Simultaneous and Fast SPE-HPLC Analyses of Nine Anti-Hypertensive Drugs in Human Plasma

Imran Ali*[1], Kamlesh K. Dutta[2,3], A.K. Jain[3], Mohd. Asim[1] and Syed Dilshad Alam[1]

[1]Department of Chemistry, Jamia Millia Islamia (Central University), New Delhi-110025, India
[2]Quest Pharmaceuticals Pvt. Ltd. Birgunj, Nepal
[3]School of Basic and Applied Sciences, Galgotias University, Gautam Budh Nagar - 203201, India

Address for Correspondence

Department of Chemistry, Jamia Millia Islamia (Central University), New Delhi-110025, India.
E-mail: drimran_ali @yahoo.com

ABSTRACT

Objectives: Increasing cardiovascular patients is a matter of global issue. Various drugs are being used to cure these ailments with different mode of actions. The present article describes simultaneous and Fast SPE-HPLC analyses of nine anti-hypertensive drugs in human plasma.

Methods: Cardiovascular drugs such as amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, furosemide, telmisertan, losartan and olmesartan were separated by HPLC on Sushell C_8 (150 x 4.6 mm, 2.6 μm) column. The mobile phase was phosphate buffer (65% with 0.8% TEA) in acetonitrile; 0.5 mL/min flow rate, 230 nm detection and 40±1℃ temperature.

Results: The values of retention, separation and resolution factors were from 0.19-3.40, 1.20-3.60 and 2.43-12.37, respectively. The values of retention times, tailing factor and number of theoretical plates ranged from 2.64-11.60, 1.20-1.34 and 25883-144283, respectively. The percentage bindings with plasma determined by SPE were 40, 35, 70, 90, 70, 90, 90, 90, and 90%, respectively. The developed SPE-HPLC methods were applied successfully for monitoring these drugs into human plasma.

Keywords: HPLC, Plasma, SPE, Cardiovascular, Sunshell.

INTRODUCTION

Nowadays, about 33.3% populations in 194 countries are suffering from various cardiovascular diseases[1]. These ailments comprise hypertension, angina pectoris, cardiac arrhythmias, thyrotoxicosis, migraine headaches and glaucoma[2, 3]. These diseases are due to abnormal changes in various biological activities. Therefore, the different drugs are used to cure cardiovascular diseases. For example,

carvedilol and metoprolol are β-blockers relaxing blood arteries resulting into decrease in blood pressure[4]. On the other hands, amiloride, hydrochlorothiazide and furosemide are diuretics reducing blood volume and decreasing blood pressure[5,6]. Losartan, olmesartan and telmisartan are angiotensin used to control blood pressure[7]. Amlodipine is calcium channel blocker, used to low blood pressure and also to cure from angina pectoris[8]. Sometimes, medicinal practitioners prescribe the combination of three or more drugs. Besides, the combination therapy may lead some side effects due to the formation of other molecules by the interactions of these drugs in human body. The pharmacokinetics and pharmacodynamic of the reported drugs functions differently and vary in combination therapies. Therefore, there is a great need to develop cardiac HPLC method, which can detect these drugs simultaneously. These drugs were mixed in human plasma and kept at 37ºC for 24 hours to determine interaction products; if any.

A thorough search of literature was carried out through Scifinder, Scopus, Science direct and peer reviewed Journals[9-26]. It was observed that there are some HPLC methods for analyses of these drugs, but no one describes HPLC separation of all these molecules simultaneously. Besides, these methods are time and costly chemical consuming. Additionally, the limits of detections of these HPLC methods are quite high. Besides, solid phase extraction (SPE) method for the simultaneously extraction of these drugs is not available so far. In view of these facts, the attempts have been made to develop and validate SPE and HPLC methods for the separation and identification of all these drugs simultaneously in human plasma. Besides, the efforts were also made to determine any new by products during combination therapy. The results of these findings are reported herein.

EXPERIMENTAL

Chemicals and reagents

The standards of amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, furosemide, telmisertan, losartan and olmesartan were purchased from different manufacturers (Figure 1). The suppliers of these drugs were Anphar Laboratory, Gandhi Nagar, India (amiloride HCl), Sun Pharmaceuticals, Ahmed Nagar, India (metoprolol tartrate), CTX Life Science Pvt. Ltd., Surat, India (hydrochlorothiazide), Aurobindo Pharma-ceuticals, Hyderabad, India (carvedilol), Prudence Pharmaceuticals, Ankleshwar, India (amlodipine besylate), Manglam Drug Organics, Mumbai, India (furosemide), Harika Drug Pvt. Ltd., Hyderabad, India (telmisartan), Vashudha Pharmaceuticals, Hyderabad, India (losartan potassium) and Nutra Specialities Pvt. Ltd., Bharathi Nagar, India (olmesartan). Acetonitrile and triethyl amine of AR grade were purchased from Merck, India. Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$) of AR grade and ortho phosphoric acids were purchased from Merck, Mumbai, India. Fresh Frozen Human Plasma (Mfg. License No. 504) was collected from Rotary Blood Bank, New Delhi, India. Millipore water was prepared using a Millipore Milli-Q (Bedford, M.A., U.S.A.). pH meter was used for adjusting mobile pH.

Instruments used

HPLC system used was of Shimadzu (Kyoto, Japan) consisting of system controller pump (model: SCL-10AVP), liquid chromatograph (model: LC-10ATVP) and CLASS VP software, with UV-Vis detector (model: SPD-10A). Sunshell C_8 column (150 x 4.6mm, 2.6 µm) of Chromanik Japan was used for this purpose.

Preparation of standard solutions

The standard solutions of individual (1.0 mgmL^{-1}) and the mixture (0.0001-0.025 mgmL^{-1}) of these molecules were prepared in methanol. The stock solutions were protected from light sample vials by covering with aluminium foil and stored at 4ºC. A grade bulb pipettes and 10.0 mL volumetric flask were used for serial dilutions of these molecules to obtain the required concentration ranges (0.001-0.025 mgmL^{-1}).

Extraction of the drugs from plasma by solid phase extraction (SPE)

To determine the generation of any new molecule in the human body during combination therapy, these drugs were mixed individually and in mixture with human plasma. 1.0 mL (1.0 mgmL^{-1}) of these drugs was mixed with 5.0 mL fresh frozen human plasma individually and their mixture, separately and respectively. These samples were kept in an incubator for 37ºC for 24 hrs. For extraction purpose, acetone (15.0 mL) was mixed with each sample vial and kept for 30 minutes. These samples were centrifuged at 10,000 rpm (11,180 g) for 10.0 min to separate the supernatant. The supernatant was evaporated to dryness under vacuum. The residue was re-dissolved in 10.0 mL phosphate buffer (20 mM, pH 7.0), respectively. Sep-Pac C$_{18}$ cartridges (1.0 mL Waters, Milky Way, USA) were pre-conditioned with 2.0 mL methanol and 5.0 mL Millipore water, separately and respectively. The buffers containing individual and their mixture drugs were passed through the cartridges with 0.1 mLmin^{-1} flow rate, followed by cartridges washing with 2.0 mL Millipore water at 0.1 mLmin^{-1} flow rates. Further, these cartridges were dried by passing hot air. The elution of the reported drugs was carried out using 10.0 mL methanol at 0.1 mLmin^{-1} flow rate. The eluted methanol solutions containing the cardiac drugs were concentrated under vacuum to 0.5 mL, separately and respectively. These samples were further used for HPLC analyses.

HPLC conditions

All the experiments were carried out by HPLC system as mentioned above. The aliquots of 5.0 µL of standard solutions of each drug and their mixture (0.005 mgmL^{-1} of each drug in methanol) were loaded onto HPLC instrument, separately and respectively. The mobile phase used was phosphate buffer (65% with 0.8% TEA) in acetonitrile with flow rate of 0.5 mL/min in isocratic mode. UV detection was achieved at 230 nm with temperature at 40±1ºC. Fresh mobile phase was prepared, filtered and degassed daily prior to use. The chromatographic parameters such as retention (k), separation (α) and resolution (R$_s$) factors for the reported drugs were calculated. The order of eluted peaks was ascertained by running each individual drug. The qualitative and quantitative analyses were optimized by considering retention times and peak areas, respectively. The number of theoretical plates and tailing factor were also calculated. The HPLC method was optimized and validated. The developed HPLC method was applied for analyses of these drugs in human plasma samples.

Validation

HPLC method was validated by calculating different HPLC parameters[27-32]. For method validation linearity, limit of detection (LOD), limit of quantitation (LOQ), specificity, precision, accuracy, robustness and ruggedness were determined. The LOD and LOQ were determined by injecting lowest concentration of these drugs. The results of the statistical analyses of the experimental HPLC data such as relative standard deviation (SD), correlation

coefficients (CC) and confidence limits (CL) were calculated by Microsoft Excel software program. Excellent linearity of the calibration graphs and the negligible scatter of experimental points were used for calculations of correlation coefficients and relative standard deviations. Robustness of method was determined by versatility of the experimental factors that affected the peak areas.

Linearity

The linearity of this method was confirmed by least squares linear regression analysis of calibration curve. Besides, the linearities of calibration curves (peak area *vs.* concentration) for amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, furosemide, telmisertan, losartan and olmesartan standards were optimized and compared by varied concentration ranges. Equal volume (5.0 µL) of the standards as described above was loaded onto HPLC instrument. The HPLC chromatograms of the reported drugs were developed separately and respectively. The calibration curves of amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, furosemide, telmisertan, losartan and olmesartan were constructed using the observed peak areas *vs.* nominal concentrations of the reported drugs.

Detection and quantitation limits

The limits of detection (LOD) and quantitation (LOQ) for the reported drugs were determined as three and five times to the baseline noise, respectively. As per the United States Pharmacopoeia this standard was followed[33].

Specificity

The specificity of the method was ascertained by observing any interference in HPLC results due to the presence of some impurities in the standard samples. The standard samples were mixed with very low amount of crude reported drugs to make it impure.

Precision

Precision data was calculated at three different concentrations i.e. 0.001, 0.005 and 0.025 mgmL^{-1} of all the reported drugs. Five sets of HPLC runs were carried out for all these three concentrations.

Accuracy

Accuracy of HPLC method was determined by different concentrations of the reported molecules. Three concentrations used were 0.001, 0.005 and 0.025 mgmL^{-1}. The HPLC runs were optimized five times (n = 5). The accuracies were determined by interpolation of five replicates peak areas of these reported drugs.

Robustness

Robustness of HPLC method was determined by changing a slight variation in the chromatographic experimental conditions such as flow rate, temperature, mobile phase composition and wavelength. The retention time, peak area and shape were analyzed under the established and slightly varied experimental conditions.

Ruggedness

Ruggedness of the method was ascertained by changing the experimental conditions such as several operators and different days.

RESULTS AND DISCUSSION

The results and discussion section is divided into two parts *i.e.* solid phase extraction and HPLC experiments. These are discussed below.

Solid phase extraction

Solid phase extraction method was used to separate the drug mixture and any

other new molecules or interferences from plasma samples. To determine the efficiency of the reported SPE methods the percentage recoveries of each cardiovascular drug were calculated. The percentage recoveries of amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, frusemide, telmisertan, losartan and olmesartan were determined by running the blank experiments. The calculated percentage recoveries of these cardiovascular drugs from plasma are given in Table 1. A perusal of this table indicates that the values of the percentage recoveries of amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, fusemide, telmisertan, losartan and olmesartan were 60, 65, 30, 10, 30, 10, 10, 10, and 100%, respectively. The remaining amounts of these drugs (bound to plasma proteins) were 40, 35, 70, 90, 70, 90, 90, 90, and 90%, respectively. The optimization of SPE was achieved by varying different experimental conditions such as concentrations and pHs of phosphate buffer and the flow rates of plasma samples, phosphate buffer and eluting solvents. Instead of, other eluting solvents such as methanol, ethanol, ethyl acetate and dichloromethane were also tested. Thus, by exhaustive experimentation, the best eluting solvent was methanol. It is observed that the polarity of methanol is good enough to elute these cardiovascular drugs from C_{18} cartridge. The maximum percentage recoveries of cardiovascular drugs were achieved using phosphate buffer (20.0 mM, pH 7.0) separately and respectively, at 0.1 mL min^{-1} flow rate. The values of RSD, correlation coefficient (R) and confidence level for these drugs ranged from 1.2-1.6, 0.9994-0.9995 and 99.2-99.5, respectively (Table 2).

Chromatography

The chromatographic parameters such as retention (k), separation (α), and resolution (Rs) factors were calculated for amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, frusemide, telmisertan, losartan and olmesartan. The values of these parameters are given in Table 3. The values of retention, separation and resolution factors were ranged from 0.19-3.40, 1.20-3.60 and 2.43-12.37, respectively. The values of retention times, tailing factor and number of theoretical plates ranged from 2.64-11.60, 1.20-1.34 and 25883-144283, respectively. The HPLC chromatograms of these cardiovascular drugs in standard and plasma samples are given in Figure 2 and 3, respectively. It is clear from these figures that the reported drugs were base line separated. The identification of the separated drugs was determined by running and comparing the retention times of the individual amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, frusemide, telmisertan, losartan and olmesartan drugs, respectively. There was no extra peak in plasma samples, which confirmed no drug-drug interaction for the reported cardiovascular drugs.

HPLC method optimization

To optimize the HPLC conditions, different combinations of solvent systems were tried. Besides, the alteration in flow rate, detection wavelength and amount injected was also carried out. The pHs of the mobile phase were varied using triethyl amine. Additionally, optimization was also achieved using some additives such as diethyl- and triethyl amines in the mobile phases. Owing to exhaustive experimentation, the most excellent HPLC conditions were developed and reported herein.

The effect of acetonitrile on the separation of these drugs was carried out. The amounts of acetonitrile were varied from 10-50 mL. It was observed that the peaks were broad from 10-30 mL.

Contrarily, the peaks merged into one another at high value of acetonitrile (40-50 mL). As a result 35 mL acetonitrile was found suitable for the maximum separation. The flow rate of the solvent system varied from 0.2 to 1.0 mLm^{-1}. It was observed that at low flow rates (0.2, 0.3 and 0.4 mLm^{-1}), the peaks were poorly resolved with higher retention times. Contrarily, at increase flow rates to 0.6-1.0 mLm^{-1} the peaks were merged into one another. Briefly, the peaks were well resolved at 0.5 mLmin^{-1} flow rate, which was considered as the best one.

Validation

The results of HPLC validation parameters such as linearity, LOD, LOQ, specificity, precision, accuracy, robustness and ruggedness are given herein.

Linearity

The linearity of calibration curves (peak area *vs.* concentration) for amiloride HCl, metoprolol tartrate, hydrochloro-thiazide, carvedilol, amlodipine besylate, frusemide, telmisartan, losartan potassium and olmesartan standards were checked over the concentration ranges of 4.018-6.026 µgmL^{-1}, 4.504-6.756 µgmL^{-1}, 3.910-5.866 µgmL^{-1}, 3.936-5.894 µgmL^{-1}, 4.019-6.029 µgmL^{-1}, 3.917-5.875 µgmL^{-1}, 4.038-6.058 µgmL^{-1}, 3.936-5.904 µgmL^{-1} and 4.099-6.149 µgmL^{-1}, respectively. The plotted curves were linear over these concentration ranges (n=5) for the reported cardiovascular drugs. The peak areas of amiloride HCl, metoprolol tartrate, hydrochlorothiazide, carvedilol, amlodipine besylate, frusemide, telmisartan, losartan potassium and olmesartan were plotted *vs.* their respective concentrations. The linear regression analysis was estimated on obtained curves. The correlation coefficient (r) (n=5) for amiloride HCl, metoprolol tartrate, hydrochlorothiazide, carvedilol, amlodipine besylate, frusemide, telmisartan, losartan

potassium and olmesartan were found to be 0.9997, 0.9998, 0.9995, 0.9998, 0.9992, 0.9998, 0.9997, 0.9993 and 0.9998 respectively. The values of RSD and confidence levels were in the range of 0.323-0.722% and 98.277-100.964% across the concentration ranges studied.

Detection and quantitation limits

The LOD and LOQ of amiloride HCl, metoprolol tartrate, hydrochloro-thiazide, carvedilol, amlodipine besylate, frusemide, telmisartan, losartan potassium and olmesartan were 0.2273 and 0.6886, 0.2285 and 0.6924, 0.3019 and 0.9147, 0.1674 and 0.5072, 0.3945 and 1.1955, 0.2015 and 0.6107, 0.2202 and 0.6671, 0.3559 and 1.0785, 0.2150 µg/mL and 0.6514 µg/mL, respectively.

Specificity

The method was quite specific as can be seen from Figure 3. The retention times of all molecules were almost similar in both standard solutions and plasma samples. There was no effect of the added impurities in the standards on the retention times and peak shapes of these molecules. These findings indicated good specificity of the reported method.

Precision

Precision data was calculated at three different concentrations i.e. 0.001, 0.005 and 0.025 mgmL^{-1} of all the reported drugs. Five sets of the chromatographic runs were carried out for all three concentrations. The values of RSD and confidence levels were in the range of 0.323-0.722% and 98.277-100.00%, respectively.

Accuracy

Accuracy of HPLC method was ascertained using different concentrations of the reported molecules. Three concentrations used were 0.001, 0.005 and

0.025 mgmL^{-1}. The chromatographic runs were carried out five times (n = 5). The accuracies were determined by interpolation of five replicates peak areas of these molecules. The values of absolute errors were ranged from 0.1.5-0.5.

Robustness

Robustness of HPLC method was determined by changing a slight variation in the chromatographic experimental conditions. The varied experimental conditions were mobile phase composition, flow rate, temperature and wavelength. The retention time, peak area and shape were analyzed under the established and slightly varied experimental conditions.

Ruggedness

Ruggedness of the method was determined by changing the experimental conditions such as several operators and different days.

Application of the developed SPE-HPLC methods to the real world samples

The developed and validated SPE and HPLC methods were applied for monitoring the reported drugs into human plasma samples. It was observed that there was no extra peak in the chromatograms (Figure 3), indicating good selectivity of SPE method. Besides, the absence of any extra peak confirms no new molecule and metabolic product during combination therapy. The HPLC results in terms of retention, separation, resolution factors and symmetry of the eluted peaks were similar to those of the standard samples. These findings showed that the reported SPE and HPLC methods were selective, efficient, rugged and reproducible.

CONCLUSION

The reported SPE and HPLC methods were selective, efficient, rugged,

economic, eco-friendly and reproducible for the separation and identification of amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, frusemide, telmisertan, losartan and olmesartan in human plasma. There was no extra peak in plasma samples, which confirmed no drug-drug interaction for the reported cardiovascular drugs. Besides, the absence of any new peak established no metabolic product. The separation and identification of these nine cardiovascular drugs is reported first time so far. Therefore, SPE and HPLC methods can be applied for analyses of these nine cardiovascular drugs. The developed SPE and HPLC methods were applied successfully for monitoring these drugs into human plasma.

ACKNOWLEDGEMENT

REFERENCES

1. Global Health Observatory: (GHO). World Health Organization, 2012.
2. Rekhi, G. S.; Jambhekar, S.S.; Souney, P. F.; Williams, D. A. A fluorimetric liquid chromatographic method for the determination of propranolol in human serum/plasma. *J. Pharm. & Biomed. Anal.* 1995, 13, 1499-1505.
3. Ali, I.; Gaitonde, V. D.; Aboul-Enein, H.Y.; Hussain, A. Chiral separation of β-adrenergic blockers on CelluCoat column by HPLC. *Talanta* 2009, 78, 458-463.
4. Press Release, FDA Approves First Generic Versions of Coreg, U.S. Food and Drug Administration, 2007.
5. Duarte, J. D.; Cooper-DeHoff, R. M. Mechanisms for blood pressure lowering and metabolic effects of thiazide and

thiazide-like diuretics. *Expert Rev. Cardiovasc. Ther.* 2010, 8, 793-802.

6. Rodgers, H. C.; Knox, A. J. Pharmacological treatment of the biochemical defect in cystic fibrosis airways. *Eur. Respir. J.* 2001, 17, 1314-1321.

7. Aulakh, G. K.; Sodhi, R. K.; Singh, M. An update on non-peptide angiotensin receptor antagonists and related RAAS modulators. *Life Sci.* 2007, 81, 615-639.

8. Wang, J. G. A combined role of calcium channel blockers and angiotensin receptor blockers in stroke prevention. *Vasc. Health Risk Manag.* 2009, 5, 593-605.

9. Youssef, R. M.; Maher, H. M.; El-Kimary, E. I.; Hassan, E. M.; Barary, M. H. Validated stability-indicating methods for the simultaneous determination of amiloride hydrochloride, atenolol, and chlorthalidone using HPTLC and HPLC with photodiode array detector. *J. AOAC Int.* 2013, 96, 313-323.

10. El-Gindy, A.; Emara, S.; Mostafa, A. HPLC and chemometric-assisted spectrophotometric methods for simultaneous determination of atenolol, amiloride hydrochloride and chlorthalidone. *IL Farmaco* 2005, 60, 269-278.

11. Carr, K.; Rane, A.; Frölich, J. C. Method development by RP-HPLC for estimation of frusemide and its application. *J. Chromatogr.* 1988, 145, 421-427.

12. Nagori, B. P.; Solanki, R. RP-HPLC Method for Simultaneous Estimation of Frusemide and Amiloride Hydrochloride in Tablet Formulation. *Ind. J. Pharm. Sci.* 2010, 72, 384-387.

13. Chandra Bose, R. J.; Sivanseyal, G.; Duraisamy, K. K.; Surender, N. S.; Ramasamy, P. Validated RP-HPLC method for the simultaneous estimation of ramipril and metoprolol tartrate in bulk and tablet dosage form. *Asian J. Biochem. & Pharma. Res.* 2011, 2, 171-177.

14. Baranowska, I.; Markowski, P.; Baranowski, Development and validation of an HPLC method for the simultaneous analysis of 23 selected drugs belonging to different therapeutic groups in human urine samples. *J. Anal. Sci.* 2009, 25, 1307-1313.

15. Ali, I.; Alam, S. D.; Farooqi, J. A.; Nagae, N.; Gaitonde V. D.; Aboul-Enein, H. Y. A Comparison of β-Blockers Separation on C18 and C28 Columns in Human Plasma., *Anal. Methods* 2013, 5, 3523-3529.

16. Uslu, B.; Ozden, T. HPLC and UPLC methods for the simultaneous determination of enalapril and hydrochlorothiazide in pharmaceutical dosage forms. *Chromatographia* 2013, 76, 1487-1494.

17. Ketema, G.; Sankar, D. G. RP-HPLC Method for Estimation of Carvedilol in Pharmaceutical Dosage Forms. *Am. J. Pharm Tech Res.* 2012, 2, 513-521.

18. Galanopouloua, O.; Rozoub, S.; Antoniadou-Vyza, E. HPLC analysis, isolation and identification of a new degradation product in carvedilol tablets. *J. Pharma. & Biomed. Anal.* 2008, 48, 70-77.

19. Raju, V. B.; Rao, A. L. Simultaneous estimation of perindopril and amlodipine in combined dosage form by RP-HPLC method. *Int. J. Chem. Sci.* 2011, 9, 1290-1297.

20. Yeole, M. P.; Asnani, A. J. Simultaneous Determination of Telmisartan and Amlodipine in Tablets by Reverse Phase High Performance Liquid Chromatography. *Res. J. Pharm & Technol.* 2011, 4, 75-77.

21. Wankhede, S. B.; Tajne, M. R.; Gupta, K. R.; Wadodkar, S. G. RP-HPLC

method for simultaneous estimation of telmisartan and hydrochlorothiazide in tablet dosage form. *Indian J. Pharm. Sci.* 2007, 69, 298-300.

22. Bhat, L. R.; Godge, R. K.; Vora, A. T.; Damle, M. C. A Validated RPHPLC Method for Simultaneous Determination of Telmisartan and Hydrochlorothiazide in Pharmaceutical Formulation. *J. Liq. Chromatogr. Relat. Technol.* 2007, 30, 3059-3067.

23. Thomas, A. B.; Chavan, U. B.; Nanda, R. K.; Kothapalli, L. P.; Jagdale, S. N.; Dighe, S. B.; Deshpande, A. D. Simultaneous RP-HPLC analysis of atenolol, hydrochlorothiazide, and losartan potassium in a tablet formulation. *Acta Chromatographica* 2010, 22, 219-226.

24. Panchal, H. J.; Suhagia, B. N. Simultaneous analysis of atorvastatin calcium and losartan potassium in tablet dosage forms by RP-HPLC and HPTLC. *Acta Chromatographica* 2010, 22, 173-187.

25. Pathak, S. U.; Saple, S. R.; Vaidya, V. V.; Kekare, M. B. Development and Validation of RP-HPLC Methods for Simultaneous Estimation of Amlodipine, Hydrochlorothiazide and Olmisartan Medoxomil in Tablets Formulation. *Anal. Chem.: An Indian J.* 2011, 10, 624-627.

26. Sagirli, O.; Önal, A.; Toker, S. E.; Şensoy, D. Simultaneous HPLC Analysis of Olmesartan and Hydrochlorothiazide in Combined Tablets and in vitro Dissolution Studies. *Chromatographia* 2007, 66, 213-218.

27. Federal Register (FDA), Validation of Analytical Procedures, 1995, 60, 11260.

28. Federal Register (FDA), Validation of Analytical Procedures: Methodology, 1997, 62, 27463.

29. Federal Register (FDA), Analytical Procedures and Methods Validation: Chemistry, Manufacturing and Controls Documentation, 2000, 65, 52776.

30. FDA, Reviewer Guidance. "Validation of Chromatographic Methods." Center for Drug Evaluation Research (CDER), Washington, USA, 1994.

31. FDA, "Guidelines for submitting samples and analytical data for method validation, Rockville, MD." Center for Drugs and Biologics Department of Health and Human Services, Washington, USA, 1987.

32. USP 25–NF 20, Validation of Compendial Methods Section (1225) (United States Pharmacopeal Convention, Rockville, Maryland, USA, 2256, 2002.

33. The United State Pharmacopeia (24th Ed.). United States Pharmacopeial Convention, Rockville, MD, 2000.

**Table 1.** The percentage recoveries of cardiovascular drugs from human plasma

Name of drugs	% Recovery by SPE from human plasma	% of drug interactions with human plasma
Amiloride HCl	60.0	40.0
Metoprolol tartrate	65.0	35.0
Hydrochlorothiazide	30.0	70.0
Carvedilol	10.0	90.0
Amlodipine besylate	30.0	70.0
Frusemide	10.0	90.0
Telmisatan	10.0	90.0
Losartan potassium	10.0	90.0
Olmesartan	10.0	90.0

Table 2. Validation data of SPE method

| Drugs	%RSD	Correlation Coefficient (r)	Confidence Level (%)
Amiloride HCl	1.5	0.9995	99.5
Metoprolol tartrate	1.6	0.9995	99.5
Hydrochlorothiazide	1.2	0.9994	99.3
Carvedilol	1.3	0.9994	99.3
Amlodipine besylate	1.6	0.9995	99.5
Frusemide	1.2	0.9993	99.2
Telmisatan	1.3	0.9994	99.3
Losartan potassium	1.5	0.9995	99.5
Olmesartan	1.5	0.9995	99.5

Table 3. The values of retention times (Rt), capacity factor (k), separation factor (α) and resolution factor (Rs), tailing factor (T) and number of theoretical plates (NTP)

Drugs	Rt	k	α	Rs	T factor	NTP/meter
Amiloride HCl	2.64	..	..	..	1.33	025883
Metoprolol tartrate	3.13	0.19	..	2.86	1.34	034283
Hydrochlorothiazide	3.58	0.36	1.91	2.52	1.34	042686
Carvedilol	6.011	1.28	3.59	12.37	1.33	084358
Amlodipine besylate	6.64	1.52	1.19	2.93	1.29	096847
Furosemide	7.59	1.88	1.24	4.12	1.25	106327
Telmisatan	8.46	2.21	1.18	3.55	1.26	120859
Losartan potassium	10.86	3.11	1.41	8.78	1.20	144150
Olmesartan	11.60	3.40	1.09	2.43	1.19	144283
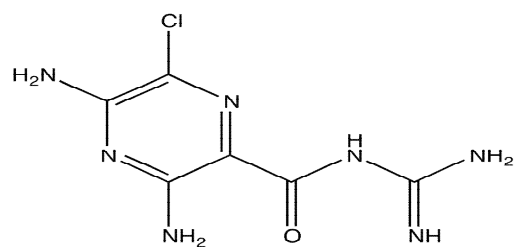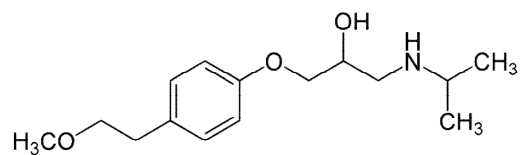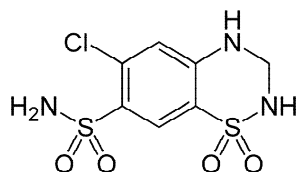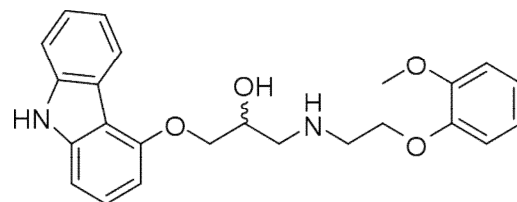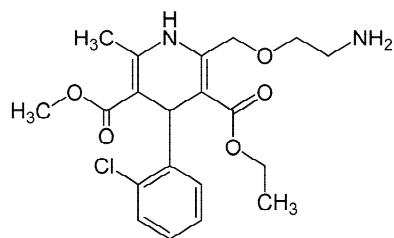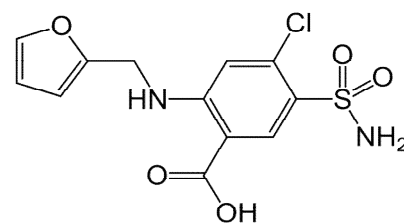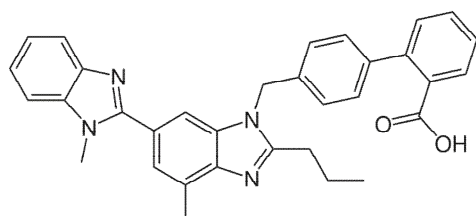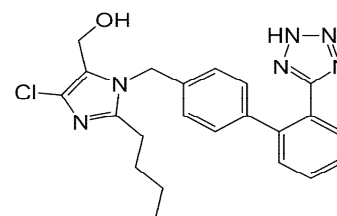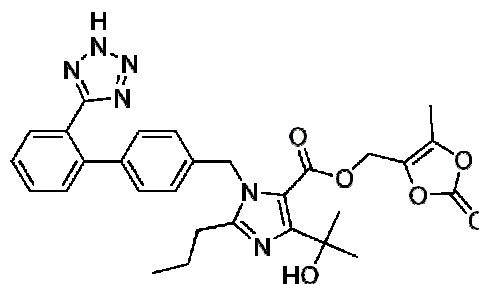
Figure 1. Chemical structure of cardiovascular drugs
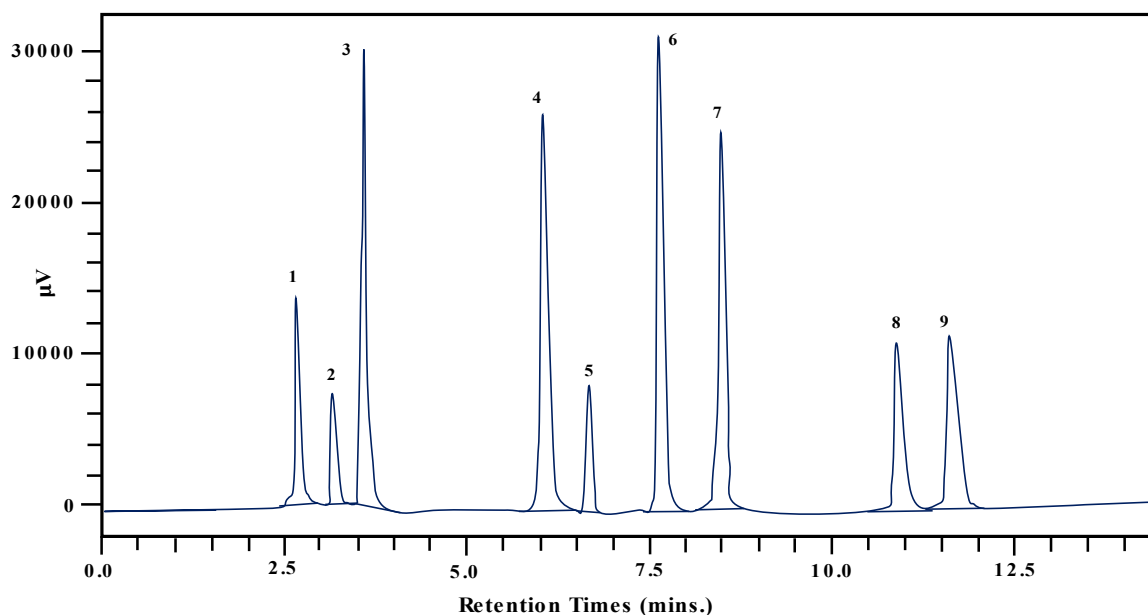
Figure 2. HPLC chromatogram of standard cardiovascular drugs (1-AmilorideHCl, 2-Metoprololtartarate, 3-Hydrochlorothiazide, 4-Carvidilol, 5-Amlodipine, 6-Furosemide, 7-Telmisertan, 8-Losartan and 9-Olmesartan)
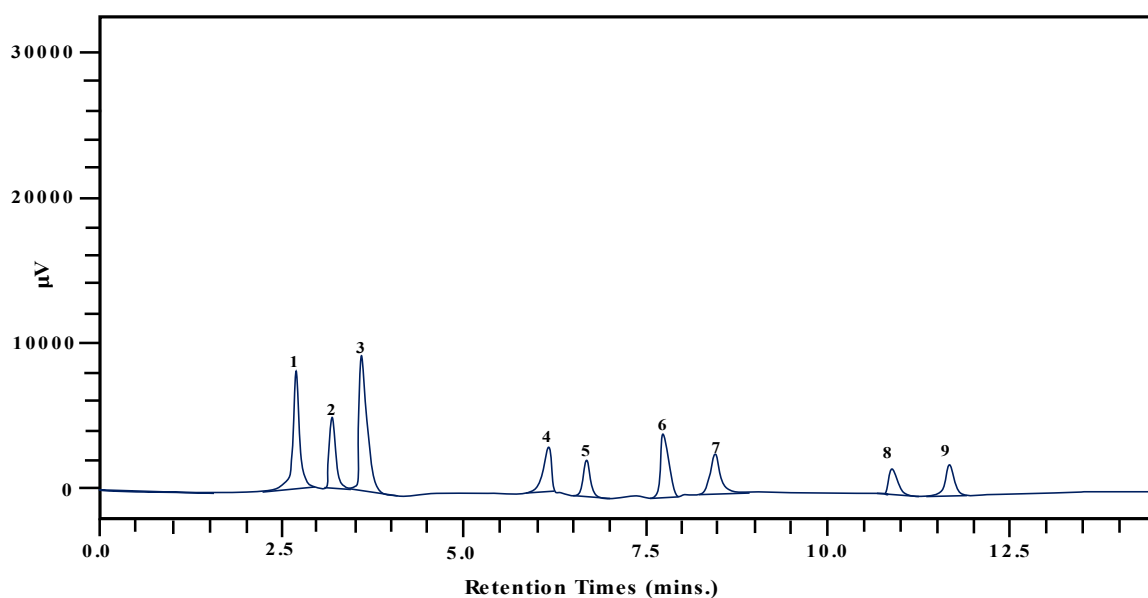
Figure 3. HPLC chromatogram of cardiovascular drugs in human plasma (1-AmilorideHCl, 2-Metoprololtartarate, 3-Hydrochlorothiazide, 4-Carvidilol, 5-Amlodipine, 6-Furosemide, 7-Telmisertan, 8-Losartan and 9-Olmesartan)