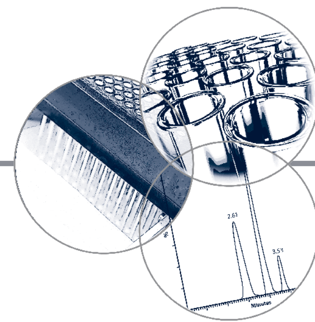

A validated micellar LC method for simultaneous determination of furosemide, metoprolol and verapamil in human plasma

An accurate and precise micellar LC method coupled with UV and fluorimetric detectors was developed and validated for the simultaneous analysis of furosemide, metoprolol and verapamil in human plasma. The total analysis time was 25 min (12 min for sample preparation and 13 min for drug separation). All drugs possessed linear behavior (r > 0.999 for calibration curves) in their therapeutic concentrations. The mean drug recoveries were 101.9, 100.1 and 100.2% for furosemide, metoprolol and verapamil, respectively. The accuracies (relative error %) were less than 15% for all drugs. Intra- and inter-day precisions (RSD%) were less than 15% and the stability data were acceptable according to the US FDA guideline for bioanalytical method validation.

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Cardiovascular diseases have been the leading cause of global morbidity and mortality for over a century and their management is the focus of research efforts by biomedical researchers [1]. Among the cardiovascular diseases, hypertension is one of the primary causes of much inconvenience and death every year [2]. Arrhythmias (disorders of heart rhythms), which are common problems in hypertensive patients, are difficult to manage and different classes of drugs (calcium-channel blockers [verapamil] and β-blockers [metoprolol]) are used for pharmacotherapy of arrhythmias. β-blockers and diuretics (e.g., furosemide) are common in hypertension therapy and combinations of these drugs (verapamil–furosemide or metoprolol–furosemide) for the treatment of arrhythmias in hypertensive patients are frequently used.

Verapamil hydrochloride (Figure 1A) is a phenylalkylamine calcium-channel blocking agent used as an anti-arrhythmia and hypotensive agent [3]. Metoprolol (Figure 1B) is a β-1 selective aryloxy propanolamine used in the treatment of some cardiovascular disorders such as hypertension, arrhythmia and heart failure [4]. Furosemide (Figure 1C) is a loop diuretic anthranilic acid derivative (5-aminosulfonyl-4-chloro-2-[(2-furanylmethyl) amino] benzoic acid) used in the treatment of congestive heart failure and edema [5].

Determination and quantification of drugs in **biological fluids** are essential in pharmaceutical, toxicological, doping and clinical chemistry research. The therapeutic efficacy is related to the concentration of drugs in biological fluids and tissues. The simpler and more rapid methods for simultaneous analysis of several drugs (especially the drugs that are used concurrently, such as furosemide–metoprolol or furosemide–verapamil), or the drugs that have severe contraindications [6,7] and possible toxicity (e.g., verapamil–metoprolol), are interesting for **therapeutic drug monitoring** purposes.

A number of HPLC methods have been developed (Table 1) in human plasma, urine or other biological samples [3,4,8–15]. In most cases, a pretreatment procedure (e.g., liquid–liquid extraction or SPE) is needed for the developed methods. In addition, in most cases for simultaneous analysis, gradient elution has been used. One of the successful analysis methods for biological samples is **micellar LC** (MLC), which has been applied to the analysis of different classes of drugs, especially cardiovascular drugs [16]. In this method an aqueous micellar media modified with a short-chain alcohol (e.g., butanol or pentanol) is used as mobile phase instead of hydro-organic mobile phases. The surfactant (e.g., sodium dodecyle sulphate [SDS] or Brij) is dissolved in water of a concentration higher than the critical micelle concentration and the resultant solution after pH modification is mixed with the desired concentration of the organic modifier and used as mobile phase. The main advantage of this method is the ability to solubilize proteins. This property enables researchers to inject biological samples directly (i.e., by dilution or filtration) to the HPLC system

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Key Terms

Biological fluids: Body fluids are liquids excreted or secreted from inside the body of a living person.

Therapeutic drug monitoring: The quantitative measurement of a specific drug concentration in plasma, to use in the management of a patient's drug therapy.

Micellar LC: A form of reversed-phase LC that uses aqueous micellar solutions as the mobile phase.

without time-consuming pretreatments (i.e., complete protein precipitations or extraction) [17]. In addition because of the divers interaction between analyte and mobile phase it is possible to separate a divers set of analytes with different pKa and logP values. MLC have been applied for analysis of diuretics [18], β-blockers [19], anticonvulsants [20], verapamil [3], quinolines [21], tamoxifen [22], disopyramide, lidocaine and quinidine [23].

A MLC method has been developed and validated for the determination of verapamil in plasma and urine samples [3] with a LLOQ of 100 ng/ml in plasma. The sample preparation method in this study was ten-times dilution of plasma sample. There was no validated method for the quantification of furosemide using this method in plasma sample. Furosemide has been quantified by MLC after derivatization and a linear range of 6 to 82 µg/ml and LOD of 0.15 µg/ml have been reported [15]. There is no study for metoprolol in plasma samples using MLC but the application of this method for the determination and quantification of metoprolol along with some other β-blockers in urine samples has been studied and a linear range of 0.05 to 0.5 µg/ml has been reported for metoprolol [10].

Simultaneous separation of these drugs using MLC method has not been reported and the present study provided a fully validated and simple isocratic MLC method for the determination of furosemide, metoprolol and verapamil in human plasma. The developed method is applicable for therapeutic drug monitoring of the mentioned drugs.

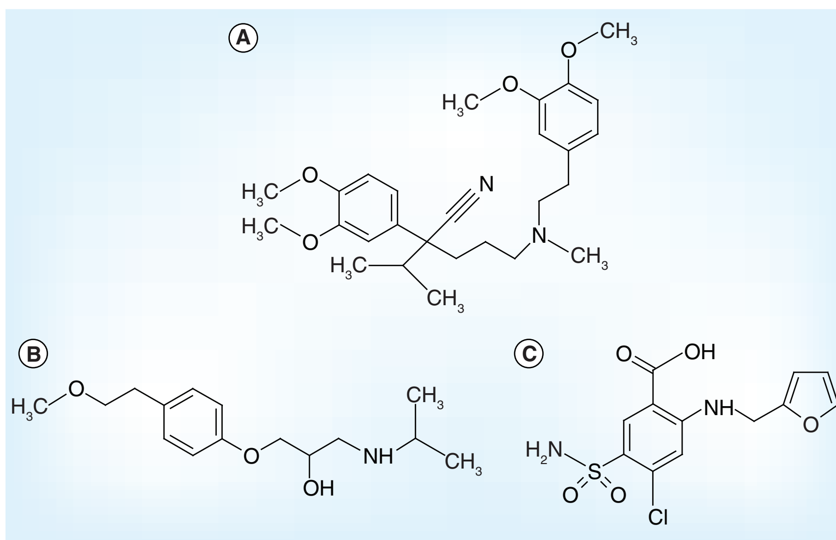

Figure 1. (A) Verapamil, (B) metoprolol and (C) furosemide.

Experimental

■ Chemical & reagents

Furosemide, verapamil and metoprolol kindly gifted by Daroupakhsh Pharmaceutical Co. (Tehran, Iran); Na_2HPO_4, H_3PO_4 and triethyl amine (TEA) were purchased from Merck (Darmstadt, Germany); HPLC-grade ACN, 1-butanol and MeOH were purchased from Scharlau (Barcelona, Spain); SDS was purchased from Acros organics (NJ, USA), and fresh, double-distilled water (prepared daily in the laboratory) was used in the study. MeOH solutions of the studied drugs were prepared using 25 mg of each drug in 25 ml MeOH and refrigerated at 4°C as stock solutions.

■ Instrumentation

The Knauer (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 Shimadzu RF-551 spectroflourimeter, a data processor using EzChrom software and a MZ ODS precolumn cartridge followed by a C18 ODS-3 (5 µm) MZ analytical column (150 × 4.6 mm) incubated in a space column oven (Grace Vydac Inc., Worms, Germany). The powders were weighted using a Mettller Toledo AB204-S (Mettller Toledo Inc., USA) analytical balance. The solvents were filtered through a 0.45 µm membrane filter (Millipore Corp., Billerica, MA, USA) using a Millipore vacuum pump (Millipore Corp.). The samples were vortexed using a LABTRON shaker model LS-100 and centrifuged by a Sanyo Micro-centaur (MSBO10.CX2.5) centrifuge (UK). A Metrohm (744) pH meter equipped with a glass electrode (a AgCl reference system and KCl 3 M as electrolyte), was used to measure the pH of solutions. A Liarre Strasonic 18–35 ultrasonic bath was used to degas the mobile phase prior to use.

■ Sample preparation

Working standard solutions were prepared daily by dilution of the stock solutions with water and then plasma. Combined working standards containing metoprolol, verapamil and furosemide were prepared similar to working standards. Spiked plasma samples were prepared daily by dilution of a 2 µg/ml of spiked plasma sample by blank plasma. Stability assays were performed using the quality control (QC) samples, which were prepared by spiking the low, middle and high concentrations (according to the calibration curve) of each analytes.

Table 1. Summary of previous studies.

Drug name	Sample	Method	Sample preparation	Linear range (ng/ml)	Ref.
Metoprolol and bisoprolol	Human plasma	HPLC–FL	LLE	6.25–200	[8]
Metoprolol	Human plasma (pediatric sample)	HPLC–FL	SPE	2.4–195.2	[9]
Metoprolol and a metabolite	Human plasma and urine	HPLC–FL	SPE	1–400	[4]
Metoprolol and other β-blockers	Human urine	MLC–FL	Dilution	50–500	[10]
Verapamil and norverapamil	Specimen blood, kidney, liver	HPLC–FL	LLE, SPE	–	[11]
Verapamil	human plasma	HPLC–DAD	SPE	100	[12]
Verapamil	Human urine and serum	MLC–FL	Dilution	100–2000	[3]
Furosemide	Human urine and plasma	HPLC–FL	LLE	–	[13]
Furosemide and amiloride	Human plasma	HPLC-UV	LLE	7.8–1000	[14]
Furosemide	Human urine	MLC-UV	Drug derivatization and dilution	6000–82000	[15]

DAD: Diode array detector; FL: Fluorimetric; LLE: Liquid–liquid extraction; MLC: Micellar LC.

■ Plasma sample collection & pretreatment

Drug-free plasma samples were supplied by Iranian blood transfusion research center (Tabriz, Iran) and aliquot into polypropylene microtubes and frozen at -20°C until analysis. acetonitrile 500 µl was added to a polypropylene microtube containing 500 µl plasma spiked with drug mixture, and after 20 s vortexing followed by 10 min equilibration time, the mixture was centrifuged for 10 min. The supernatant, a semi-clear liquid, was transferred to another microtube and injected to the chromatographic system.

In order to check the applicability of the developed method to the analysis of real unknown samples, six different plasma samples obtained from the cardiac patients admitted to the emergency unit of Madani Hospital (Tabriz, Iran) were analyzed. All samples were collected from patients who had signed a written consent form; these were approved by the Ethics Committee of Tabriz University of Medical Sciences and stored in heparinized tubes and centrifugated at 13,000 (9447 × g) rpm for 10 min. The supernatant was transferred to a microtube and frozen at -20°C until analysis.

■ Chromatographic conditions

The separations were done using a mobile phase of buffer (0.15 M SDS, 25 mM Na_2HPO_4)/1-butanol/TEA (93/6/1, v/v/v). The buffer pH was adjusted to 3.00 ± 0.05 using H_3PO_4. The chromatographic separation was performed at 40.0 ± 0.5°C with the flow rate of 2 ml/min. Verapamil and metoprolol were detected by spectrofluorimeter (excitation 230 nm and emission 311 nm) and furosemide was detected by UV detector at 240 nm. The equilibration time of the mobile/stationary phases was 120 min and after 120 min, the recycled mobile phase was used for 3 working days and was refreshed after 3 days. The loop volume that was used for injections was 20 µl.

■ Assay validation

Partial validation experiments were carried out according to the US FDA recommendations [24]. The calibration, linearity, LOD, LLOQ and ULOQ, intra- and inter-day precisions, accuracy, recovery, selectivity, stability (room temperature, refrigerator [4°C and freeze–thaw]) and robustness were tested for each drug.

The mean of three calibration curves (produced on three different days) was used for the validation studies. The mean of three replicates was used for all quantifications. The lowest concentration of calibration curve was selected as the LLOQ while the RSD% of three replications was less than 20% and for ULOQ the highest concentration of calibration curve selected while the RSD% of three replicates was less than 15% was, according to FDA recommendations.

Results & discussion

■ Optimization of separation

Chromatographic conditions

Both verapamil and metoprolol possess positive charge according to their pKa values in acidic solutions, whereas for furosemide the neutral form is predominant in this pH range, and then it is obvious that the initial separation can be obtained using the acidic mobile phases (2–6). Considering the logP values of metoprolol (1.79) and verapamil (3.90) it is predicted that these drugs could be separated using a micellar media with moderate SDS concentration. In addition, it is proved that the addition of short-chain alcohols is essential to improve efficiency

for micellar mobile phases [16,25]. We selected 1-butanol because of its better miscibility with micellar solution in comparison with 1-pentanol. Studies showed that the column temperature is an effective parameter on the efficiency in micellar mobile phases; according to these considerations, different mobile phases were designed based on the pH (2–6), column temperature (25–40°C), SDS concentration (150–250 mM), 1-butanol (3–6% v/v), TEA (0–1% v/v) and flow rate (1.0–2.2 ml/min). The retention time and resolution were checked to select the optimized condition. We began the initial experiments with 1-butanol-250 mM SDS buffer (4–96% v/v). After checking the pH (2–6) we found that the drugs are eluted with higher resolutions in pH 3. The next experiments based on the combined effects of pH (2 and 3) and temperature (25–40°C) showed that pH 3 at 40°C resulted in better resolution and more stable baseline. Our experiments showed that higher temperatures result in lower retention times and narrower peaks accordingly. Further experiments to optimize the SDS concentrations showed that a lower concentration (i.e., 150 mM) gives the desired resolution and retention times at higher flow rates (i.e., >2 ml/min) and higher SDS concentrations are not needed. 1-butanol volume percentage optimization showed that increased concentration leads to decreased retention time, but we stopped it at 6% v/v to avoid micelle structure modification. Our experiments about the effect of TEA confirmed the previous study [25] about the positive effect on separation efficiency and 1% v/v added to the final mobile phase. Finally, a mobile phase of 1-butanol/buffer (150 mM SDS/25 mM Na_2HPO_4)/TEA (6/93/1%, v/v/v) was selected, with the buffer pH of 3 and the flow rate of 2 ml/min. The column was incubated at 40°C during analysis and all mobile phases were allowed to equilibrate for 120 min. The injected volume was 20 µl and the loop was washed using the mobile phase before each injection. The retention times of analytes in this condition were 3.6, 6.4 and 11.6 min for furosemide, metoprolol and verapamil, respectively. The sample chromatograms are provided in Figure 2.

Plasma sample preparation

The main advantage of the MLC method is possibility of direct injection (e.g., after dilution or filtration), of the biologic samples to the chromatographic system, as claimed in the literature [17].

In some cases dilution is not applicable because of the low therapeutic concentration of the target drugs. In this study we encountered such a problem and our experiments showed that using a partial precipitation method is enough to prepare plasma samples for injection and there is no need for complete precipitation, which is essential in conventional HPLC in order to avoid column clogging. In this method, 500 µl of acetonitrile was added to a 500 µl plasma sample and after vortexing for 1 min, the mixture was centrifuged for 10 min (12000 rpm [8050 × g]) and the supernatant was injected into the chromatographic system. The total sample preparation time was 12 min, which is acceptable for bioanalytical methods. The quenching effect of acetonitrile on the fluorescence emission of the drugs was not a limiting factor for our analysis.

Assay validation

Linearity & calibration curves

Calibration curves were constructed by plotting the response function (peak area) against the corresponding concentrations at five increasing levels for verapamil (0.100–0.800 µg/ml), metoprolol (0.100–0.800 µg/ml) and furosemide (0.5–4.0 µg/ml) on three different days. The linearity was evaluated by the correlation coefficients (r > 0.999 for all calibrations). The details of mean calibrations and corresponding validation parameters (LOD, LLOQ and ULOQ) along with therapeutic levels of the studied drugs [26] are summarized in Table 2. The RSD% (precision) and relative error (RE)% (accuracy) values for calibration data are reported in Table 2.

Selectivity & specificity

Six randomly selected drug-free human plasma samples were injected and the results showed that there are no matrix contributing peaks for metoprolol and verapamil, for furosemide we found a plasma peak just after the furosemide peak. The resolution between this peak and furosemide peak was better for frozen samples. Figure 2 A1,A2 and B1,B2 show the desired chromatograms. In order to check the selectivity of the proposed method we checked some other cardiovascular drugs (i.e., propranolol, atorvastatin, amiodaron, diltiazem, hydrochlorothiazide, amiloride, carvedilol, amlodipine, losartan, lovastatin, simvastatin, lisinopril, captopril, enalapril, atenolol and nifedipine) and some commonly used substances such as caffeine, ibuprofen, acetaminophen, diazepam and salicylic acid.

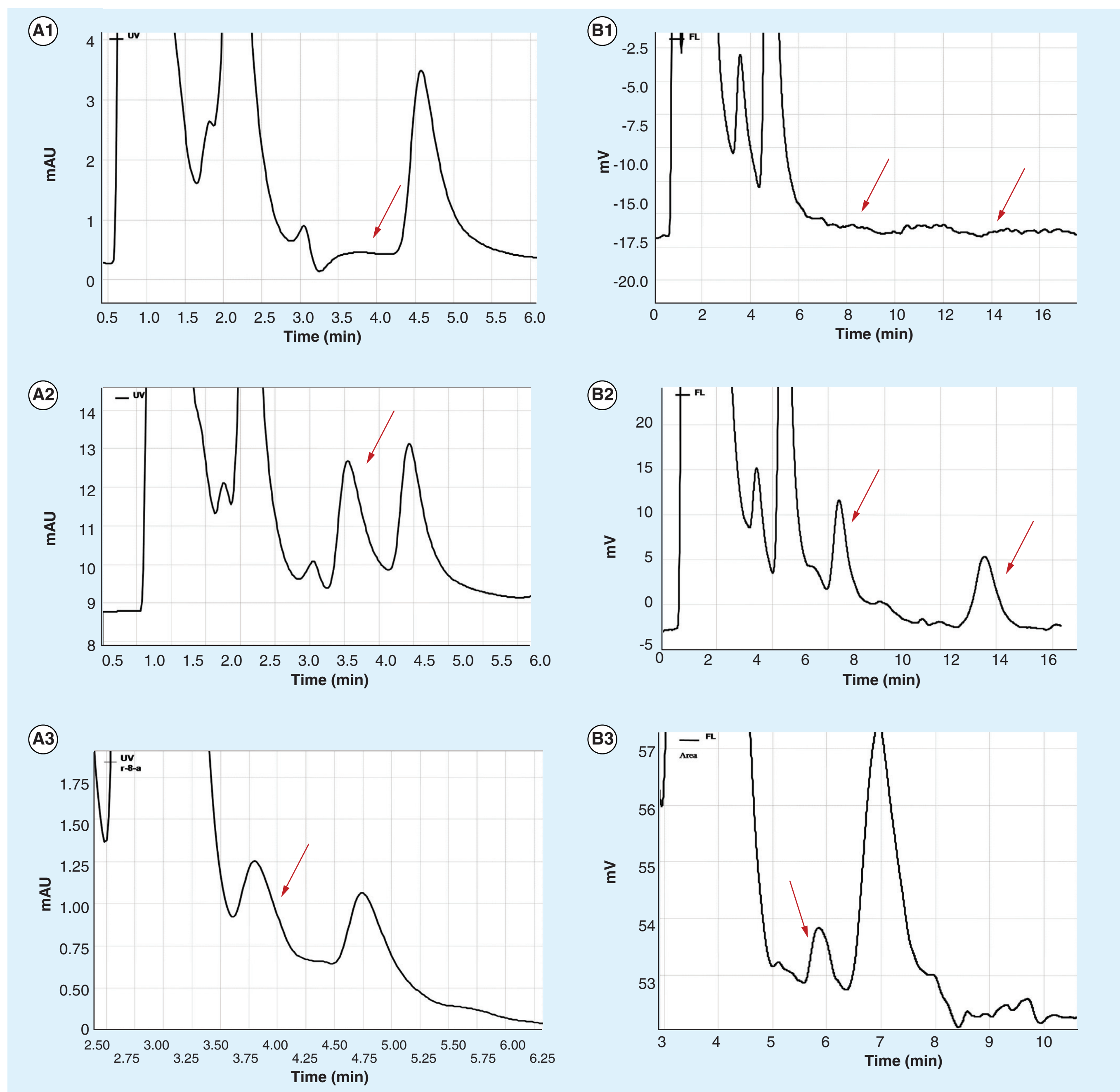

Figure 2. Chromatograms of free drug plasma samples (A1 and B1), 0.4 µg/ml spiked plasma sample (A2 for furosemide and B2 for metoprolol and verapamil) and real plasma sample (A3 for furosemide and B3 for metoprolol).

The experiments showed that although some of them eluted (losartan, salicylic acid diltiazem, propranolol, amlodipine and carvedilol), with the developed mobile phase they do not interfere with the desired drugs and their elution times are before or after furosemide, verapamil or metoprolol.

■ Precision & accuracy

The results of intra-assay precision and accuracy of calibration standards are shown in Table 3. All values for LLOQ and higher concentrations were below 15%. Inter-assay and intra-assay precision along with accuracy for QC samples are listed in Table 4. The similar results obtained for both these validation experiments demonstrated that the developed method is both accurate and precise.

■ Recovery

The mean recoveries (recovery ranges) for verapamil, metoprolol and furosemide were

Table 2. Validation data of the proposed method for quantification of metoprolol, verapamil and furosemide in human plasma with pKa, logP and therapeutic range of drugs in plasma.

Parameters	Verapamil	Metoprolol	Furosemide
Linear range (µg/ml)	0.1–0.8	0.1–0.8	0.5–4
Slope	1.88	1.56	0.33
Slope (standard error of mean)	0.02	0.02	0.00
Intercept	-37.98	-48.17	68.83
Intercept (standard error of mean)	9.67	8.48	9.06
Correlation coefficient	0.9998	0.9997	0.9997
Number of data points	5	5	5
LOD (µg/ml)	0.015	0.016	0.081
LOQ (µg/ml)	0.051	0.054	0.272
LLOQ (µg/ml)	0.100	0.100	0.500
pKa	8.97	9.17	3.9
logP	3.90	1.79	2.0
Therapeutic range (µg/ml)	0.05–0.25	0.02–0.50	1.0–6.0

100.2 (97.7–103.0)%, 100.1 (97.5–104.5)% and 101.9 (93.5–108.1)%, respectively.

Stability

According to the stability experiments the plasma samples were stable after 12 h at room or refrigerator temperature and also the freeze–thaw cycles caused no significant instability. The highest deviation was obtained for furosemide LLOQ at room temperature (RE[%] = 14.6%), which is acceptable (<20%) for biological samples according to FDA recommendations. The only recommendation is to analyze the plasma samples within 1 h after precipitation with acetonitrile in order to avoid possible degradation of the drugs in the presence of acetonitrile. The stability results are summarized in TABLE 5.

Drugs are not stable in ACN-containing solution for more than 120 min. The instability was more obvious for lower concentrations, especially for verapamil. We checked the possibility of the sample dilution with water instead of precipitation and we found that it is possible to use at least five-times dilution with water for higher concentrations (e.g., peak plasma concentration of the studied drugs).

Table 3. Intra-assay precision and accuracy of calibration standards.

Drug name	Nominal conc. (µg/ml; n = 3)	Found conc. (µg/ml; n = 3)	Precision (RSD%)	Accuracy (RE%)
Metoprolol	0.1	0.105	6.9	5.3
	0.2	0.199	9.5	-0.5
	0.4	0.391	3.2	-2.3
	0.6	0.598	0.8	-0.4
	0.8	0.803	3.7	0.4
Verapamil	0.1	0.106	8.9	6.2
	0.2	0.197	1.8	-1.7
	0.4	0.396	2.3	-0.9
	0.6	0.596	4.9	-0.7
	0.8	0.806	5.3	0.6
Furosemide	0.5	0.528	7.3	5.7
	1.0	0.969	2.9	-3.1
	2.0	2.049	4.2	2.5
	3.0	3.024	5.9	0.8
	4.0	4.045	3.5	1.1

RE: Relative error.

Robustness

The robustness of the method was checked by making slight variations to the chromatographic parameters: pH (2.9, 3.0 and 3.1), column temperature (39.5, 40.0 and 40.5°C), mobile phase flow rate (1.9, 2.0 and 2.1 ml/min), 1-butanol concentration (5, 6, 7% v/v) and three replicate injections of 0.4 µg/ml spiked plasma sample, were studied under the desired conditions. The results showed negligible differences in peak area and retention times. There is just a considerable effect for 1-butanol variation, which will be avoidable by preparing high volumes (e.g., 500 ml) of the mobile phase.

Application to real samples

In order to check the applicability of the developed model in the analysis of real unknown samples, six different plasma samples were analyzed. The frequently administered combination of these drugs was metoprolol–furosemide, and in one case metoprolol–verapamil had been prescribed despite their severe contraindication. FIGURE 2 A3 AND B3 shows two chromatograms of analyzed samples. The only problem occurred with furosemide, where a matrix peak coeluted, making the quantitative measurement difficult for some samples. The quantities of the detected drugs were sometimes below the LLOQ of our method and because of that in some cases the RSD% values were high, but as can be seen in the chromatograms (FIGURE 2) the peaks were completely detectable.

Conclusion

A rapid and cost-effective benefit MLC–UV fluorimetric method has been developed to separate and quantify three frequently used cardiovascular drugs (i.e., furosemide, metoprolol and verapamil) from human

Table 4. Assay precision and accuracy of quality control samples.

Drug name	Conc. (µg/ml)	Intra-assay precision (RSD%)	Inter-assay precision (RSD%)	Accuracy (RE%)
Metoprolol	0.1	2.4	4.7	10.3
	0.4	3.4	2.3	-5.0
	0.8	1.4	0.4	-1.1
Verapamil	0.1	5.3	5.9	5.1
	0.4	3.7	0.9	0.5
	0.8	3.9	0.6	2.8
Furosemide	0.5	2.4	10.6	8.1
	2.0	1.9	2.7	6.6
	4.0	3.8	3.5	2.1

RE: Relative error.

plasma. The developed method is applicable for pharmacokinetic, pharmacodynamic and therapeutic drug monitoring studies. The main advantages of the developed method are simple sample preparation and minimal use of organic solvents. In comparison with previous MLC methods for the analysis of mentioned drugs, the method has the advantage of being able to simultaneously analyze the studied drugs, in addition to the validation of the method according to FDA guidelines. The developed method can be used as a routine analytical method in drug analysis laboratories.

Future perspective

The use of MLC method for the routine analysis of some drugs in therapeutic drug monitoring laboratories (i.e., drugs with fluorescence emission or high therapeutic concentrations) will be possible. In addition, simpler sample pretreatment methods can be developed by using MLC.

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Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Table 5. Stability data for studied drugs.

Drug	Conc. (µg/ml)	Freeze–thaw stability		Room temperature stability		4°C stability	
		Conc. found (µg/ml)	Accuracy (RE%)	Conc. found (µg/ml)	Accuracy (RE%)	Conc. found (µg/ml)	Accuracy (RE%)
Metoprolol	0.1	0.109	9.4	0.103	2.7	0.105	5.5
	0.4	0.384	-4.0	0.399	-0.1	0.403	0.8
	0.8	0.814	1.8	0.859	7.3	0.782	-2.3
Verapamil	0.1	0.116	15.7	0.091	-8.8	0.112	12.4
	0.4	0.374	-6.4	0.378	-5.4	0.387	-3.3
	0.8	0.808	1.0	0.890	11.3	0.760	-5.0
Furosemide	0.5	0.476	-4.8	0.573	14.6	0.471	-5.8
	2.0	1.852	-7.4	1.999	-0.1	1.946	-2.7
	4.0	3.848	-3.8	4.304	7.6	3.893	-2.7

RE: Relative error.

Executive summary

- An isocratic micellar LC method has been developed for the simultaneous analysis of cardiovascular drugs (verapamil, metoprolol and furosemide) in plasma.
- A combination of UV fluorimetric detector was used for the detection of drugs.
- The developed method has been validated according to US FDA guidelines.
- The method was applied to the analysis of some real samples.
- The micellar LC method is applicable for the analysis of cardiovascular drugs in biologic samples.
- The stability of drugs in pretreated plasma samples should be checked during method development.

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