein) immobilized column under similar chromatographic conditions. It was observed that in addition to binding to anti-HIV compounds which are reported [18] to bind to AGP in vivo, other compounds specific for HAS, i.e., gyrase inhibitors [16] and NSAIDs [17] were also bound to AGP (Table 5, Fig. 4). This may be attributed to the nonspecific binding to AGP and/or to the difference in the

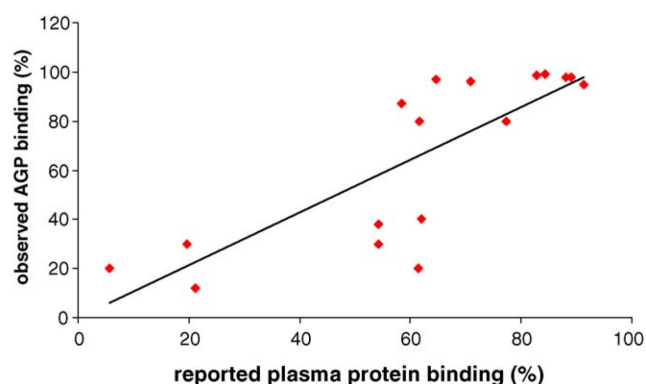

Fig. 4. Comparison of protein binding of some compounds by immobilized AGP column HPLC with the literature values.

binding properties of α1-acid glycoprotein in solution state versus immobilized state [24,49–51].

4. Conclusion

The HSA-HPLC method described in the present investigation exhibited good correlation with the reported plasma protein binding values indicating the suitability of the method for routine plasma protein binding estimation in drug discovery programmes. A comparison of the method was also made with the conventional ultra filtration technique. It was observed that both the techniques yield almost similar results except for some instances where the analytes could not be analyzed by either ultra filtration due to binding to filter membrane or did not elute from HSA column due to very strong binding. It can be concluded that both the techniques have their own limitations and advantages. HSA-HPLC is suitable for the compounds that are designed to specifically bind to HSA and could indicate underestimated plasma protein binding for compounds that bind to other plasma proteins. On the contrary, ultra filtration provides total plasma protein binding in the presence of all the plasma proteins and resembles some of in vivo processes. The current commercial HSA columns can be used in drug–protein binding while AGP columns are not as appropriate for such work [24,49,50,51]. Further, the authors recommend the use of more sensitive and faster LC–MS technique instead of HPLC for obtaining higher through put for such studies.

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