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Original Article

Novel SPE-HPLC Method for Analyses of β-Blockers in Human Plasma Using New Generation Phenyl-Ethyl Column

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ABSTRACT

Objectives: The mixture of six β-blockers was analyzed in human plasma by SPE and HPLC methods.
Methods: Timolol, atenolol, oxprenolol, alprenolol, acebutolol and carazolol were separated using Phenyl-Ethyl column (PhE, 250 x 4.6 mm, 5.0 µm). The mobile phase was phosphate buffer (50 mM, pH 3.0)-acetonitrile (70:30, v/v) at 1.0 mL/min flow rate. The detection was set at 220 nm and 27±1ºC temperature.
Results: The values of chromatographic parameters i.e. capacity (k), separation (α) and resolution factor (R*s*) were in the range of 2.76-17.72, 1.14-1.82 and 2.11-6.64, respectively. The limits of detection and quantification were 0.1-0.5 µg/mL and 0.6-0.30 µg/mL, respectively. The percentage recoveries of extraction were in the range of 32.30- 50.5%. π-π, hydrophobic and polar interactions between β-blockers and phenyl-ethyl column were responsible for the separation. The reported SPE and HPLC methods were efficient, reproducible, fast and selective. The developed and validated methods were applied for monitoring six β-blockers in human plasma. Briefly, the developed SPE and HPLC methods can be applied to monitor the reported β-blockers in any sample of biological, environmental and industrial origins.

Keywords: β-Blockers, SPE-HPLC, Phenyl-ethyl column, Human plasma, Mechanism of separation.

INTRODUCTION

Cardiovascular diseases are major causes of mortalities in the world. Cardiovascular patients may increase up to 1.56 billion by the end of 2025 globally[1]. β-Adrenergic blockers are the most recommended and prescribed drugs for the

treatment of cardiovascular diseases[2-6]. Timolol is used for the treatment of glaucoma, while atenolol is useful for the prevention of supraventricular arrhythmias[7,8]. Oxprenolol, alprenolol and acebutolol are used in the treatment of cardiac arrhythmias[9-11]. Carazolol is mostly used in veterinary sciences for artificial insemination[12]. These are considered to be the most reliable medications for cardiovascular diseases but get accumulated into body; leading to toxic adverse effects and other problems. It has been reported that long term consumption of propranolol causes diabetes[13-15]. Therefore, there is a great need for the analyses of β-blockers in human plasma with low limits of detection. Besides, pharmacokinetic and pharmacodynamic studies are also important for safety profiles. Additionally, the assessment of the potential toxic effects of drug metabolites in human beings is also required[16,17]. The analyses in biological matrices such as plasma, tissues, serum, urine are useful for clinical trials and other studies[18]. Among various analytical techniques, HPLC is considered as the best one due to its efficiency, ease of operation, rapidness, selectivity and reproducibility[19-21].

A thorough search of literature of β-blockers analyses indicated that the reported methods involve costly and hazardous solvents with moderate limits of detection[22-27]. Furthermore, it was observed that the reported methods describe moderate HPLC separation of β-blockers. Besides, most of the methods utilized traditional sample treatment methods i.e. liquid-liquid extraction. In view of these facts, there is a great need of economically and environmentally viable SPE (solid phase extraction) and high performance liquid chromatography (HPLC) methods. Certainly, such methods will reduce the economic pressure faced by the pharmaceutical industries and research laboratories for β-blockers development and analyses. Prior to HPLC analysis, sample preparation is required to remove the impurities present with the desired analytes in the biological matrices[28,29]. SPE has the merits of less organic solvents consumption, high extraction recoveries and simultaneous extraction[30]. Literature indicates some papers dealing with SPE of β-blockers. These methods employed methanol (0.1% TFA), methanol (0.3% 2-aminoheptane), methanol (0.1% propylamine) etc. as eluents. Flow rate ranged from 0.1-1.5 mL/min. While, the percentage recoveries ranged from 31.80-95.5%[31,32]. Besides, these reported SPE methods show certain limitations such as use of corrosive TFA and long run times.

Phenyl-ethyl column (PhE sunniest) of Chromanik, Japan, was used due to its different selectivities towards β-blockers. Phenyl-ethyl column has capabilities of π-π interactions, hydrogen bonding, hydrophobic interactions *via* aromatic moieties, cations and unsaturated bonds[33]; resulting into good separation. Considering the above facts, the attempts have been made to develop efficient, fast, reproducible SPE and HPLC methods with low detection limits. The developed SPE and HPLC methods were applied for the simultaneous analysis of six β-blockers in human plasma. The results of these findings are given herein.

EXPERIMENTAL

Chemicals and reagents

β-Blockers such as acebutolol, alprenolol, timolol, carazolol, oxprenolol, atenolol (**Figure 1**) were purchased from Sigma Chemical Co., Milky Way, USA. The standard solution (1.0 mg/mL) of each β-blocker was prepared in methanol. Acetonitrile, ethanol and methanol of HPLC grades, sodium dihydrogen phosphate

($NaH_2PO_4.2H_2O$) and orthophosphoric acid of AR grade were supplied by Merck, Mumbai, India. Frozen Human Plasma (Mfg. License no. 504) was purchased from Rotary Blood Bank, New Delhi, India.

Instrumentation

Solid phase extraction of the samples was carried out using solid phase extraction unit, purchased from Varian, USA. C_{18} cartridges were purchased from Waters, Milky Way, USA. The analyses were carried out on HPLC system (ECOM, Prague, Czech Republic) with solvent delivery pump (model Alpha 10), manual injector, UV detector (Indtech Instrument, Mumbai, India) and Winchrome software. pH meter of Control Dynamics (Model APX175 E/C), centrifuge of Remi (model C-30BL) was used. Deionized water was prepared using Millipore-Q, Bedford, MA, USA system.

Solid phase extraction (SPE)

Sample preparation was carried out by mixing 1.0 mL (1.0 mg/mL) of six β-blockers with fresh frozen human plasma (5.0 mL). The spiked plasma samples were kept for 30.0 min. After vortexing for 2.0 min, 25.0 mL acetone was added to sample and again kept for 30.0 min. The sample was centrifuged for 10.0 min at 10,000 rpm to separate the supernatant. The supernatant obtained was evaporated to dryness under vacuum. The residue obtained was re-dissolved in 10.0 mL phosphate buffer (25 mM, pH 9.0). Sep-Pac C_{18} cartridge (1.0 mL, Waters, Milky Way, USA) was pre-conditioned first with 5.0 mL methanol followed by 5.0 mL Millipore water. 10.0 mL Phosphate buffer containing β-blockers was loaded onto the C_{18} cartridges and passed at 0.1 mL/min flow-rate. At the same flow-rate, cartridges were washed with 2.0 mL Millipore water. After drying cartridges with hot air, β-blockers were eluted with 10.0 mL methanol containing 0.1% acetic acid at the same flow rate. Prior to its injection onto HPLC, methanolic solution of β-blocker was reduced to 0.5 mL under vaccum.

High performance liquid chromatography

HPLC analyses of six β-blockers were carried out on HPLC instrument as described above. The standard solution (1.0 mg/mL) of each β-blocker as individual and mixture was prepared in methanol. The mixture of β-blockers of the same concentration was used for the further experiment. 5.0 µL of these samples was injected onto the HPLC system. The mobile phase used was phosphate buffer (50 mM, pH 3.0)-acetonitrile (70:30, v/v). It was filtered and degassed daily prior to use in HPLC. The flow rate was 1.0 mL/min with detection at 220 nm and 27±1° C temperature. The capacity (k), separation (α) and resolution factors (R_s) were calculated. The limits of detection and quantifications were obtained as per standard protocol[34].

RESULTS AND DISCUSSION

Solid phase extraction

Solid phase extraction of all six β-blockers was performed as per the standard method. The blank experiments were performed to obtain the percentage recoveries of these β-blockers. The percentage recoveries of timolol, alprenolol, atenolol, carazolol, acebutolol and oxprenolol were 50.5, 45.0, 40.5, 39.0, 38.6 and 32.3%, respectively. All six β-blockers extracted from human plasma were identified with the retention times of their standards. The peak areas were compared with those of the standards for the quantitative estimation. The absence of any extra peak indicated SPE method as selective. To obtain maximum percentage recoveries, optimization of the various parameters was performed as given below.

Optimization

The different experimental conditions such as concentration of phosphate buffer, pH of buffer, flow rates of buffer and eluting solvents and various eluting solvents were varied. The various solvents such as acetone, ethylacetate, ethanol, dichloromethane, methanol and methanol with acetic acid (0.1%) were tried. Besides, low amounts of tetra-flouro acetic acid (TFA) and acetic acid were also added to the eluting solvent to achieve maximum percentage recoveries. The results are discussed in the following sub-sections.

Effect of phosphate buffer concentration

Optimization of solid phase extraction was performed by varying phosphate buffer concentration. Phosphate buffer concentrations were ranged from 10-35 mM. It was observed that the percentage recoveries increased by increasing buffer concentration up to 25 mM. Furthermore, no increase in percentage recoveries was observed by increasing buffer concentration (30 and 35 mM). This was due to the fact that 25 mM created the suitable ionic environment for maximum adsorption of the reported β-blockers on C_{18} material.

Effect of buffer pH

For the extraction of the desired analyte, solid phase extraction involves the adsorption of the compounds onto the C_{18} cartridge. The same is true for β-blockers. The structures of the reported molecules, types of the buffers and pHs of the buffers govern adsorption onto the sorbent. The ionic nature of these β-blockers depends on pH value. Therefore, the extent of binding and retention are governed by pH of buffer. pH values were varied from 4.0-10.0 (25 mM). The percentage recoveries increased on increasing pH of the phosphate buffer (4.0 to 9.0). However, further increase in pH to 10.0 did not increase extraction recoveries. The range of percentage recoveries was 32.30-50.50%. β-Blockers contain various functional groups such as hydroxyl, amine, oxide, etc, which form hydrogen bonds with the material of C_{18} cartridge. Low percentage recoveries at pH 2.0-8.0 might be due to poor hydrogen bondings among β-blockers and C_{18} cartridge materials. The maximum percentage recoveries at pH 9.0 might be due to stronger hydrogen bondings. This is due to the fact that the functional groups of the reported molecules are capable to form stronger hydrogen bonds at pH 9.0.

Effect of buffer flow rate

The maximum percentage recoveries were obtained by varying flow rates of buffer. Low percentage recoveries were observed at high flow rate and vice versa. Optimization of this parameter was carried out by varying flow rate at 0.1 to 1.0 mL/min. The percentage recoveries decreased at flow rate 0.1 to 1.0 mL/min in the order of 0.1> 0.2 > 0.3 > 0.4 > 0.5 > 0.6 > 0.7 > 0.8 > 0.9 > 1.0 mL/min. The percentage recoveries at 0.8, 0.9 and 1.0 mL/min were not satisfactory. Moreover, some plasma impurities along with the analytes were also eluted at high flow rates. The maximum percentage recoveries of all six β-blockers were observed at flow rate 0.1 mL/min. Therefore, 0.1 mL/min was selected as the best flow rate.

Effect of other solvents

Optimization was also performed by varying eluting solvents such as acetone, ethylacetate, ethanol, dichloromethane, methanol and methanol with acetic acid (0.1%). Besides, acetic acid and trifluoroacetic acid (0.1-0.2%) were added in these solvents to extract all six β-blockers from C_{18} cartridge. The order of percentage recoveries obtained from these solvents was methanol (acetic acid, 0.1%) > methanol >

dichloromethane > ethanol > ethylacetate > acetone. After extensive experimentation, it was concluded that the maximum percentage recoveries were obtained using methanol with acetic acid (0.1%) while minimum recoveries were obtained with acetone. Methanol has high polarity and good dielectric constant values, which are responsible for easy desorption of β-blockers from C_{18} cartridge. However, the addition of acetic acid provided more polarity to methanol; resulting into the maximum desorption of these molecules. Dichloromethane also yielded high recoveries but avoided due to its volatile nature. Other solvents such as ethanol, ethyl acetate and acetone yielded low percentage recoveries of all six β-blockers. The poor dielectric constants and high polarities of these solvents could not dissociate the bond between β-blockers and C_{18} cartridge. Therefore, methanol (0.1% acetic acid) was selected as the best eluting solvent.

Effect of eluting solvent flow rate

Flow rate of the eluting solvents is another optimization parameter. It was observed that high flow rate gave poor percentage recoveries. On the other hand, maximum recoveries were obtained at low flow rates. Therefore, all six β-blockers were passed through C_{18} cartridge at flow rate 0.1-1.0 mL/min. It was observed that there was a gradual decrease in the recoveries of β-blockers at flow rate 0.1-1.0 mL/min. This was due to incomplete desorption of β-blockers from C_{18} cartridge in eluent passed at high flow rate. At low flow rates eluent slowly passed through the cartridge desorbing β-blockers effectively. Hence, maximum recoveries were obtained at low flow rates. The percentage recoveries of all six β-blockers decreased from 0.1-1.0 mL/min. Therefore, 0.1 mL/min was selected as the best flow rate. After extensive experimentation, the best results were obtained. The maximum percentage recoveries of these β-blockers were obtained with phosphate buffer (25 mM, pH 9.0), methanol (0.1% acetic acid) as eluting solvent and 0.1 mL/min flow rates of buffer and eluting solvent.

High performance liquid chromatography

The various chromatographic parameters such as retention (k), separation (α) and resolution (R_s) factors were calculated for all six β-blockers (acebutolol, alprenolol, timolol, carazolol, oxprenolol, atenolol). The values of chromatographic parameters are listed in **Table 1**. The values of k ranged from 2.76-17.72. The values of α for timolol-atenolol, atenolol-oxprenolol, oxprenolol-alprenolol, alprenolol-acebutolol, acebutolol-carazolol were 1.8, 1.82, 1.33, 1.14 and 1.29, respectively. R_s values were 3.48, 6.64, 4.11, 2.11, 6.07. The values of α and R_s were greater than one, indicating complete separation **(Figure 2).** The order of elution was carazolol > acebutolol > alprenolol > oxprenolol > atenolol > timolol as ascertained by running experiments under identical conditions.

Optimization

The optimization was performed by varying various chromatographic parameters. The different variations carried out were content of acetonitrile in the mobile phase, flow rate of mobile phase, pH of buffer (mobile phase) and detection wavelengths. Besides, other buffers such as acetate, ammonia, citrate etc. were tried. The organic modifiers tested were methanol, ethanol and isopropanol. An exhaustive experimentation resulted into the best optimized results reported in this paper. The effects of various optimized parameters are discussed in the following sub-sections.

Effect of acetonitrile concentration

Acetonitrile concentration in the mobile phase is an important constituent, which was altered to optimize the chromatographic separation. Various combinations of mobile phase of phosphate buffer (50 mM, pH3.0)-acetonitrile were tried. To get the best results the percentage of acetonitrile varied from 20-80%. The values of k decreased on increasing the concentration of acetonitrile in mobile phase. At 10% acetonitrile concentration, all six β-blockers were separated but the peaks were broad and not well resolved. On increasing acetonitrile concentration to 20%, separation of β-blockers increased. Further increase in acetonitrile content (30 mL) resulted into sharp and well resolved peaks. It was observed that on further increasing the concentration of acetonitrile the peaks approached closer and merged into one another (difficult to separate). At 40% acetonitrile concentration there was partial separation between two peaks (alprenolol and acebutolol; retention times 14.56 and 16.01 min). At concentration 50, 60, 70, 80% only five, four, three and two peaks were observed, respectively. The values of α and Rs for all six β-blockers showed the best separation at 30% acetonitrile.

Effect of mobile phase pH

To optimize the results mobile phase pH was varied at pH 2.0, 3.0, 5.0, 7.0 and 9.0. The values of k increased from pH 2.0 to pH 9.0 but peaks were broad at pH 4.0-9.0. The separation of all six β-blockers was observed at all pHs. But at pH values 7.0 and 9.0, the peaks were broad and less resolved. Moreover, it was observed that at pH 7.0 only four peaks (timolol, atenolol, oxprenolol and carazolol) were observed with partial resolution between two peaks (alprenolol and acebutolol; at retention times 17.14 and 18.82 min). At pH 9.0, the resolution between two peaks (alprenolol and acebutolol) decreased with retention times 18.17 and 19.64 min. The values of α was maximum at pH 9.0. Furthermore, it was observed that Rs values decreased from pH 2.0 to 9.0 showing maximum resolution at pH 2.0. But high acidic conditions (pH 2.0) damaged the column, and, hence pH 3.0 was considered as the best one.

Effect of mobile phase flow rate

The optimization was performed by varying the flow rates of the mobile phase. The changes in the retention times (t_r) of β-blockers were noted at flow rates 0.5, 1.0, 1.5 and 2.0 mL/min. There was a decrease in the retention times of all six β-blockers with increase in the flow rate. At 0.5 mL/min flow rate, all six β-blockers were separated but the peaks were broad. At 1.0 mL/min the peaks were well resolved, narrow and base lined. There was partial resolution between two peaks (alprenolol and acebutolol) at 1.5 mL/min. Moreover, at 2.0 mL/min flow rate two peaks (alprenolol and acebutolol) merged and only five appeared. The maximum values were observed at 1.0 mL/min except for alprenolol-acebutolol. Besides, the maximum values of Rs. were observed at 1.0 mL/min flow rate. Therefore, 1.0 mL/min was the best flow rate for the complete separation of all the six β-blockers.

Effect of wavelength

Optimization was also performed by varying the detection wavelengths from 200 to 280 nm. The experimentation was performed at UV detection wavelength 200, 220, 240, 260 and 280 nm. The values of the limits of detection and quantification were calculated. The limits of detection and quantification obtained at detection wavelength 200, 240, 260 and 280 nm were high. On the other hand, low limits of detection and quantification were observed at 220 nm detection wavelength. The limits

of detection of these molecules were in the range of 0.1 to 0.5 µg/mL. The limits of quantization were of 0.6-0.30 µg/mL. Therefore, 220 nm was considered as the best detection wavelength.

Validation of the methods

The developed method was validated by carrying out five set (n = 5) of SPE and HPLC procedures under identical experimental conditions. The concentrations of these β-blockers (0.5, 1.0 and 1.5 mg/mL) were used to validate the method. The linearity was confirmed by least squares linear regression analysis of calibration curve[35]. The calibration curves were constructed and the linearity was in the range of 01.-2.5 mg/mL concentrations. Microsoft excel program was used for the regression analysis. The regression analysis data for SPE of all six β-blockers are given in **Table 2**. The standard deviation (SD), correlation coefficients (r^2) and confidence level (%) ranged from ±0.06-0.09, 0.9997-0.9999 and 99.6-99.82%. On the other hand, **Table 3** shows the precision data of HPLC analyses. The values of standard deviation, correlation coefficients and confidence level were in the range of ±0.04-0.05, 0.9996-0.9999 and 98.72-99.52, respectively. As determined by the least square analysis, the correlation coefficient for calibration curves were higher than 0.999. Lower limit of detection (LOD) for all the six β-blockers were in the range of 0.1-0.5 µg/mL.

Mechanism of separation

The order of elution found was carazolol > acebutolol > alprenolol > oxprenolol > atenolol > timolol. This order of elution can be explained by supramolecular interactions occurring between these drugs and the phenyl group of the stationary phase. These drugs **(Figure 1)** have aromatic rings, unsaturated bonds, amine, amide and hydroxyl groups; responsible for various interactions with the phenyl groups present in the stationary phase (PhE)[36]. The various interactions shown by these groups are aromatic π-π interactions (A), aromatic-unsaturated bond π interactions (B), aromatic-amide π-π interactions (C), cationic-π interactions due to quaternary amine (D) and cationic-π interactions due to protonated hydroxyl groups (E)[37-43]. The strength of these interactions are in the order of A > B > C > D > E. Under the experimental conditions, amine and hydroxyl groups were protonated providing surface for cationic-π interactions.

Timolol eluted earlier than atenolol due to poor interactions in the former. Timolol has only A and D interactions while atenolol exhibited A, C, D and E types of interactions. Atenolol was eluted followed by oxprenolol due to stronger bonding in the later. Oxprenolol had A, B, D and E interactions, which are stronger than the bondings in atenolol. Of course, both β-blockers had four interactions but interaction B is stronger than C. Oxprenolol was eluted followed by alprenolol, although these possess similar interactions. The strength of interaction A in alprenolol is greater than in oxprenolol due to the presence of two oxygen atoms in oxprenolol. Acebutolol was eluted after alprenolol, which showed maximum interactions A, B, C, D and E. Carazolol was the last eluted β-blocker due to strongest π-π interactions between carbazole moiety and stationary phase.

Application of SPE-HPLC method in real world samples

The validity of the developed method was applied to analyze the reported β-blockers in human plasma. The qualitative and quantitative analyses of β-blockers were carried out by using the above mentioned SPE and HPLC conditions. The chromatograms of β-blockers in human plasma are shown in **Figure 3**. The

quantitative analyses of β-blockers in human plasma were carried out by comparing their peak areas with those of standards. For calculation of concentrations of β-blockers in human plasma, five sets of SPE and HPLC experimentations were carried out under identical experimental conditions. It is clear from **Figure 3** that the developed SPE and HPLC methods are selective as no impurity peak was observed.

CONCLUSION

The simultaneous analysis of six β-blockers was performed using SPE-HPLC method. The developed SPE-HPLC method was fast, selective, reproducible, efficient and rugged. π-π Interactions between phenyl-ethyl column and β-blockers were responsible for good separation. The limits of detection were quite satisfactory. The developed method also utilized low amount of costly and hazardous solvent such as acetonitrile. Therefore, the developed method is economical and environmentally safe. The developed and validated method was applied to monitor β-blockers in human plasma samples. Briefly, the developed SPE and HPLC methods can be applied to monitor the reported β-blockers in any sample of biological, environmental and industrial origins. Moreover, the reported single SPE and HPLC method can be used for any of six β-blockers; saving economy of analyses. The reported method was compared with the available methods in to the literature (Table 4). It is clear from this table that the present work is better than the reported ones.

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Table 1. Chromatographic parameters- capacity factors (k), separation factors (α), resolution factors (R*s*) of all six β-blockers

S. No.	β-Blocker	k	α	R_s
1.	Timolol	2.76	1.80	3.48
2.	Atenolol	4.96	1.82	6.64
3.	Oxprenolol	9.05	1.33	4.11
4.	Alprenolol	12.00	1.14	2.11
5.	Acebutolol	13.68	1.29	6.07
6.	Carazolol	17.72	-	-

Table 2. Regression analysis data for the extraction of six β-blockers

S. No.	β-blockers	Recovery	Extraction (% Recovery)		Confidence level CL (%)
			Standard deviation (SD)	Correlation coefficient (r^2)	
1.	Timolol	50.50	±0.07	0.9999	99.80
2.	Atenolol	40.50	±0.08	0.9998	99.71
3.	Oxprenolol	32.30	±0.06	0.9999	99.82
4.	Alprenolol	45.00	±0.07	0.9988	99.60
5.	Acebutolol	38.60	±0.06	0.9998	99.73
6.	Carazolol	39.00	±0.09	0.9997	99.62

Table 3. Chromatographic and precision data of six β-blockers

S. No.	β-blockers	Rs	SD	Correlation Coefficient	Confidence level (%)
1.	Timolol extracted (Timolol standard)	3.48 (3.49)	± 0.05 (±0.05)	0.9998 (0.9999)	99.52 (99.63)
2.	Atenolol extracted (Atenolol standard)	6.64 (6.64)	± 0.04 (±0.05)	0.9996 (0.9999)	99.25 (99.46)
3.	Oxprenolol extracted (Oxprenolol standard)	4.11 (4.11)	± 0.05 (±0.05)	0.9997 (0.9999)	99.10 (99.31)
4.	Alprenolol extracted (Alprenolol standard)	2.11 (2.10)	± 0.05 (±0.05)	0.9997 (0.9999)	98.85 (99.06)
5.	Acebutolol extracted (Acebutolol standard)	6.07 (6.07)	±0.04 (±0.05)	0.9996 (0.9999)	98.72 (99.11)
6.	Carazolol extracted (Carazolol standard)	-	-	-	99.02 (99.23)

Table 4. A comparison of β-blockers separation by SPE and HPLC

S. No.	β-Blockers	Matrices	Sample preparation technique	Stationary phase	Mobile phases	Separation Times and flow rates	Detection limits	Drawbacks	Refs.
1.	Atenolol, practolol, metoprolol,oxprenolol and propranolol	-	-	C_{18}	Methanol-acetonitrile-phosphate buffer (10 mM, pH 3.0) (15:15:70, v:v:v)	15.0 min. at 1.0mL/ min	0.25-50 ng/mL	Sample preparation technique is not given, Method was applied in real samples.	[44]
2.	Nadolol, pindolol, Acebutolol, timolol, metoprolol, oxprenolol, labetolol, propranolol and alprenolol.	Urine and serum	Automated In-Tube SPME	C_{18}	Acetonitrile-methanol-water-acetic acid (15:15:70:1), pH =4.0	25 min. at 0.2-0.45 mL/min	0.1-1.2 ng/mL	High run time, costly paraphernalia (LC-MS).	[45]
3.	Alprenolol, atenolol, metoprolol, nadolol, pindolol,	Cultured corneal epithelium	-	C_8	Gradient elution of solution A: water containing	30 min. 1.0 mL/ min	0.7 and 1.3 nM	Sample preparation technique is not given. High run	[46]

	propranolol, sotalol and timolol				0.03% (v/v) of trifluoroacetic acid, and solution B: water-acetonitrile (50:50, v/v) containing 0.03% (v/v) of trifluoroacetic acid.			time.	
4.	Bisoprolol and metoprolol	Plasma	LLE	C_{18}	Acetonitrile-water, triethylamine - orthophosphoric acid (18:82, 20:80, v/v)	8.0 min. at 1 mL/min	3.13 ng/mL	Utilized tedious and expensive sample preparation technique. Involved and high amount of orthophosphoric acid in mobile phase (dangerous to column life).	[47]
5.	Atenolol, sotalol, diacetolol, carteolol, nadolol, pindolol, acebutolol, metoprolol, celiprolol, oxprenolol, labetalol, propranolol and tertatolol	Plasma	LLE	C_{18}	Gradient of acetonitrile-phosphate buffer (pH 3.8)	26 min. at 1.0 mL/ min	5 -10 ng/mL	Utilized tedious and expensive sample preparation technique, Run time is high.	[24]
6.	Acebutolol, atenolol, metoprolol and sotalol	Water	SPE	C_{18}	Gradient of acetonitrile and 1% acetic acid	11.2 min. at 250 µL/min	-	Expensive involving high quantity of acetonitrile and acid, Detection limit is not given, costly	[48]

								paraphernalia (LC-MS).	
7.	Atenolol, sotalol, metoprolol, bisoprolol, propranolol and carvedilol,	Whole blood	SPE	C_{18}	Gradient of- (A): 10 mM ammonium formate adjusted to pH 3.1 with formic acid and (B): acetonitrile	9.6 min. at 0.3 mL/min	-	High quantity of acetonitrile, acid are used (dangerous for column life), method is expensive, Detection limit is not given, costly paraphernalia (LC-MS).	[49]
8.	19 beta-blockers	Urine	SPE	C_{18}	Gradient of – (solvent A): 0.1% formic acid in water and (solvent B): methanol	7.59 min. at 0.5 mL/min	3-36 ng/mL	Very low difference in retention times, resolution poor, costly paraphernalia (LC-MS).	[50]
9.	Acebutalol, metoprolol, bopindolol, oxprenolol, bufuralol, bisoprolol, indinolol, carazolol and alprenolol	Plasma	SPE	C_{28}	Phosphate buffer (20mM, pH 7.0)-acetonitrile (75 : 25, v/v)	51.40 min. at 0.5 mL/ min	1.0- 10.0 ng/mL	High run time hence the method is costly.	[22]
10.	Carteolol, carvedilol, clenbuterol, clorprenaline, ephedrine, mexiletine, propafenone, propranolol, salbutamol, and timolol.	Plasma	Online SPE	C_{18}	Gradient elution of acetonitrile and 10mM ammonium acetate buffer (pH 3.5)	7.19 min. at 1 mL/ min	0.2 ng/mL – 1.0 ng/mL	Very low difference in retention time, poor resolution, costly paraphernalia (LC-MS).	[51]
11.	Atenolol, sotalol, nadolol, pindolol,	Bovine eye tissues	LLE	C_{18}	Gradient of (A): 5 mM ammonium formate in	13.0 min. 0.4 mL/min	-	sample preparation method is tedious. Very	[52]

	timolol, metoprolol, betaxolol and propranolol				water (pH 3.5 adjusted with formic acid) and (B): acetonitrile:methanol (75:25) containing 0.02% triethyl amine (pH 4.0; adjusted with formic acid)			low difference in retention time, poor re4solution, Detection limits are not given, costly paraphernalia (LC-MS).	
12.	32 β-blocker	Urine.	-	C_{18}	Ammonium acetate buffer (pH = 3.5, 4mmol ammonium acetate , 1% glacial acetatic acid in distilled water)-acetonitrile	7.0 min. at 1.0 mL/min	10-100 ng/mL	Involve tedious and expensive sample preparation technique, costly paraphernalia (LC-MS).	[53]
13.	22 β-blocker	Plasma	SPE	C_{18}	Gradient of (eluent A): 5 mM aqueous ammonium formate adjusted to pH 3.0 with formic acid and (eluent B): acetonitrile	7.5 min. at 0.4-0.7 mL/min	0.001-0.1 mg/L	Involve costly solvents, Expensive method, costly paraphernalia (LC-MS).	[54]
14.	12 β -blockers	Sewage	SPE	C_8	Gradient of-(solventA): water-acetonitrile-formic acid (94.5:5.0:0.5) (v/v) and (solventB): acetonitrile-formic acid 99.5:0.5 (v/v)	24.32 min. at 0.2 mL/min	6 -11 ng/L	Long retention time. Involve high concentration of costly solvents, Expensive method, costly paraphernalia (LC-MS).	[55]
15.	15 β -blockers	Serum and urine	SPE	C_{18}	Gradient elution of a mixture of two solvents: Solvent A: 50 mL/L acetonitrile	30 min. 1.0 mL/min	-	Long retention time, costly due to high consumption of solvent,	[56]

					and solvent B:500 mL/L acetonitrile, in 50 mmol/L phosphate buffer, pH 3.0, containing 375 mg/L sodium octyl sulfate and 3 mL/L triethylamine.			Expensive method, Detection limits are not given	
16.	21 beta-blockers	Urine	LLE	C_{18}	Gradient elution of mobile phase consisted of 1% acetic acid in water and acetonitrile.	22 min. at 0.3 mL/min	5-500 ng/mL.	Involve tedious and expensive sample preparation technique, Expensive method, costly paraphernalia (LC-MS).	[57]
17.	Sotalol, carteolol, and bisoprolol	Urine	CM-LPME	C_{18}	Gradient of (A): mixture of 10 mM triethylamine and 20 mM potassium dihydrogen phosphate solution (adjusted to pH 3.0 with phosphoric acid) and (B): acetonitrile.	18 min. at 1.0 mL/min	0.005-0.01 mg/L	Only four beta-blockers, tedious sample prepn. techn, Expensive method.	[58]
18.	Timolol, atenolol, oxprenolol, alprenolol, acebutolol and carazolol	Human Plasma	SPE	Phenyl-Ethyl	Phosphate buffer (50 mM, pH 3.0)-acetonitrile (70:30, v/v)	21.90 min. at 1.0 mL/min	0.1-0.5 µg/mL	Reproducible, good separation and resolution factors, inexpensive, capable to monitor β-blockers in	Present work

								human plasma, supera-molecular level mechanism (helpful to design experiments for other β-blockers and molecules).	

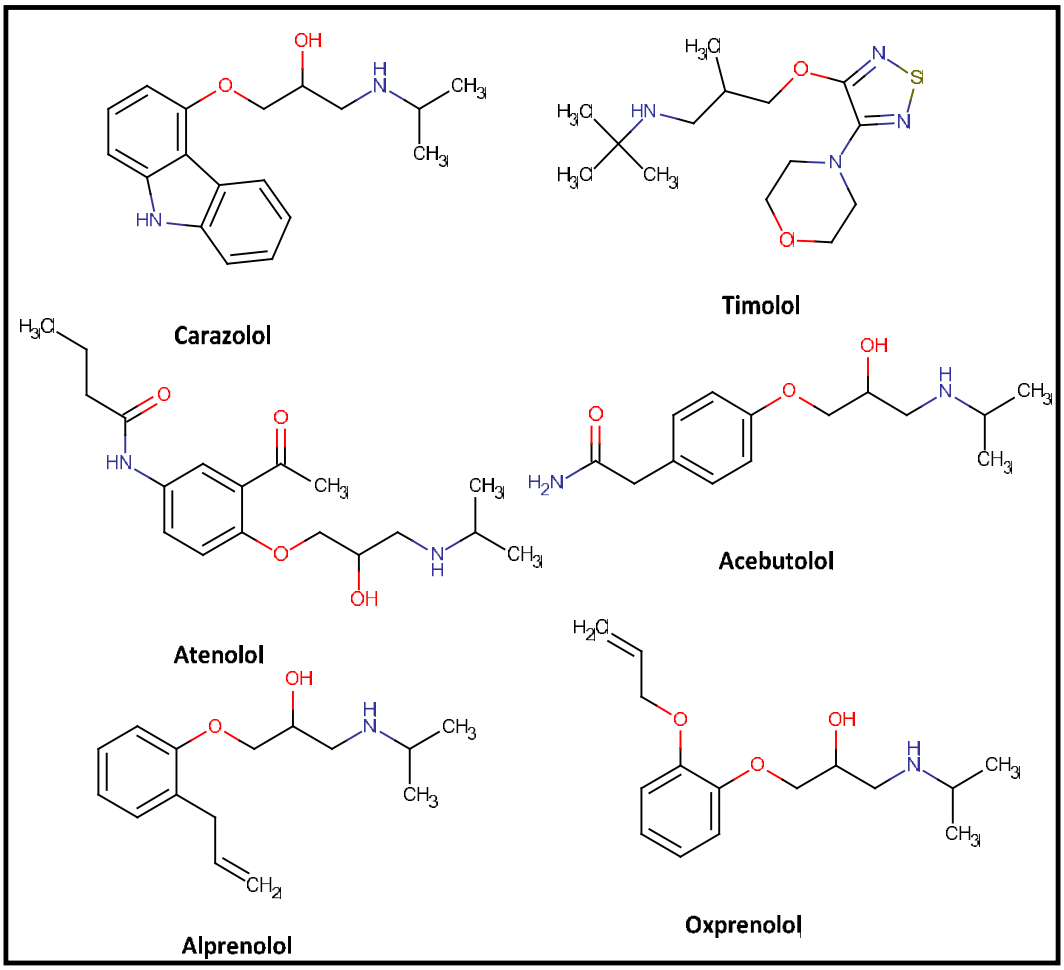

Figure 1. Chemical structures of the commonly prescribed β-blockers

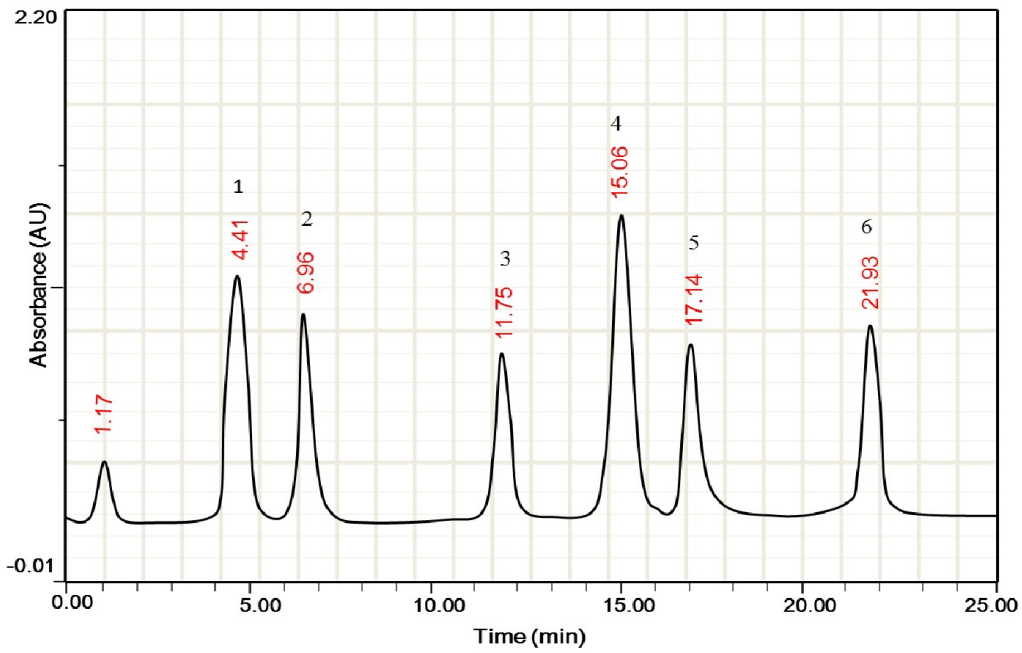

Figure 2. Chromatograms of six β-blockers in standard sample. (Experimental Conditions as given in text.)

β-Blockers: 1. Timolol, 2. Atenolol, 3. Oxprenolol, 4. Alprenolol, 5. Acebutolol and 6. Carazolol.

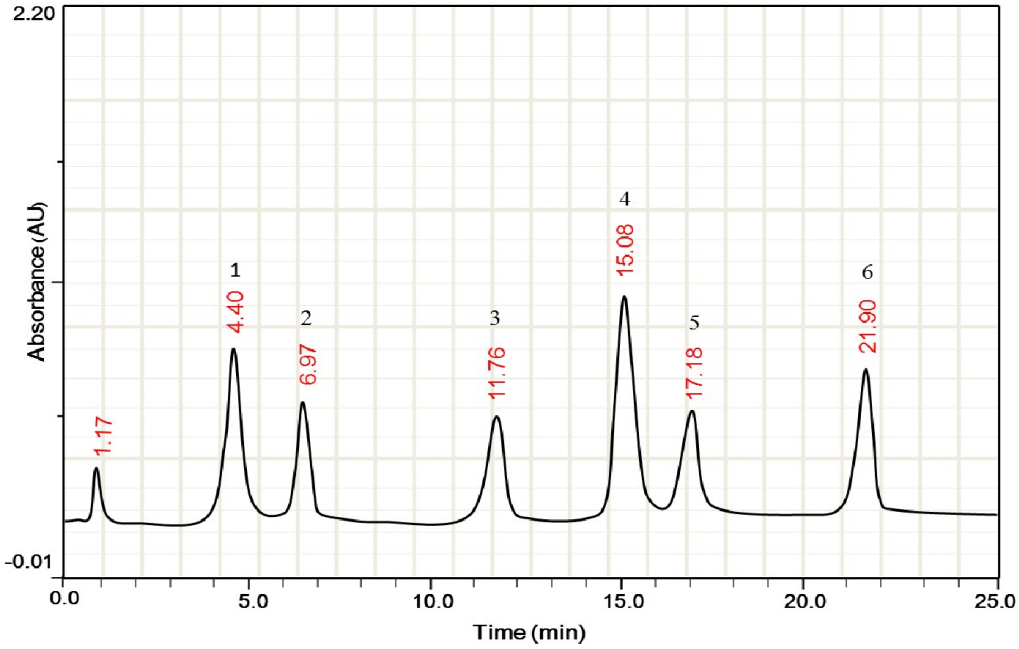

Figure 3. Chromatogram of all six β-blockers in human plasma. (Experimental conditions as given in text.)

β-Blockers: 1. Timolol, 2. Atenolol, 3. Oxprenolol, 4. Alprenolol, 5. Acebutolol and 6. Carazolol.