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RESEARCH ARTICLE

Gel permeation chromatographic method for drug protein binding studies

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ABSTRACT

The estimation of individual plasma protein in free and bound form with analytes has importance in pharmacokinetics study. Albumins and globulins are most abundantly found in plasma and plays crucial role in plasma protein binding. The present communication deals with the development of gel permeation chromatographic method for the estimation of plasma protein binding studies for ketoprofen, tapentadol and furaltadone. The method was developed using WATER system with Ultrahydrogel column (7.8 x 300 mm i.d.), refractive index detector (1.00 to 1.75 RIU) and Rheodyne injection valve fitted with a 20µl sample loop using 0.1% sodium nitrate as a mobile phase. The method was developed by studying binding above drugs with most of plasma proteins. These complexes have shown linearity in range of 100 to 300µg/ml of these drugs. The developed method has been validated using USFDA guidelines. The developed bioanalytical method is accurate, precise, and selective and sensitive for quantification of plasma proteins bound drugs. The further optimization of method using other standard plasma proteins will explore its applicability in pharmacokinetic and biopharmaceutical studies of most of drugs.

Keywords: Bovine serum albumin; gel permeation chromatography; plasma protein binding; human serum albumin.

1. Introduction

The plasma protein binding is one major determinant of the distribution of chemicals in the body. The data on plasma protein binding of pharmaceuticals is limited and for some drugs, minimal information which like percentage bound or fraction unbound in plasma is available [1, 2]. Thus, there is a need to generate data on plasma protein binding of drugs which can be used to characterize protein binding, distribution and availability of chemicals *in vitro* [3]**.** In serum and other complex biological samples, a few proteins often constitute a high percentage of the total protein. These high-abundance proteins can mask the numerous proteins of lower abundance in proteomics analyses, whether intact or in tryptic digests. Size-Exclusion Chromatography (SEC) was used to estimate the molecular weights of the proteins and peptides in the samples [4, 5].

Gel permeation chromatography (GPC) has become the most widely used technique for determination of molecular weight distributions [6, 7]. The apparent size and the shape

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of the polymers in solution is the basis for separation in size exclusion chromatography [8, 9]. This simple separation mechanism relies on protein size differences, packing material pore size and particle size as well as the operating conditions such as sample concentration and volume for the successful resolution of protein mixtures [10]. Size exclusion packing material has evolved from the large particle polysaccharide soft gels to small, rigid packing that offers enhanced resolution and significantly faster separation [11-13]. The principle feature of SEC is its gentle non-adsorptive interaction with the sample, enabling high retention of biomolecular activity [8, 10].

Bovine serum albumin (also well-known as Fraction "V" or BSA) is a serum albumin protein that has several biochemical applications including ELISA (Enzyme-Linked Immuno Sorbent Assay), Immuno-Blot Technique and Immuno Histochemistry [14]. BSA is also commonly used to determine quantity of other proteins, by comparing an unknown quantity of protein to known amounts of BSA due to its stability, its lack of effect in many biochemical reactions, and its low cost [15]. BSA is commonly used as protein standard in protein assays and as a molecular weight standard for SDS-PAGE and GPC [16, 17].

Electrophoresis, ultrafiltration, equilibrium dialysis was previously used to determine protein binding and based on measurements of freely dissolved concentrations, required specialized analytical equipment and experience and time consuming. Nowadays, there are various methods HPLC, GPC etc. available for the separation of different proteins. The present communication deals with development of GPC method for estimation of human serum albumin (HSA). The method has been developed by using BSA and applied for estimation of HSA obtained from human blood.

2. Materials and Methods

2.1 Materials

2.1.1. Reagents and chemicals

Sodium nitrate, Potassium dihydrogen orthophosphate, Acetonitrile of HPLC grade was purchased from Finar Chemicals. BSA (fraction V) was obtained from Hi-Media Chemicals. In present work, we have targeted three drugs for the protein binding study. Drugs i.e. Ketoprofen, Tapentadol, Furaltadone were procured from pharmaceutical company as a gift sample. Human blood was obtained from Shahu Blood Bank, Kolhapur.

2.1.2. Instrument

Gel Permeation Chromatography: The WATER GPC system is equipped with, Ultrahydrogel TM 500 column (7.8 x 300mm i.d.), Refractive Index detector (1.00 to 1.75 RIU) and Rheodyne injection valve fitted with a 20 µl sample loop operating at temperature of 15-40^{0}C. The chromatographic and the integrated data were recorded using IEEE-488 (bus interface) computer system. The GPC chromatographs were processed by using BREEZE 2 software.

2.1.3. UV-Visible Spectroscopy

The instrument used for the present study was PC based Jasco V-630 UV-Visible double beam Spectrophotometer with 1 cm matched pair quartz cell and spectral bandwidth of 2 nm.

Spectrophotometric method for protein binding study of ketoprofen

Preparation of standard solution

2.1.3.1. Preparation of albumin- Ketoprofen complex in KCl

Egg albumin was separated from the eggs, 5ml of this egg albumin was added in 1% KCl (25 ml) and mixed thoroughly. 10µg/ml of the Ketoprofen was then mixed with the above solution. The resultant solution was titrated with 1N HCl until change in p^H. In this mixture again add 5ml HCl (1N). This solution was then extracted with the 5 ml of chloroform. Two layers, aqueous and organic were then separated. Organic layer was transferred to the watch glass and evaporated. Reconstitute the dried solid extract with 5ml of 1% sodium bicarbonate solution and volume was adjusted with water. UV absorbance of the resultant solution was then plotted at specified wavelength.

2.1.3.2. Preparation of albumin-Ketoprofen complex in NaCl

Egg albumin was separated from the eggs, 5ml of this egg albumin was added in 1% NaCl (25ml) and mixed thoroughly. 10µg/ml of the Ketoprofen was then mixed with the above solution. The resultant solution was titrated with 1N HCl until change in pH. In this mixture again add 5ml NaCl (1N). This solution was then extracted with the 5ml of chloroform. Two layers, aqueous and organic were then separated. Organic layer was transferred to the watch glass

and evaporated. Reconstitute the dried solid extract with 5ml of 1% sodium bicarbonate solution and volume was adjusted with water.

Above solutions were scanned in the wavelength range of 200-400nm and the calibration curve was constructed shown in Tables 1, 2 and Figures 1, 2.

Table 1. Absorbance of albumin-Ketoprofen complex in KCl and NaCl.

Analyte	Absorbance of sample containing KCl	Absorbance of sample containing NaCl
Albumin-Ketoprofen complex	0.0012	-0.0010

2.1.3.3. Calibration curve for Ketoprofen

The data for calibration curve is given in Table 4 for Ketoprofen while spectrum is shown in Figure 1. Calibration curves are shown in Figure 2 for Ketoprofen.

Table 2. Linearity of Ketoprofen at 240nm

Sr. No.	Concentration (µg ml^{-1})	Absorbance
1	5	0.0891
2	10	0.451
3	20	1.014
4	30	1.51
5	40	2.0751
6	50	2.61

2.2 Methods

2.2.1. Chromatographic conditions

The separation of fractionated plasma and analysis of BSA was carried out on WATER system with Ultra hydrogel TM 500 column (7.8 x 300mm, i.d.) as stationary phase and 0.1% sodium nitrate as mobile phase using Refractive Index detector and Rheodyne injection valve fitted with a 20µl sample loop operating at temperature of 15-40°C. The flow rate of 0.7mL/min was maintained throughout analysis.

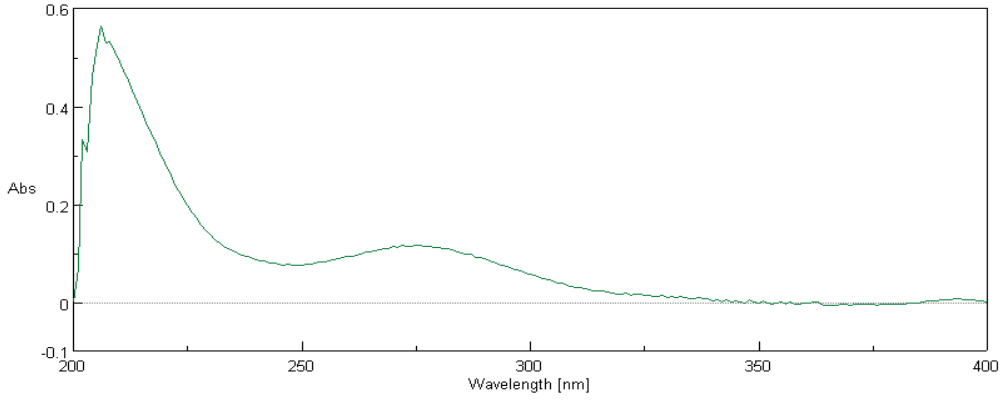

Figure 1. Spectrum of Ketoprofen at 240nm

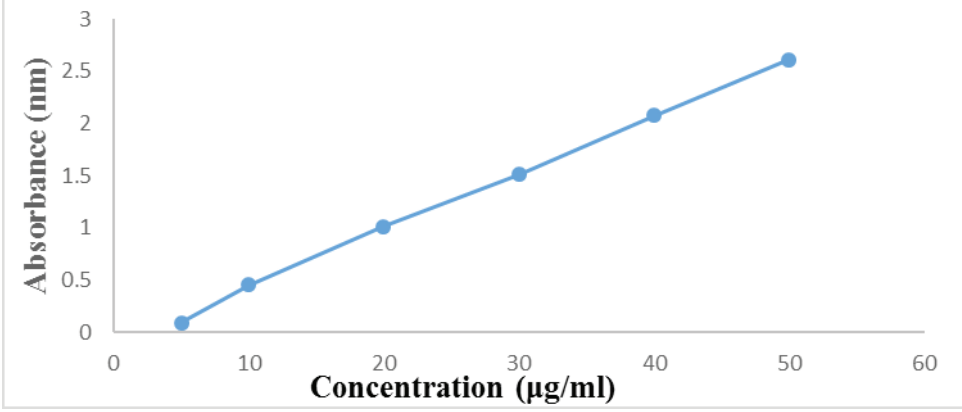

Figure 2. Calibration plot of Ketoprofen at 240nm

2.2.2 Standard Stock Solution of BSA

Accurately weighed 100mg of sodium nitrate was dissolved in a 100ml of double distilled water. This solution was sonicated for 15min and used a mobile phase. This mobile phase was used for preparation of standard solutions and analysis. Accurately weighed 100mg of BSA was dissolved in sufficient mobile phase to produce 100mL of total volume. The resultant solution was sonicated for 20min. After sonication, it is filtered through Whatman filter paper and used thereafter for analysis.

2.2.3 Fractionation of Plasma [18]

Accurately weighed 1.34g of potassium dihydrogen orthophosphate dissolved in sufficient water to produce 100mL of total volume. Its pH was adjusted to 6.6 using hydrochloric acid and ammonia. This solution was then sonicated for 15 min. This solution was then used as a mobile phase. To 200µL of plasma, 400µL of acetonitrile was added with gentle mixing. The mixtures were kept at room temperature for 30 min. and then vortexed for 5 min. at 6000 rpm. The supernatants were removed via pipette (except for the lowest 10-20µL) and the volumes reduced to 50µL in a SpeedVac. The resulting samples were reconstituted to the original concentration in serum by addition of 150µL of mobile phase.

2.2.4 Calibration Curve of BSA

Different dilutions of BSA i.e. 50, 75, 100, 150, 200µg/mL were prepared by using the standard stock solution of BSA in mobile phase. These solutions were then injected into WATER GPC system and chromatograms were recorded. The BSA showed linearity over concentration range of 50-200µg/mL. The results of calibration curve are shown in Table 3 and Figure 3.

2.2.5 Analysis of HSA from Fractionated Plasma

The fractionated plasma was taken as such for analysis. This solution was then injected into WATER GPC system and chromatogram was recorded. Figure 3 shows chromatogram of all fractionated proteins in plasma with HSA at same retention time as that of BSA.

2.2.6 Chromatographic estimation of fractionated plasma

A 15µl of reconstituted plasma solution was injected into the injection port of chromatographic system having fixed volume loop injector. Chromatograms were noted and percent relative standard deviation was calculated shown in table 3 and 4 and chromatogram showed in figure 3.

Table 4. % of R. S. D. of fractionated plasma (plasma: ACN, 200:200 µl).

Analyte	% R.S.D
Albumin	3.495
Ig A	7.207
Ig M	5.752
Transferrin	1.948
α1-Antitrypsin	2.946
α1-Lactalbumin	2.647

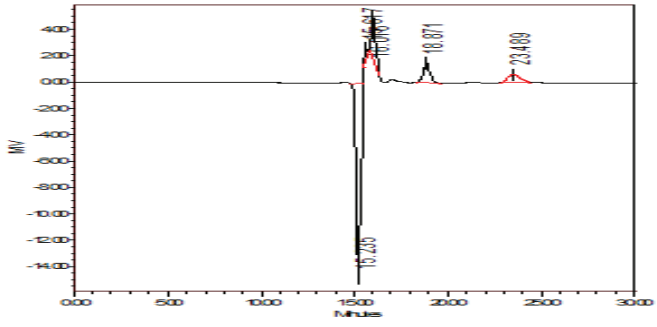

Figure 3. Chromatogram of fractionated plasma

Table 3. Different chromatographic parameters of fractionated plasma (plasma: ACN 200:200µl).

Name of proteins	RT (min)	Area (µV* sec)	% Area	Height	% Height	Width (sec.)
Albumin	14.879	122941	88.55	-67220	91.34	76
Ig A	15.625	59943	0.12	2713	3.07	54
Ig M	16.631	2141	88.55	104	0.12	43
Transferrin	17.836	13477	3.17	4333	4.90	90
α-1 Antitrypsin	21.017	2341	0.11	43	0.05	120
α - Lactalbumin	22.431	12830	7.14	494	0.45	98

3. Drug protein binding study using GPC

3.1. Optimization of mobile phase

From the literature survey, mobile phase compositions were selected. Finally, mobile phase containing 100Mm KH_2PO_4 was found to give best resolution. Before analysis, the mobile phase was filtered through a 0.45µ nylon filter and then degassed ultrasonically for 15 min.

3.2. Preparation of 100 Mm KH_2PO_4

6.8gm of accurately weighed KH_2PO_4 was taken in 500ml volumetric flask. The volume was adjusted to 500ml using filtered double distilled water or HPLC grade water. The pH of the solution was adjusted to 6.6. The resultant solution then sonicated for 15 -20 min.

3.3. Preparation of drug plasma complex:

3.3.1. Preparation of Ketoprofen, Tapentadol and Furaltadone plasma complex

1mg of accurately weighed Ketoprofen, Tapentadol and Furaltadone was dissolved to 10ml of filtered double distilled water or HPLC grade water. To this sample solution, 100µl of human plasma was added. The resultant mixture was then mixed thoroughly by moving it clockwise and anticlockwise direction. 1ml of ACN was then added to above solution. The resultant solution was then filtered through Whatman filter paper.

3.3.1.1. Chromatographic estimation of Ketoprofen, Tapentadol and Furaltadone plasma complex

A 15µl of above plasma-Ketoprofen solution was injected into the injection port of chromatographic system having fixed volume loop injector. Chromatograms was plotted on gel permeation chromatography. Table 5 shows Different Chromatographic parameters of Ketoprofen, Tapentadol and Furaltadone (100µg/ml)-plasma complex. Table 6 shows AUC of fractionated protein and Ketoprofen, Tapentadol and Furaltadone (150-300µg/ml)–protein complex. Table 7 shows Percent increase in AUC of Ketoprofen, Tapentadol and Furaltadone (150-300µg/ml) - protein complex. While chromatograms are shown in Figures 4, 5 and 6.

3.3.1.2. Determination of % increase in AUC of Ketoprofen, Tapentadol and Furaltadone (100-300µg/ml)-plasma complex

As the concentration Ketoprofen increases there is increase in the binding of drug to Albumin. It means that concentration of Ketoprofen is directly proportional to the albumin binding at least up to 300µg/ml. After the saturation of albumin binding sites, binding of Tapentadol with IgM is effective up to 200µg/ml. Further increase in the drug concentration does not affect the Ig M binding of drug. The increase in the concentration of Ketoprofen leads to binding with transferrin up to at least 300µg/ml of drug concentration. The statistical data obtained after replicate determinations (n = 9) is shown in respective tables.

Table 5. Different chromatographic parameters of Ketoprofen, Tapentadol and Furaltadone (100µg/ml)- plasma complex.

Analyte	RT (min.)	Area	% Area	Height	% Height	Width
Ketoprofen						
Albumin	15.75	819789	92.89	-11746	95.42	186
Ig M	16.62	264730	3.87	-189	3.18	104
Transferrin	18.75	24955	2.46	195	2.50	97
Tapentadol						
Albumin	15.50	950462	100	-15741	100	241
Furaltadone						
Albumin	16.27	255138	71.76	-8854	78.05	75
Ig M	17.19	51962	14.62	-1627	14.35	84
Transferrin	19.91	48428	13.62	862	7.60	118

Table 6. AUC of fractionated protein and Ketoprofen, Tapentadol and Furaltadone (150-300 µg/ml)–protein complex.

Analyte	Area of fractionated protein	Area of Keto. – protein complex 100 µg/ml	Area of Keto. – protein complex 150 µg/ml	Area of Keto. – protein complex 200 µg/ml	Area of Keto. – protein complex 250 µg/ml	Area of Keto. – protein complex 300 µg/ml
Ketoprofen						
Ig G	2192	-	-	-	-	-
Albumin	124491	859789	949752	899307	1142572	1490381
Ig A	60135	-	-	-	-	-
Ig M	1958	264730	61344	804510	347964	91429
Transferrin	12793	24955	34011	39079	41560	43891
α1-Antitrypsin	3052	-	-	-	-	-
α1-Lactalbumin	13750	-	-	-	-	-
Tapentadol						
Ig G	2192	-	-	-	-	-
Albumin	124491	910985	1248548	1252889	1329874	930563
Ig A	60135	-	-	-	-	-
Ig M	1958	-	-	-	-	69402
Transferrin	12793	-	-	40924	49975	72389
α1-Antitrypsin	3052	-	-	-	-	-
α1-Lactalbumin	13750	-	-	-	-	-
Furaltadone						
Ig G	2192	-	-	-	-	-
Albumin	124491	284813	413064	542498	542365	542485
Ig A	60135	-	-	-	-	-
Ig M	1958	56845	65689	87671	91210	91210
Transferrin	12793	47218	96703	135602	221437	383075
α1- Antitrypsin	3052	-	-	-	-	-
α1- Lactalbumin	13750	-	-	-	-	-

Table 7. Percent increase in AUC of Ketoprofen, Tapentadol and Furaltadone- protein complex.

Analyte	% increase in AUC at 100 µg/ml	% increase in AUC at 150 µg/ml	% increase in AUC at 200 µg/ml	% increase in AUC at 250 µg/ml	% increase in AUC at 300 µg/ml
Ketoprofen					
Ig G	-	-	-	-	-
Albumin	84.25	87.36	84.90	88.95	92.78
Ig A	-	-	-	-	-
Ig M	98.94	95.41	99.80	99.08	96.38
Transferrin	52.30	54.04	56.90	58.96	72.69
α1- Antitrypsin	-	-	-	-	-
α1- Lactalbumin	-	-	-	-	-
Tapentadol					
Ig G	-	-	-	-	-
Albumin	83.95	89.15	89.71	90.07	85.89
Ig A	-	-	-	-	-
Ig M	-	-	-	-	97.53
Transferrin	-	-	66.74	71.85	79.92
α1-Antitrypsin	-	-	-	-	-
α1-Lactalbumin	-	-	-	-	-
Furaltadone					
Ig G	-	-	-	-	-
Albumin	54.20	71.13	75.04	74.83	75.03
Ig A	-	-	-	-	-
Ig M	94.08	96.59	97.60	97.81	97.81
Transferrin	28.04	64.50	74.92	85.24	91.89
α1-Antitrypsin	-	-	-	-	-
α1-Lactalbumin	-	-	-	-	-

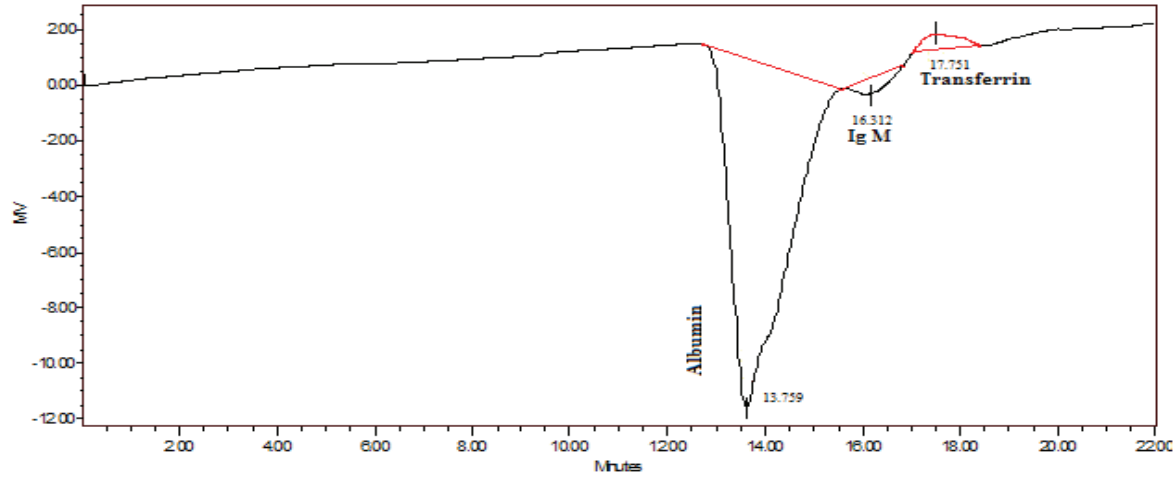

Figure 4. Chromatogram of Ketoprofen (100µg/ml)-plasma complex.

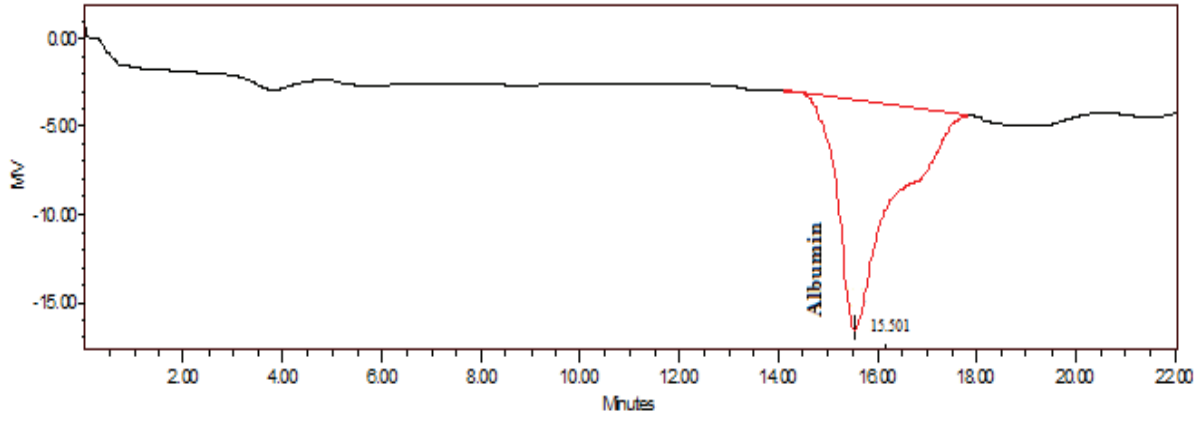

Figure 5. Chromatogram of Tapentadol-plasma complex at 100µg/ml.

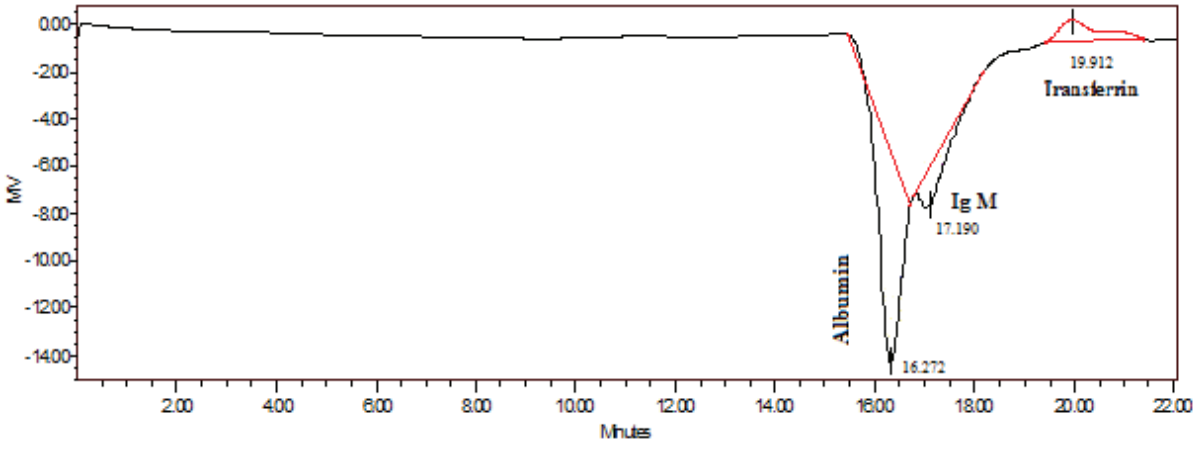

Figure 6. Chromatogram of Furaltadone (100µg/ml) -plasma complex.

4. Validation of developed methods by USFDA guidelines

The proposed gel permeation chromatography method was validated by guidelines given by USFDA.

4.1. Selectivity

Separate specificity, in relation to endogenous plasma components, was validated by analysis of series of samples without drug. The retention times for investigated Ketoprofen, Tapentadol and Furaltadone were found to be different than that of plasma components. Hence, it shows selectivity of method for elution of these three drugs in plasma.

4.2. Precision Study

The precision study was carried out for three samples of drugs with three concentrations asper specified in accuracy study for three successive days at two different times. The intra-day and inter-day precision study was carried out. The prepared plasma-drug samples of analytes were stored in deep freezer at about -17°C in between analysis. The results precision study are shown in Table 8.

4.3. Extraction Recovery Study

This study represents the efficacy of an analytical method in the variant limit. Recovery is the response of detector obtained from an amount of analyte added and recovered from the sample. The results of recovery study were compared with analytical results for extracted samples at three different concentrations which were equivalent to LLOQ, MQC and HQC and with three unextracted concentrations that represent 100% recovery. The % recovery was calculated by formula,

% recovery= (Mean response of extracted samples/Mean response of unextracted samples) X 100. [21] Results are shown in Table 9.

Table 8. Precision results analytes

Analyte	Precision		Drug sample concentration (µg)	% Conc. Found Mean[1] ± SD[2]
Ketoprofen	Inter-Day	D1	50	89.36±1.352
		D2	100	90.12±3.124
		D3	150	91.45±1.247
	Intra-Day	T1	50	89.24±5.451
		T2	100	89.87±4.325
		T3	150	91.72±1.244
Tapentadol	Inter-Day	D1	50	88.24±3.245
		D2	100	88.94±1.642
		D3	150	89.36±7.124
	Intra-Day	T1	50	89.01±3.210
		T2	100	89.79±1.024
		T3	150	90.78±2.427
Furaltadone	Inter-Day	D1	50	90.12±4.012
		D2	100	89.45±3.412
		D3	150	88.91±4.157
	Intra-Day	T1	50	91.02±5.621
		T2	100	89.76±1.356
		T3	150	89.01±2.157

[1]Average calculated at three levels 3X5 of fifteen determinations; [2]Standard deviation; D= Day; T= Time

Table 9. Extraction recovery study and quality control sample analysis

Drug Sample	Conc. (µg/ml)	% Conc. Estimated Mean[A]±SD[B]	% Recovery Estimated Mean[C]±SD[B]
Ketoprofen	50	86.26±2.141	87.12±1.254
	150	86.91±1.245	87.84±1.201
	300	87.12±1.341	88.74±1.347
Tapentadol	50	87.12±1.371	88.47±2.047
	150	87.98±1.240	89.14±2.347
	300	88.75±1.025	90.12±1.035
Furaltadone	50	86.78±2.140	88.01±2.304
	150	87.54±1.031	88.79±1.670
	300	88.34±1.672	89.38±2.457

[A]Average of five determinations; [B] Standard Deviation; [C] Average of four determinations

4.4. Linearity and Range Study

The response factors were calculated and found to be directly proportional to concentration of analytes over the range of 100-600µg/ml. Study was carried out for three successive days. The calibration curve was plotted by least square method. Table 10 shows the results of linearity study by regression analysis.

Table 10: Results of linearity study for plasma analysis

Parameters	Ketoprofen	Tapentadol	Furaltadone
Range µg/ml	100-600	100-600	100-600
Regression Eq^{n}.		Y=A+B*C	
Intercept (A)	5.247×10^{-2}	9.897×10^{-2}	4.687×10^{-2}
Slope (B)	3.342×10^{-3}	2.174×10^{-3}	4.385×10^{-3}
SE[1] of Intercept	4.137×10^{-4}	2.651×10^{-4}	5.398×10^{-4}
SE[1] of Slope	3.175×10^{-5}	1.025×10^{-5}	3.387×10^{-5}
Correlation coefficient (r)	0.9985	0.9992	0.9994

Y= Unit of response factor; C= Conc. in µg/ml; [1]Standard error

4.5. Lower Limit of Quantitation (LLOQ)

The lowest serum concentrations were quantified with coefficient of variation which was less than 20%. The LLOQ values for Ketoprofen, Tapentadol and Furaltadone were found to be 50, 50 and 25µ/ml, respectively.

4.6. Stability study

This study was carried out at different time and atmospheric conditions for analysis of stock samples of unextracted plasma, newly produced samples. The study was carried out asper the guidelines by recording 6 readings mean and their standard deviation [22].

4.6.1. Stock Solution Stability Study

The prepared stock solutions were stored at -17°C for about 3 days. After 3rd day analysis of samples were done and compared with newly prepared stock samples. It was found that good recovery for Ketoprofen, Tapentadol and Furaltadone samples 99.65±1.24; 99.04±1.38 and 98.95±1.305 separately.

4.6.2. Freeze and Thaw Stability Study

This study was carried out by exposing the samples of analyte at high, medium and low temperatures to know the effect of freeze and thaw cycles on the stability of analyte samples. Samples were analyzed after completion of each cycle and compared with newly prepared samples for experimental concentrations. The % recovery for Ketoprofen, Tapentadol and Furaltadone samples was found to be 98.76±1.24; 99.24±1.15 and 99.41±1.34 respectively.

4.6.3. Short-Term Stability Study

For determination of short-term stability samples were stored at ambient temperature (20°C) for 12hrs. Samples of low, mid and high concentration were used to determine short-term stability in plasma. After 12hrs. Samples were analyzed and compared with freshly prepared samples. The % recovery of Ketoprofen, Tapentadol and Furaltadone samples was found to be 99.86±3.24; 100.21±3.12 and 99.96±2.034 respectively.

4.6.4. Post-Preparative Stability Study

This study was carried out by keeping low, mid and high concentration samples at 4°C about 4hrs. After 4hrs. Samples were analyzed and compared with the newly prepared samples. It was found that good % recovery of samples 100.34±2.014; 99.98.79±1.36 and 100.45±3.247 for Ketoprofen, Tapentadol and Furaltadone respectively.

5. Result and Discussion

The binding of drugs to plasma protein especially albumin and α-glycoprotein is one of factor that influences drug disposition. The activity and toxicity of drug are related to unbound concentration of drug rather than total concentration hence it becomes compulsory to determine plasma protein bound concentration of drugs [3]. For this purpose, there should be method that will completely resolve all plasma proteins and estimation of bound drug with individual plasma protein mostly HSA will be possible.

The separation, identification and quantification of HSA will be crucial due to difficulties in purification and high cost of HSA. Hence in present communication, we have developed method using BSA as it can be used as standard for gel permeation chromatography due to its stability, its lack of effect in many biochemical reactions, and its low cost. The compositions of two proteins are very similar. The HSA contains 609 amino acids in sequence with only 585 amino acids in the final product observed in human blood whereas BSA contains 607 amino acids in sequence with only 585 amino acids in the final product observed in the

cow blood. They have only one difference as BSA has two tryptophan residues whereas HSA has only one. This change in structure is responsible for having only difference in spectrofluorometric properties, not other physicochemical properties. Hence its use will not make any difference as a standard in plasma protein binding studies or protein assay.

The methods reported for estimation of HSA have utilized SDS-PAGE, Electrophoresis, Ultrafiltration, Equilibrium Dialysis and HPLC (3, 4, 5, 6, 10, 11, 15, and 20). But these methods were complex as compared to GPC. In present communication, GPC method has been developed and it has been validated for estimation of HSA obtained from human plasma. The method has been optimized on Ultrahydrogel TM 500 column with varying concentrations of sodium nitrate in water but finally, effective resolution has been obtained with 0.1 % sodium nitrate with 20 µL of injection volume and at flow rate of 0.7 mL/min. The resolution has been achieved on saccharide column i.e. Ultrahydrogel whereas previously reported methods were found to resolve all plasma proteins on peptide columns but all peaks are not properly resolved compared to reported method. The calibration curve has been plotted using standard BSA concentrations in range of 50-200µg/mL. The human plasma has been obtained from blood bank and it has been fractioned as specified in above procedure. The separation of all plasma proteins has been observed as shown in chromatogram (Figure 4). The peak of HSA was observed at retention time 15.23 in fractionated plasma. It has been confirmed as area of this peak was highest within all peaks area in fractioned plasma and it has been retained at same retention time as that of BSA. Thus, this method is useful for estimation of HSA in free or bound form.

The drugs selected for protein binding studies were Ketoprofen (anti-inflammatory), Tapentadol (narcotic analgesics) and Furaltadone (anti-infective). Ketoprofen and Furaltadone bind with albumin, IgM and transferrin while Tapentadol binds only with albumin fractions of plasma proteins. UV- Spectrophotometric study of the above mentioned drug Ketoprofen along with albumin was also recorded and it shows good correlation. Proteins from the human and other than human sources were also studied by using GPC. Chromatogram of all these proteins shows satisfactory separation according to its molecular weight.

6. Conclusion

In this work, development of an *in-vitro* model for protein binding studies of some chemotherapeutic agents have been developed. The developed method is simple, accurate, precise, sensitive, robust and selective for estimation of HSA in its free and bound form present in purified or fractionated plasma samples and also address following analytical issues related to drug-protein binding and validation of above developed method. Thus this method has its application in plasma protein binding, biopharmaceutical and pharmacokinetic studies of various types of drugs.

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