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On-line column-switching high-performance liquid chromatography analysis of cardiovascular drugs in serum with automated sample clean-up and zone-cutting technique to perform chiral separation

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Abstract

A selective and highly reproducible, multi-column HPLC method is described for the analysis of the following cardiovascular drugs: lidocaine, pindolol, metoprolol, oxprenolol, diltiazem and verapamil, in serum. Column-switching devices are employed in combination with advanced separation media technologies for the automated analysis of samples containing complex matrices. The method consists of on-line sample clean-up using a restricted access sorbent, HPLC analysis of the drugs on a microsphere non-porous silica RP-18 column, and front-cutting to perform the chiral separation of pindolol enantiomers on a second HPLC system. Simultaneous control of the two HPLC systems and data analysis is achieved from a single centralized software. The R.S.D. values of the peak areas for spiked serum are less than 1% for metoprolol and oxprenolol, 2–5% for lidocaine, diltiazem and verapamil, and 1.2 and 2.4% for the two pindolol enantiomers. Recoveries, limits of detection and linearities are provided.

Keywords: Column switching; Enantiomer separation; Lidocaine; Beta-blockers; Diltiazem; Verapamil

1. Introduction

In the analysis of complex biological samples, an efficient HPLC separation combined with a sophisticated mode of detection does not always provide the required sensitivities and selectivities. In such instances, attention must be drawn also to the pretreatment part of an analytical method. With the larger series of sample to be processed, alternatives to traditional sample preparation techniques are increasingly sought. On-line column-switching devices combined with advanced separation media technologies represent a powerful and reliable solution to the automated clean-up and trace enrichment of drugs in complex biological samples, with a consequent improvement in the analytical process [1,2].

Among the column-switching techniques which transform off-line multi-step methods into fully automated procedures, zone-cutting is found to be one of the most useful and versatile. This technique involves the transfer of a fraction of the chromatographic effluent to a second column in order to perform a further separation of components [2].

The introduction of recent separation media technologies has been essential to the development of new column-switching applications. For example, restricted access (RA) sorbents, in the form of extraction cartridges fitted to the switching valve of a sampling injector, have been shown to provide a highly efficient on-line clean-up of protein-containing samples, before HPLC analysis using conventional columns [3–6]. Designed for the deproteinization of biological samples, RA sorbents are characterized by a hydrophillic external surface incor-

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porating pores coated with a reversed-phase, or more specific phase dedicated to selective extractions [7,8]. Interfering proteins are excluded from the pores by hydrophillic and size exclusion interactions, and washed off directly. Drug analytes are retained on the bonded phase of the sorbent pores before elution onto the analytical column.

The chiral chromatographic separation of β-blockers, one of the most widely prescribed and therapeutically important groups of drugs, has been extensively reviewed [9]. In general, the use of chiral stationary phases is the preferred approach to chiral separation. For the separation of β-blockers enantiomers, the use of the α_1-acid glycoprotein (AGP) column has been widely documented [10].

In the pharmacokinetic and pharmacodynamic study of cardiovascular drugs, it is important to monitor drug levels both quickly and accurately. In addition, where treatments involve the combined administration of several drugs, the analytical method should ideally permit the simultaneous determination of all the analytes present in the sample. This paper presents a column-switching method for the direct HPLC analysis of a number of cardiovascular drugs in serum, including: one antiarrythmic drug, lidocaine; three β-blockers, pindolol, metoprolol and oxprenolol; and two calcium-channel inhibitors, diltiazem and verapamil. With use of a sampling injector equipped with two independent six-way valves, a rapid and efficient on-line sample clean-up is performed using a RA cartridge. The different drugs are then separated by HPLC, and the sampling injector performs a selective front-cut of the pindolol fraction for enantiomeric analysis on a second HPLC system.

2. Experimental

2.1. Materials

Chemicals were of analytical grade and solvents were of HPLC grade. The standards (lidocaine, pindolol, metoprolol, oxprenolol, diltiazem and verapamil) and the reagents (phosphoric acid 85% v/v; mono and dibasic potassium phosphate anhydrous) were obtained from Sigma (St. Quentin Fallavier, France). Lyophilized human drug-free serum was obtained from Bio-rad (Ivry sur Seine, France), and redissolved in water before its use, according to the manufacturer's recommendations. Water and acetonitrile were obtained from J.T. Baker (Paris, France). Phosphate buffers of pH 2.5 and pH 7.0 were prepared with mono- and dibasic potassium phosphate, respectively, and adjusted with phosphoric acid. All buffers and HPLC eluents were prefiltered (0.2 μm) and degassed with helium.

2.2. Instrumentation

2.2.1. Sampling injector for column switching

Column switching was carried out using a Gilson 233 XL Dual-valve Sampling Injector (Gilson Medical Electronics, Villiers le Bel, France), consisting of a large-capacity XYZ sampling injector, fitted with two automated six-port Model 7010 valves (Rheodyne, Berkeley, CA, USA) and a Model 402 syringe pump equipped with 1 ml and 100 μl syringes. The sampling injector was configured for on-line clean-up with HPLC analysis, and zone-cutting for performing a second HPLC analysis (Fig. 1). The left valve of the injector was fitted with a Pinkerton GFF2 restricted access sorbent cartridge from Interchim (Montluçon, France). The right valve was fitted with a LiChrospher RP-18 enrichment cartridge from Merck (Nogent sur Marne, France). Control was from the 720 (V2.0) keypad software.

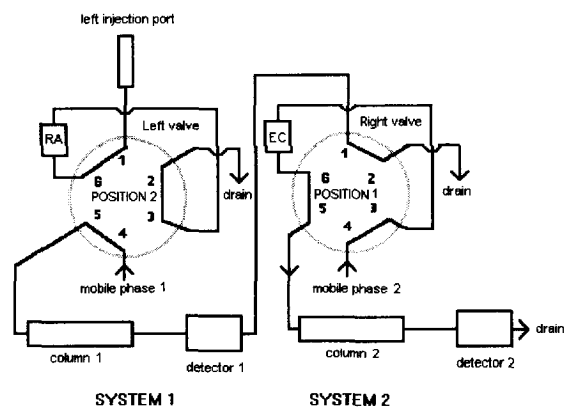

Fig. 1. Valve system diagram for on-line sample clean-up and front-cutting technique (initial conditions). RA: restricted access sorbent cartridge. EC: enrichment cartridge.

2.2.2. HPLC

The 233 XL sampling injector was interfaced to two HPLC systems (Gilson Medical Electronics). System 1, installed on the left valve of the sampling injector (Fig. 1), consisted of two Model 306 pumps fitted with 5SC pump heads, a Model 805 manometric module, a Model 811C mixer module equipped with 700 μl chamber, a Model 119 UV–Vis detector equipped with a high pressure cell. The HPLC column used in system 1 was a Micra RP-18 non-porous silica column from Bischoff (Leonberg, Germany). System 2, installed on the right valve of the sampling injector, consisted of one Model 307 pump fitted with 10WSC pump head and a Model 119 UV–Vis detector. The column used in system 2 was a Chiral-AGP column from Interchim (Montluçon, France). Simultaneous control of both HPLC systems and data handling was provided by UniPoint (V1.1) System Software.

2.3. Methodology

The sample preparation and chromatographic conditions of the method were optimized using analytical standards in 10 m*M* phosphate buffer of pH 7.0. Each standard was prepared from a stock solution of 2.5 mg/ml in methanol, and diluted with the buffer to the following concentrations: lidocaine (25 μg/ml), pindolol (5 μg/ml), metoprolol (20 μg/ml), oxprenolol (20 μg/ml), diltiazem (10 μg/ml) and verapamil (10 μg/ml). The method was then applied to spiked serum samples containing lower analyte concentrations to obtain the analytical results described in the following section. When stored at 4°C and protected from light, samples were stable for a week. After filtration (0.2 μm), sample was loaded onto the 233 XL sampling injector for clean-up and analysis according to the two methods described in Fig. 2 and Table 1. Sample (20 μl) was injected via the left injection port of the sampling injector onto the RA sorbent cartridge. Cardiovascular drugs were retained on the cartridge whilst the proteinaceous fraction was washed away. The left valve of the sampling injector was then switched, and HPLC mobile phase 1 transferred the analytes from the cartridge onto analytical column 1. Following detection, pindolol was trapped on the enrichment cartridge (EC), then the right valve of the injector was switched and the front-cut was back-flushed using mobile phase 2 onto column 2 for separation of the enantiomers.

SYSTEM 1

Sample Preparation (left valve)

Cartridge:	Pinkerton GFF2, 5 μm (14 x 4.6 mm)
Sample:	20 μl
Eluents:	Reservoir filled with water Conditioning and washing eluent prepared with 10 mM phosphate buffer at pH 7.0

HPLC

Column 1:	Micra NPS RP-18, 1.5 μm (33 x 4.6 mm)
Mobile phase 1:	Binary gradient at 1.0 ml/min

time (min)	% acetonitrile	% 10mM phosphate buffer (pH 2.5)
0	0	100
2	0	100
7	30	70
10	30	70
11	0	100

Temperature:	25°C
Detection:	UV at 220 nm (0.01 AUFS, 10 mV)

SYSTEM 2

Sample Preparation (right valve)

Cartridge:	LiChrospher RP-18, 25 μm (25 x 4.6 mm)
Sample:	Front-cut from system 1

HPLC

Column 2:	Chiral-AGP, 5 μm (100 x 4.0 mm)
Mobile phase 2:	30 mM phosphate buffer at pH 7.0–acetonitrile (9:1) at 1 ml/min
Temperature:	25°C
Detection:	UV at 220 nm (0.001 AUFS, 10 mV)

Fig. 2. System 1 and system 2 conditions.

3. Results and discussion

3.1. Optimization of sample clean-up and drugs separation with system 1

In the optimization of the sample clean-up step, various RA sorbents were tried. The cartridge was selected according to the following criteria: (i) efficient removal of matrix interferents using a minimum of washing solvent, and (ii) retention of all analytes on the cartridge before complete transfer to the analytical column using HPLC mobile phase 1. The Pinkerton GFF2 cartridge was found to provide the best selectivity and reproducibility. Washing conditions were optimized using analyte concentrations well above their typical therapeutic ranges in

Table 1
Sequence of 233 XL valve positions and description of the corresponding events

Step	Event	Valve position (left/right)
1	RA cartridge conditioning (left valve) 2×944 μl, 0.5 ml/min	2/1
2	Sample loading via left injection port	2/1
3	RA cartridge washing 800 μl, 0.5 ml/min	2/1
4	Analytes transfer to column 1	1/1
5	Front-cut adsorption on EC cartridge (right valve)	1/2
6	Front-cut transfer to column 2	1/1

serum (from 5 to 25 μg/ml) in order to set the maximum limits. The study showed that the breakthrough volumes using 10 m*M* phosphate buffer were highest at an optimum pH value of 7.0, ranging from 750 μl for lidocaine (at pH 3.0 the breakthrough volume was only 100 μl) to over 1.6 ml for diltiazem and verapamil. A minimum volume of 800 μl of 10 m*M* phosphate buffer was required to completely remove interfering proteins from 20 μl of serum. Hence, for the washing of the RA cartridge, conditions were set at 800 μl of 10 m*M* phosphate buffer of pH 7.0 (see step 3 in Table 1).

The objective for the HPLC separation was to achieve the best resolution of all analytes in the shortest possible time, using minimal organic modifier to enable the trapping of pindolol on the enrichment cartridge of system 2. The use of conventional reversed-phase columns (C_{18} and C_8) was unsatisfactory since acetonitrile concentrations of over 10% were required to achieve acceptable retention times (using 10% acetonitrile, the first peak, lidocaine, is detected only after 5.7 min). Thus a microsphere non-porous silica C_{18} column was selected which provided a high-resolution, high efficiency separation using far less organic modifier than with the use of conventional porous silica columns [7]. The separation shown in Fig. 3 was performed with a gradient elution (Fig. 2). Due to the chemical nature of the analytes, the pH of the aqueous mobile phase was set at 2.5 to obtain the narrowest peak width and to achieve the best resolution between the lidocaine and pindolol; at pH 3.0 the resolution (R_S) was 0.8, peaks were broader and retention times were longer. To achieve maximum recovery from the enrichment cartridge, the acetonitrile concentration was kept as low as possible during the first few minutes of pindolol trapping. The mobile phase gradient was then adjusted to optimize the separation of diltiazem and verapamil (peaks 5 and 6, respectively), to give a final concentration of 30% acetonitrile after 7 min. This concentration of organic modifier was sufficient to completely release the two last eluting analytes from the Pinkerton GFF2 cartridge. In Fig. 3, a comparison is provided of chromatograms obtained using standards diluted in 10 m*M* phosphate buffer of pH 7.0, spiked serum and drug-free serum. These results demonstrate an excellent correlation between standards and spiked serum of identical concentration (respectively lidocaine: 10 μg/ml, pindolol: 2 μg/ml, metoprolol: 4

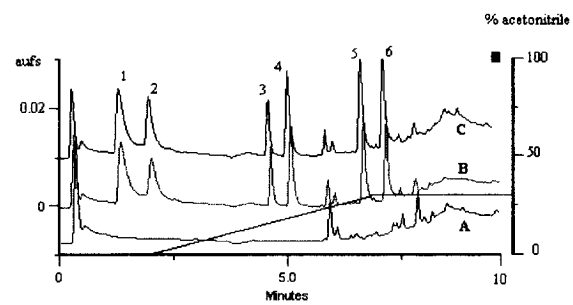

Fig. 3. On-line clean-up and analysis of cardiovascular drugs in serum with HPLC system 1. 1=lidocaine, 2=pindolol, 3=metoprolol, 4=oxprenolol, 5=diltiazem, 6=verapamil. (A) drug-free serum, (B) standards in buffer, (C) spiked serum.

μg/ml, oxprenolol: 4 μg/ml, diltiazem: 2 μg/ml and verapamil: 4 μg/ml), with no major interference observed from the drug-free serum.

3.2. Front-cutting with optimization of enrichment and separation of pindolol enantiomers

Two solutions exist for the trapping of analytes using the zone-cutting technique: the use of a sample loop (of 500 μl to over 1 ml) or an enrichment cartridge. The sample loop approach was unsuitable due to the large differences in pH value of HPLC mobile phases 1 and 2 (pH values of 2.5 and 7.0, respectively). An enrichment cartridge was therefore used to minimize the trapping volume. The Li-Chrosorb RP-18 cartridge gave the best results in terms of the retention and recovery of pindolol. Indeed, this sorbent provided a correct retention during the loading of the eluent from system 1 (the breakthrough volume was over 2 ml for pindolol at 5 μg/ml), with a rapid release of enantiomers during elution with mobile phase 2 which contained 10% acetonitrile. The retention time of pindolol on HPLC system 1 was 1.95 min. Therefore, to allow a margin security in the switching of the right valve of the sampling injector, loading onto the enrichment cartridge was started at 1.50 min, with injection onto system 2 at 3.80 min (steps 5 and 6 in Table 1).

The strategy for the separation of pindolol enantiomers using a Chiral-AGP column was taken from the literature [10,11] and adapted to this application. A critical point in the separation was the buffer concentration. In initial tests using 10 m*M* phosphate buffer, a splitting of enantiomer peaks was observed, probably as a result of the incompatibility of the different pH values of system 1 and system 2 mobile phases. This problem was resolved by increasing the buffer concentration to 30 m*M*. A pH value of 7.0 for mobile phase 2 was found to provide the best enantiomeric resolution with acceptable retention times. Fig. 4 shows the excellent correlation of chromatograms achieved for pindolol enantiomers of identical concentration in a standard solution and spiked serum with no interference observed from the drug-free serum.

The time between the injection of one sample and the next one was only 16 min. This short processing time was achieved by operating the sampling injector in its concurrent mode and working the two systems simultaneously (including HPLC analysis and re-conditioning).

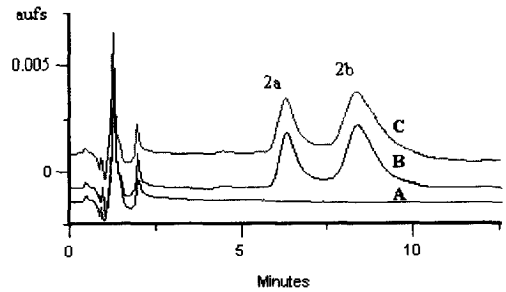

Fig. 4. Chiral analysis of the front-cut with HPLC system 2. 2a and 2b pindolol enantiomers. (A) drug-free serum, (B) standards in buffer, (C) spiked serum.

3.3. Analytical data

Table 2 shows a summary of the reproducibility and the reliability of this technique. The R.S.D. ($n=10$) of retention time were less than 1.2% for all analytes; and the R.S.D. of peak area were below 1% for metoprolol and oxprenolol, and between 2 to 5% for other compounds. Recoveries (calculated as percentage of spiked standards injected onto RA cartridge vs. spiked standards injected directly onto HPLC column) were satisfactory for all drugs, except lidocaine, which has a high polarity and was therefore not completely retained by the RA cartridge. Despite a low recovery, an acceptable R.S.D. of peak area for lidocaine was obtained. Table 3 gives LOD and linearities of cardiovascular drugs in serum using the proposed technique. Most data meet the typical therapeutic ranges. Limits of detection varied from 20 to 300 ng/ml. Since standards for the pindolol enantiomers were commercially unavailable, their corresponding LOD could not be determined and were thus estimated at approx. 10 ng/ml. LOD of diltiazem and verapamil were a bit high due to minor interfering peaks appearing as a result of the gradient elution of system 1 (Fig. 3). To lower these values and hence extend the linearity ranges for diltiazem and verapamil, it is suggested that isocratic con-

Table 2
Analytical data of the automated determination of cardiovascular drugs in serum with system 1 and 2

Analyte	t_R (min)	Conc. (μg/ml)	R.S.D.$_{t_R}$[a] (%)	R.S.D.$_{area}$[b] (%)	Recovery (%)
Lidocaine	1.33	10	0.6	4.4	80
Pindolol[c]	1.95	2	1.2	3.2	–
Metoprolol	4.60	4	0.2	0.4	98
Oxprenolol	5.04	4	0.2	0.6	100
Diltiazem	6.66	2	0.1	2.0	100
Verapamil	7.16	4	0.1	4.9	100
Pindolol a[d]	6.25	–	0.6	1.2	93
Pindolol b[d]	8.28	–	0.9	2.4	98

[a] R.S.D. of retention time ($n = 10$).
[b] R.S.D. of peak area ($n = 10$).
[c] Total pindolol analysed from system 1.
[d] Pindolol enantiomer analysed from system 2.

Table 3
Limits of detection and linearities of cardiovascular drugs in serum with system 1

Analyte	LOD[a] (ng/ml)	Linearity[b]			Range of linearity (μg/ml)
		Slope	Intercept	r	
Lidocaine	300	19688	2739	0.9993	1–20
Pindolol[c]	20	157	971	0.9995	0.05–1.0
Metoprolol	20	59	1049	0.9998	0.05–1.0
Oxprenolol	30	69	−2824	0.9997	0.08–1.0
Diltiazem	60	106	−8750	0.9992	0.15–2.0
Verapamil	80	41	−12189	0.9988	0.20–3.0

[a] Limit of detection at a signal-to-noise ratio of 3.
[b] Calibration data (based on areas) obtained from UniPoint system software; r = correlation coefficient; 4 calibration points.
[c] Total pindolol.

ditions be adopted for these specific compounds. The upper values for linearity were well above those found in a typical therapeutic range and are hence not reported here.

4. Conclusion

This work demonstrates the advantages of combining versatile on-line column switching devices with recent separation media technologies. A fully automated method is described for high-throughput sample clean-up and analysis, and is applied in the determination of cardiovascular drugs in serum to give accurate and reliable results. Ideally suited to the pharmacokinetic and pharmacodynamic studies involved in drug monitoring, the method can be easily adapted to other drugs and metabolites with minimal method development. Furthermore, since metabolites generally exhibit similar chromatographic characteristics to their parent compounds, with only slight adjustment of mobile phase conditions, the same methodology can be applied in both the separation of drugs and their metabolites.

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