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Optimization and Validation of An Isocratic HPLC-UV Method for the Simultaneous Determination of Five Drugs Used in Combined Cardiovascular Therapy in Human Plasma

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The validation of a simple isocratic HPLC-UV method for simultaneous quantification of 5 drugs (used in combination therapy protocols in cardiovascular disorders) in spiked human plasma was reported in this paper. A MZ-analytical column (15 mm × 4.6 mm, 5 µm) was used to separate carvedilol, losartan, diltiazem, furosemide and propranolol under an isocratic condition of acetonitrile/2-propanol/15 mM phosphate buffer (pH = 2) (32.5/2.5/65 v/v/v) mobile phase. The sample preparation consisted of a protein precipitation procedure using a mixture of acetonitrile and zinc sulphate solution prior to injection of sample to the chromatographic system. The maximum wavelength for the all detections was 225 nm. Method was validated according to the food and drug administration guidance for bio-analytical method validation. Method showed acceptable precision, accuracy and linearity [carvedilol (0.025-0.800 µg/mL), losartan (0.050-0.800 µg/mL), diltiazem (0.050-0.800 µg/mL), furosemide (0.025-0.800 µg/mL) and propranolol (0.025-0.800 µg/mL)]. The method was robust and reproducible and the mean recoveries were in the range 99.0-104.4 %.

Key Words: HPLC-UV, Cardiovascular, Plasma, Carvedilol, Losartan, Dilitiazem, Furosemide, Propranolol.

INTRODUCTION

Cardiovascular diseases (CVDs) are a group of heart and blood vessels disorders including hypertension, arrhythmia and high cholesterol level, which affects quality of life of many people each year[1]. Tobacco use, unhealthy diet, low physical activity and harmful use of alcohol increase the risk of CVDs including heart attacks and strokes. Beyond trying to select the healthier life style, medical therapies are needed to improve the patients who suffer from a cardiovascular disease. The studies showed significant cardiovascular risk associated with hypertension and control and management of hypertension has impressive effects on the health status. Mono therapy approach to manage the hypertension couldn't provide desired results and less than of one third of the hypertensive patients achieved the desired blood pressure[2]. The combination of thiazides, β-blockers, acetyl choline esterase (ACE) inhibitors and calcium channel blockers are the well studied combination therapy and showed that lowering the dose of these drugs by combining two or more will lead to higher efficacy (about 5 times) and lower the side effects, beyond these, the newer classes such as angiotensin II receptor antagonists also used in combination with other classes but their effects have not well evaluated yet[3].

Quantification of the mentioned cardiovascular drug families was studied widely and a brief summary of these reports (which included 1 of the 5 studied drugs) are listed in Table-1. Recently the quantification of a set of drugs used in combined drug therapy of CVDs was studied with two different methods[4,5]. The selected drugs in these studies were belonged to diuretic (chlorthalidone) - angitensin II antagonist (valsartan) and statin (fluvastamin) combination protocol. The review of the published papers showed that several analytical methods were developed for simultaneous determinations of β-blockers (including propranolol and carvedilol), calcium channel blockers (including diltiazem), diuretics (including furosemide) and angiotensin II receptor antagonists (including losartan) which are usually used in combination therapy protocols, where simultaneous analyses of these four families have been studied rarely. Regarding to the different mechanism of actions (Table-2) of these 5 drugs, their combination in different ways, are among the interested therapy strategies for CVDs. In the present study, an isocratic HPLC-UV method was developed for simultaneous determination of propranolol, carvedilol, diltiazem, furosemide and losartan in human plasma. The method was validated according to the food and drug administration (FDA) guidance for bio-analytical method validation[6].

TABLE-1
PREVIOUS QUANTIFICATION METHODS OF CARDIOVASCULAR DRUGS USING DIFFERENT CHROMATOGRAPHIC METHODS

Drug name	Analysis method	Sample	Ref.
Losartan and it's metabolite	SPE/LLE-HPLC-UV	Plasma	7
Losartan and it's metabolite	HPLC-UV	Urine, plasma	8
Losartan and it's metabolite	HPLC-Fluorescence	Urine, plasma	9
Furosemide	HPLC	Urine and plasma	10
Losartan and it's metabolites	LC-Mass	Plasma	11
Diltiazem and it's metabolites	HPLC-UV	Plasma	12
Propranolol, atenolol	HPLC-Fluorescence	Plasma	13
Losartan	SPE-HPLC-UV	Plasma	14
Losartan, hydrochlorthiazide	HPLC-UV	Serum	15
Losartan, valsartan, irbesartan, candesartan	HPLC-Fluorescence	Plasma	16
Atenolol, metoprolol	HPLC-Chemilumincence	Urine	17
Diltiazem,	HPLC	Plasma	18
Losartan and it's metabolite	LC-Mass	Plasma and urine	19
Diltiazem	SPE-LLE-HPLC-UV	Plasma	20
Furosemide	HPLC-Amperometry	Milk	21
Diltiazem and it's metabolites	LC-Mass	Plasma	22
Atenolol, sotalol, di-osteolol, exprenolol, metoprolol, celiprolol, labetalol, propranolol, tertalol, betaxolol, ...	HPLC-Photo diode array	Plasma	23
Losartan	HPLC-UV	Plasma	24
Furosemide along with other 10 drugs	HPLC-Photo diode array-Fluorescence	Urine	25
Furosemide	HPLC-Fluorescence	Urine and plasma	26
Propranolol, atenolol, metoprolol	LC-APCI-MS	Postmortem human fluid and tissue specimens	27
Losartan, hydrochlorthiazide	SPE-HPLC-Mass	Plasma	28
Diltiazem, propranolol, verapamile, carvedilol, atenolol, bisoprolol, metoprolol, amidarone, mexiletine, sotalol	LC-Mass	Plasma	29
Carvedilol	HPLC-Fluorescence	Plasma	30
Carvedilol	HPLC-Fluorescence	Plasma	31
Carvedilol	LC-Mass	Plasma	32
Acebutolol, metoprolol, propranolol, labetalol	LC-Mass	Plasma	33
Diltiazem and it's metabolites	LC-ESI-Mass	Plasma	34
Acebutolol, propranolol, nadolol, esmolol, oxprenolol	SPE-LC-Mass	Urine and serum	35
Furosemide, propranolol, carvedilol aling with 20 other drugs	HPLC-DAD	Urine	36
Diltiazem and it's metabolites	LC-MS-MS	Plasma	37
Losartan, hydrochlorthiazide	LC-MS-MS	Plasma	38
Losartan and it's metabolite	Multiplexed LC-MS-MS	Plasma	39

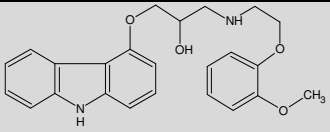
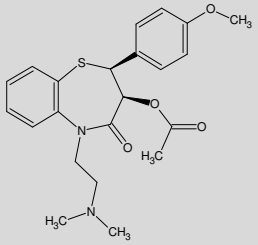
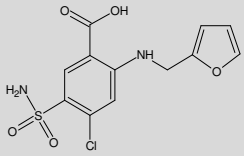
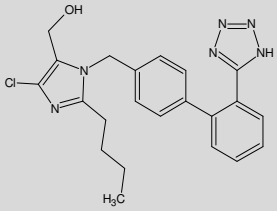
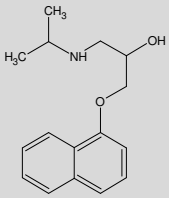
EXPERIMENTAL

Carvedilol EP, propranolol hydrochloride, diltiazem hydrochloride, losartan, furosemide, diazepam and clonazepam USP Kindly gifted by Sobhan Pharmaceutical Co. (Rasht, Iran), acetaminophen and ibuprofen were gifts from Dana Pharmaceutical Co. (Tabriz, Iran), caffeine, aspirin, salicylic acid, di-sodium hydrogen phosphate, phosphoric acid purchased from Merck, HPLC grade acetonitrile, 2-propanol and methanol were purchased from Scharlau (European Union), zinc sulphate from AJAX Chemicals (Australia) and doubly distilled water (prepared daily in the laboratory) were used in the study.

The Kanuar (Berlin, Germany) chromatographic system equipped with a WellChrom Maxi-Star K-1000 pressure pump, an online Biotech 2003 multichanel degasser, a WellChrom K-2500 spectrophotometer, a data processor using EuroChrom 2000 software, MZ ODS pre-column cartridge followed by a C18 ODS-3 (5 μm) MZ analytical column (150 mm × 4.6 mm) incubated in a space column oven (Grace Vydac Inc., Worms, Germany). The powders were weighted using a Metller Toledo AB204-S (Metller Toledo Inc. USA) analytical balance. The solvents were filtered through a 0.45 μm membrane filter (Millipore Corp., Billerica, Massachusetts) using a Millipore vacuum pump. The samples were vortexed using a LABTRON shaker model LS-100 and centrifuged by a Sanyo Micro-centaur (MSBO10.CX2.5) centrifuge (U.K.). A Metrohm (744) pH meter equipped with a glass electrode (a AgCl reference system and KCl 3 M as electrolyte), was used to measure pH of solutions. A Liarre Strasonic 18-35 ultrasonic bath was used to degas the mobile phase prior to use.

Standard solutions and spiked plasma samples: 25 mg of each drug weighted and dissolved in 25 mL acetonitrile/water (50-50 v/v) solvent mixture and refrigerated at 4 °C as stoke solutions. The working standard solutions were prepared daily by dilution of stoke solutions using the mobile phase. Combined working standards containing losartan, diltiazem, carvedilol, propranolol and furosemide were prepared similar to working standards. Spiked plasma samples were prepared daily by adding 200 μL of working standards to 200 μL plasma samples. After vortexing the spiked plasma for 20 s, it was equilibrated for 10 min before each analysis. Calibration standards were prepared by spiking the plasma samples with the working standard solutions (0.025-0.800 μg/mL). Stability assays were done using the quality control (QC) samples which were prepared by spiking the low, middle and high concentrations of each analytes.

TABLE-2
PHYSICO-CHEMICAL AND PHARMACOLOGICAL PROPERTIES OF STUDIED DRUGS

Drug	IUPAC name	Structure	Mechanism of action	pKa*	log P*
Carvedilol	(±)-[3-(9*H*-Carbazol-4-yloxy)-2-hydroxypropyl][2-(2-methoxy-phenoxy)ethyl]amine		Non-selective β-α-1-blocker	8.03	4.11
Diltiazem	*cis*-(+)-[2-(2-Dimethylaminoethyl)-5-(4-methoxyphenyl)-3-oxo-6-thia-2-azabicyclo[5.4.0]undeca-7,9,11-trien-4-yl]ethanoate		Calcium channel blockers	8.94	3.63
Furosemide	4-Chloro-2-(furan-2-ylmethyl-amino)-5-sulfamoylbenzoic acid		Loop diuretic	9.79	3.00
Losartan	(2-Butyl-4-chloro-1-beta-1*H*-imidazol-5-yl)methanol		Angiotensin II receptor antagonist	4.24	3.56
Propranolol	(*RS*)-1-(Isopropylamino)-3-(1-naphthyloxy)propan-2-ol		Non-selective β-blocker	9.15	3.10

*pKa and log P values calculated using ACDLabs software.

Plasma sample collection and preparation: Drug free plasma samples were donated by Iranian Blood Transfusion Organization Research Center and aliquot in polypropylene micro tubes and freezed at -20 °C until analysis. To a 1.5 mL polypropylene micro tube containing 400 µL plasma spiked with drug mixture, 180 µL acetonitrile added and after 20 s shaking using vortex, 20 µL zinc sulphate (1.16 M) solution was added following by 20 s shaking. After 20 min the mixture were centrifuged for 12 min (12000 rpm). The supernatant which was a clear liquid were transferred to another micro tube and 20 µL injected to the chromatographic system.

Chromatographic conditions: The separations were done using a mobile phase of acetonitrile/2-opropanol/buffer (32.5/2.5/65 v/v/v). The buffer (15 mM Na_2HPO_4) pH was adjusted to 2.00 ± 0.05 pH unit using H_3PO_4. The chromatographic separation was performed at 25.0 ± 0.5 °C. The flow rate was 0.9 mL/min. All drugs were detected at 225 nm. The equilibration time of the mobile phase was 1 h and after 1 h, the recycled mobile phase was used for 2 working days and was refreshed after 2 days.

Assay validation: In order to check the suitability of the developed method the calibration, linearity, lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), intra and inter day precisions, accuracy, recovery, selectivity and specificity, stability (room temperature, short term, long term and freeze thaw) and robustness were tested for each drug concerning FDA recommendations[6].

The calibration curve was prepared for each drug in the therapeutic drug concentration of the spiked plasma for each drug. The mean of three replications were used for all quantifications. The lowest concentration of calibration curve was considered as LLOQ while the RSD of three replications was less than 20 % and for ULOQ the highest concentration of calibration curve while the RSD of three replicates was less than 15 %.

The concentration range of calibration curves for all drugs was expected as linear range. Inter and intra day precisions were assessed by 5 replicate of low, medium and high concentrations of drugs in 3 different days. The RSD of replicates for 1 day and different days were assessed as inter and intra day precisions, respectively. The accuracy was obtained by comparing the results of 5 different plasma samples in low, medium and high concentrations with true values, and relative errors (RE) were reported as accuracy.

The recovery of the protein precipitation was calculated using the mean of 5 replicates of three concentrations for each drug using following equation:

$$\text{Recovery (\%)} = \frac{\text{Measured concentration}}{\text{Nominal concentration}} \times 100$$

The selectivity was checked by comparing the chromatograms of seven different blank plasma samples with spiked samples containing LLOQ concentration for each drug.

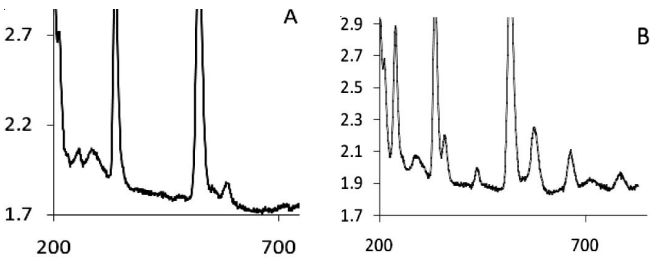
The short term stabilities of drugs in plasma sample were obtained by quantification of drugs in the same three concentrations of plasma samples in 3 days, similarly the long term stability assessed after 2 weeks and the freeze thaw stability was assessed after 3 freeze and thaw cycles in 12 h intervals during 3 days. The room temperature stability was assessed after 24 h of samples remained at room temperature.

The robustness of the method was checked based on four effective parameters (pH, column temperature, organic modifier concentration and flow rate). Each parameter was varied in three levels and the effects on the peak retention times and areas were studied.

RESULTS AND DISCUSSION

Optimization of separations

Chromatographic conditions: 30 Different mobile phases were designed based on the pH, column temperature, buffer concentration, acetonitrile concentration, 2-propanol concentration and flow rate. The retention time and resolution were checked in order to select the optimized condition. The initial experiments were begun by acetonitrile/10 mM buffer (40:60 v/v). After checking the pH (at the range of 2-6) it's found that the drugs are eluted with higher resolutions and lower retention time in pH < 3. The next experiments based on the combined effects of pH (2 and 3) and temperature (25-35 °C) showed that the lower pH at 25 °C results in better resolution and more stable baseline. Therefore pH = 2 and 25 °C was selected and then the acetonitrile (30:40 v/v) and 2-propanol (0-5 v/v %) concentrations were optimized. The best results were obtained from mobile phase composed of acetonitrile/buffer/2-propanol (32.5/65/2.5 v/v/v). Employing these conditions, the buffer concentration (10-20 mM) and flow rate (0.8-1.2 mL/min) were optimized where buffer concen-trations of 15 mM and flow rates of 0.9 mL/min were the best conditions. Finally, the mobile phase of acetonitrile/buffer (15 mM)/2-propanol (32.5/60/2.5 v/v/v) the flow rate of 0.9 mL/min is selected. The column was incubated at 25 °C during analysis and all mobile phases were allowed to equilibrate for 60 min. The injected volume was 20 µL and the loop was washed using the mobile phase before eachinjection. The elution order of drugs in this condition is shown in Fig. 1.



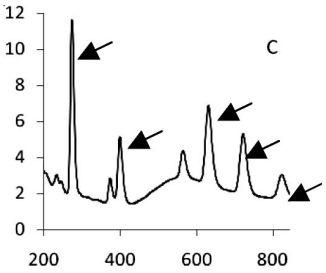


Fig. 1. Chromatograms of blank plasma sample (A), a 0.05 µg/mL spiked plasma sample (B) and 0.800 µg/mL spiked plasma sample (C). The black arrows show the peaks of propranolol, diltiazem, carvedilol, furosemide and losartan from left to right

Protein precipitation of spiked plasma: The protein precipitation method was optimized according to the clarity of the supernatant. In order to do this initially we checked the organic solvent precipitation method. The organic modifiers of the mobile phase (acetonitrile and 2-propanol) were used as precipitants. 200 µL of the binary organic solvent was added to 400 µL of the spiked plasma sample in the concentration range of 100-0 % of each organic solvent and found that the addition of 2-propanol to the acetonitrile could improve the precipitation while the neat 2-propanol was better precipitant but the injection of the resulted supernatant was lead to inappropriate peak shape. In the next step, in order to improve the precipitation of acetonitrile the addition of zinc sulphate (1.16 M) (5-50 % v/v) was tested. The results showed that the clarity was improved and the supernatant protein peak areas were decreased significantly comparing with 2-propanol modification. Then the spiked sample proteins precipitated by adding 180 µL acetonitrile followed by 20 µL zinc sulphate (1.16) solution. Fig. 2 shows the supernatant clarity of the discussed precipitation protocols.

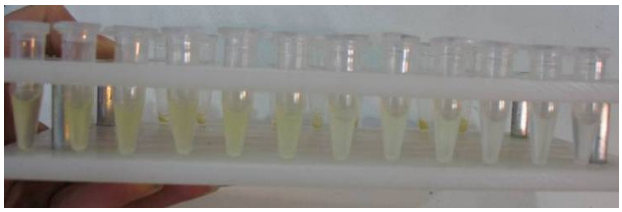


Fig. 2. Supernatant clarity of the 2-propanol (left) and zinc sulphate (right) modified, acetonitrile precipitation

Assay validation

Calibration curves: Response function (peak area) was plotted against the corresponding concentration for each drug and linearity was evaluated by calculating the correlation coefficient and other validation parameters which are summarized in Table-3. The linear range of all drugs covered their plasma therapeutic concentration ranges. Linear limit of quantification of the drugs were 0.025 µg/mL for carvedilol, furosemide and propranolol and 0.050 µg/mL for losartan and diltiazem. The upper limit of quantification were 0.800 for all drugs. The results are acceptable for the therapeutic drug monitoring application of the method.

Precision and accuracy: The results of intra assay precision and accuracy of calibration standards are shown in Table-4. All values for LLOQ and higher concentrations were below 20 % and 15 % respectively. This finding is in agreement

TABLE-3
VALIDATION REPORT OF THE PROPOSED METHOD FOR QUANTIFICATION OF CARVEDILOL, PROPRANOLOL, FUROSMIDE, LOSARTAN, DILTIAZEM

Parameters	Carvedilol	Diltiazem	Furosemide	Losartan	Propranolol
Linear range (µg/mL)	0.025-0.800	0.050-0.800	0.025-0.800	0.050-0.800	0.025-0.800
Slop	1.748	1.012	1.484	0.769	2.214
Slop S.E.	0.040	0.010	0.019	0.004	0.033
Intercept	0.024	-0.017	0.000	0.000	0.046
Intercept S.E.	0.015	0.004	–	–	0.014
Correlation coefficient	0.998	0.999	0.999	0.999	0.999
Number of data points	7	6	7	6	7
ULOQ (µg/mL)	0.800	0.800	0.800	0.800	0.800
LLOQ (µg/mL)	0.025	0.050	0.025	0.050	0.025
Therapeutic range (µg/mL)	0.020-0.160	0.100-0.250	1.000-6.000	0.200-0.650	0.020-0.300

*The LOD for these drugs were calculated as 3*Baseline noise.

TABLE-4
INTRA ASSAY PRECISION AND ACCURACY OF CALIBRATION STANDARDS

Drug	Nominal concentration (µg/mL) (N = 3)	Found concentration (µg/mL) (N = 3)	Precision (RSD %)	Accuracy (RE %)
Carvedilol	0.025	0.016	5.6	-37.2
	0.050	0.044	3.2	-11.5
	0.100	0.094	7.5	-6.5
	0.200	0.215	4.1	7.4
	0.400	0.414	1.7	3.4
	0.600	0.615	2.6	2.5
	0.800	0.781	2.8	-2.4
Diltiazem	0.050	0.047	5.1	-5.8
	0.100	0.099	5.3	-0.8
	0.200	0.201	3.3	0.6
	0.400	0.392	3.1	-2.1
	0.600	0.612	1.7	2.0
	0.800	0.794	2.9	-0.8
Furosemide	0.025	0.026	8.4	4.2
	0.050	0.048	15.9	-3.9
	0.100	0.099	6.6	-0.9
	0.200	0.205	8.3	2.6
	0.400	0.411	2.1	2.7
	0.600	0.611	3.4	1.8
	0.800	0.786	2.4	-1.8
Losartan	0.050	0.054	11.4	7.8
	0.100	0.096	9.4	-4.2
	0.200	0.202	3.4	1.0
	0.400	0.401	1.3	0.3
	0.600	0.608	2.7	1.3
	0.800	0.793	3.2	-0.8
Propranolol	0.025	0.023	5.9	-9.7
	0.050	0.046	4.0	-7.7
	0.100	0.101	5.2	1.5
	0.200	0.204	3.1	1.8
	0.400	0.395	2.9	-1.2
	0.600	0.619	2.5	3.2
	0.800	0.788	3.7	-1.5

with the criterion noticed by the guidelines[6], except for one case, *i.e.* carvedilol (0.025 µg/mL) in which 37.2 % was found. Inter and intra assay precisions along with accuracy for quality control samples are listed in Table-5. The similar results obtained for these validation experiments showed that the developed method is both accurate and precise.

Recovery: The recoveries of the investigated drugs are summarized in Table-6. The mean recoveries for all drugs in whole calibration range were acceptable (99.0-104.43 %), except carvedilol which showed lower recovery (*i.e.* 68 %) for LLOQ concentration.

TABLE-5
ASSAY PRECISION AND ACCURACY OF QUALITY CONTROL SAMPLES

Drug	Concentration	Intra-assay precision (RSD %)	Inter-assay precision (RSD %)	Accuracy* (RE %)
Carvedilol	0.025	5.59	17.08	-37.15
	0.200	4.10	12.31	7.38
	0.800	2.17	15.50	-2.40
Diltiazem	0.050	19.91	21.53	4.41
	0.200	3.29	17.07	0.56
	0.800	2.87	6.14	-0.77
Furosemide	0.025	8.44	4.68	4.17
	0.200	8.31	17.19	2.62
	0.800	2.42	3.29	-1.81
Losartan	0.050	11.36	21.57	7.79
	0.200	3.44	2.42	0.97
	0.800	3.33	12.94	-16.88
Propranolol	0.025	5.94	5.97	-9.67
	0.200	3.13	17.08	1.83
	0.800	3.73	12.31	-1.53

Accuracy calculated for 5 different samples for each concentration.

TABLE-6
ABSOLUTE AND MEAN RECOVERIES FOR STUDIED DRUGS

Drug name	Concentration (µg/mL) (N = 5)		Recovery (%)	Mean recovery (%)	Precision of recovery (RSD %)
	Nominal	Found			
Carvedilol	0.025	0.017	68.0	91.30	22.91
	0.200	0.217	108.5		
	0.800	0.778	97.3		
Diltiazem	0.050	0.056	112.0	104.43	6.43
	0.200	0.204	102.0		
	0.800	0.794	99.3		
Furosemide	0.025	0.027	108.0	102.50	4.80
	0.200	0.202	101.0		
	0.800	0.788	98.5		
Losartan	0.050	0.052	104.0	102.30	2.10
	0.200	0.206	103.0		
	0.800	0.799	99.9		
Propranolol	0.025	0.024	96.0	99.00	3.31
	0.200	0.205	102.5		
	0.800	0.788	98.5		

Selectivity and specificity: The selectivity of the developed method was checked by injecting the standard solution of frequently used drugs (acetaminophen, ibuprofen, caffeine, diazepam, clonazepam, aspirin and its metabolite salicylic

TABLE-7
FREEZE-THAW THREE TIMES, ROOM TEMPERATURE 12 h, 3 DAYS AT 4 ºC STABILITY DATA FOR STUDIED DRUGS

Drug	Concentration (µg/mL)	Freeze-thaw stability		Room temperature stability		3 days stability	
		Conc. found (µg/mL)	Accuracy (RE %)	Conc. found (µg/mL)	Accuracy (RE %)	Conc. found (µg/mL)	Accuracy (RE %)
Carvedilol	0.025	0.04	63.2	0.017	-31.4	0.03	36.0
	0.200	0.18	-11.0	0.217	8.7	0.20	-2.4
	0.800	0.70	-12.9	0.778	-2.8	0.80	-0.1
Diltiazem	0.050	–*	–	0.046	-7.5	–	–
	0.200	0.06	-71.7	0.204	1.9	0.09	-53.2
	0.800	0.58	-28.1	0.794	-0.7	0.73	-8.6
Furosemide	0.025	0.03	35.0	0.027	7.8	0.01	-46.0
	0.200	0.18	-9.1	0.202	1.1	0.14	-29.3
	0.800	0.73	-8.5	0.788	-1.5	0.76	-5.3
Losartan	0.050	0.03	-48.0	0.052	4.0	0.05	4.0
	0.200	0.19	-6.4	0.208	4.0	0.19	-6.4
	0.800	0.75	-6.0	0.793	0.9	0.90	12.0
Propranolol	0.025	0.02	-25.8	0.024	-2.4	0.03	17.8
	0.200	0.21	5.8	0.205	2.5	0.15	-25.9
	0.800	0.87	8.6	0.788	-1.5	0.91	13.8

*Diltiazem was not stable after 12 h.

acid). The results showed that except aspirin and its metabolite salicylic acid which interfere with propranolol, there are no interference for the other drugs. The specificity of the method was checked by blank injection of 7 different plasma samples and the results were compared with LLOQ spiked plasma samples data. As the chromatograms in Fig. 1 shows there is no interfering peak for the studied drugs except for diltiazem which eluted near to a protein peak and one should pay more attention to peak detection and quantification of it in LLOQ concentrations.

Stability: The stability experiments showed the plasma samples are not stable after 12 h at room temperature or in refrigerator and also the freeze-thaw cycles cause to inaccurate results for LLOQ concentrations. Concerning relatively poor stability data, it is recommended that the samples be analyzed freshly or if one has to maintain them for a week; they should be freeze at -20 °C. It is also found that; diltiazem peak vanishes after 24 h. The results are summarized in Table-7.

Robustness: The robustness of the method was checked by making slight variation on the chromatographic parameters (pH, column temperature, mobile phase flow rate, 2-propanol concentration) and 3 replicate injections of 0.200 µg/mL spiked plasma sample. The carfule review of the results revealed that most of the times the results showed negligible differences in peak area and retention times. There is a considerable effect for flow rate variation for carvedilol and diltiazem peak area which was because of plasma interference peaks that mentioned in specificity results.

Conclusion

The developed method showed acceptable selectivity, linearity, sensitivity, precision and robustness and could be applied as a routine procedure for TDM purposes (*i.e.* ensure that the plasma levels of the drugs are in therapeutic range) in quantification of the studied drugs (carvedilol, losartan, diltiazem, furosemide and propranolol). The simplicity of the method both in sample preparation step (no need to time consuming extraction methods) and chromatographic conditions (simple isocratic method) could be considered as advantages of the method and the LLOQ of the studied drugs can be improved using other detectors (*i.e.* fluorescence or mass).

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