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APPLICATION OF COLUMN SWITCHING IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF MEDROXALOL IN PLASMA

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SUMMARY

An automated high-performance liquid chromatographic (HPLC) column-switching system is described for the analysis of medroxalol, a potential antihypertensive agent, in plasma. The HPLC system uses two six-port switching valves with a Corasil® C_{18} short pre-column for an on-line sample clean-up and an SGE ODS analytical column for separation. Plasma samples were diluted with a phosphate buffer (pH 7.2) containing an internal standard and aliquots were injected directly on the HPLC system. The column-switching system was applicable to continuous analysis of hundreds of plasma samples since this technique provided very efficient on-line sample clean-up and regenerated the pre-column effectively. Results were in good agreement and the total analysis time was one third that of an alternative method.

INTRODUCTION

Medroxalol, 5-[2-[(3-(1,3-benzodioxol-5-yl)-1-methylpropyl)amino]-1-hydroxyethyl]-2-hydroxybenzamide (I, Fig. 1), is a potential antihypertensive agent which has been shown to lower blood pressure primarily by decreasing peripheral vascular resistance.

Pharmacologic studies have demonstrated that the hemodynamic changes can be attributed to α- and β-adrenergic receptor blockade as well as a direct vasodilatory action [1,2]. The intensity of β-adrenergic receptor blockade is proportional to the plasma concentration of medroxalol and the antagonism persists for 24 h after a single dose [3].

Previous analytical methods were reported for the assay of medroxalol in plasma and urine [4,5]. These methods involve two extractions with organic solvent followed by ion-pair reversed-phase chromatography. High-performance liquid chromatography (HPLC) column switching has been increasingly used instead of extraction procedures in sample pretreatment of biological fluids and other

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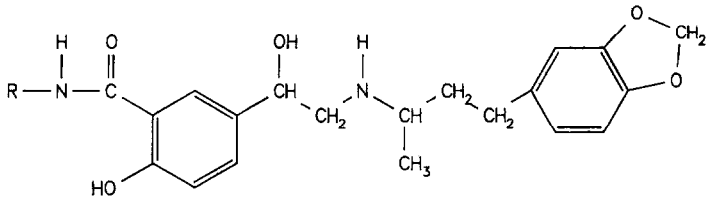

R = H : MEDROXALOL (I)

R = CH_3 : MDL 17,466 (internal standard) (II)

Fig. 1. Chemical structures of medroxalol (I) and the internal standard MDL 17,466 (II).

complex matrices for HPLC analyses. This technique has been widely used for the analysis of drugs in biological matrices [6–10], pesticides and herbicides in food [11–13], and pollutants in water and milk [14,15]. In this study, we examined the applicability of HPLC column switching for a simple on-line sample clean-up and for increased throughput in routine analysis of medroxalol in plasma. Comparison of results on selected plasma samples obtained by HPLC column switching and the current extraction method are presented in this report.

EXPERIMENTAL

Materials

Medroxalol (I) and the internal standard, 5-[2-[(3-(1,3-benzodioxol-5-yl)-1-methylpropyl)amino]-1-hydroxyethyl]-2-hydroxy-N-methylbenzamide (II, Fig. 1) were obtained from Merrell Dow Research Institute (Cincinnati, OH, U.S.A.). All organic solvents were HPLC grade and were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Buffer solutions were prepared from analytical-grade reagents (Mallinckrodt, Paris, KY, U.S.A.).

Instrumentation

The chromatographic system (Waters Assoc., Milford, MA, U.S.A.) consisted of two pumps (M510 and M590 programmable pumps equipped with four-port solvent valves), an automatic sample injector (WISP®) and two six-port automated switching valves (KIT P/n 60057). A Corasil® C_{18}, 37–50 μm, 50 × 4.6 mm I.D. column (Waters Assoc.) and an SGE ODS, 5 μm, 250 × 4.6 mm I.D. column (Scientific Glass Engineering, Austin, TX, U.S.A.) were used for the pre-column and analytical column, respectively. A guard column, Spheri-5® RP-18, 1.5-cm cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.) was connected before the analytical column. The detector was a Schoeffel FS-970 fluorimeter. The excitation wavelength was 228 nm and the emission cut-off wavelength was 389.

Sample preparation

Stock solutions of I and II were prepared in methanol (1 mg/ml as a free base). Appropriate working standard solutions were obtained by proper dilution of the stock solution with methanol. To prepare the standard calibration curve (50–1000

ng/ml), aliquots of I working standard solutions were pipetted into WISP vials and then evaporated to dryness under nitrogen. A 25-μl aliquot of II (10 μg/ml) was added followed by 0.5 ml phosphate buffer (pH 7.2) and 500 μl of control human plasma. The mixture was vortexed and a 200-μl aliquot was injected onto the column-switching system. Dilution of the plasma with the buffer allowed better sampling by the WISP autoinjector.

Unknowns were prepared similarly, substituting plasma samples for control plasma.

Column-switching operation

As shown in Fig. 2, the switching valves could be switched to any of the three positions enabling flow to be directed on the forward-flush mode from the pre-column to the waste (position 1), on the back-flush mode from the pre-column to the waste (position 2), and on the back-flush mode from the pre-column to the analytical column (position 3). Pump 1 (M510) was used to continuously deliver the mobile phase, solvent B [acetonitrile–0.1 *M* ammonium formate (28:72)] to the analytical column at a flow-rate of 1.0 ml/min. Pump 2 (programmable M590) was used to deliver either the aqueous buffer, solvent A [0.1 *M* ammonim formate] or the mobile phase to the pre-column.

Upon injection of a 200-μl plasma sample, the programmed time sequence stored in pump M590 was initiated as shown in Table I. By pumping solvent A to the pre-column, I and II were retained at the head of the pre-column while the polar endogenous components from the biological matrix were washed off and directed to the waste. To prevent peak broadening, the analytes were eluted from the pre-column to the analytical column by pumping solvent B in the back-flush mode. After the analytes were eluted from the pre-column, they were separated on the analytical column and then detected by the fluorimeter. Meanwhile the pre-column was cleaned by back-flushing with solvent B and then equilibrated with solvent A before the next injection. The regeneration of the pre-column with solvent B allowed injection of about 500 samples before replacing the column packing.

Validation study

To test the precision and accuracy of the method, a three-day validation study was conducted. A duplicate seven-point standard curve was processed each day. Two sets of unknowns were also prepared and analyzed by three analysts on different days.

The feasibility of the method was tested by analyzing some plasma samples from a single intravenous (i.v.) dose clinical study. The results were compared to those obtained by the extraction procedure [16].

Calibration and calculations

Peak-height measurements, linear regression analysis, and unknown quantitation calculations were performed using HP 3357 laboratory automation system (Hewlett-Packard, Sunnyvale, CA, U.S.A.).

Peak-height ratios (I/II) versus concentrations were plotted and the calibra-

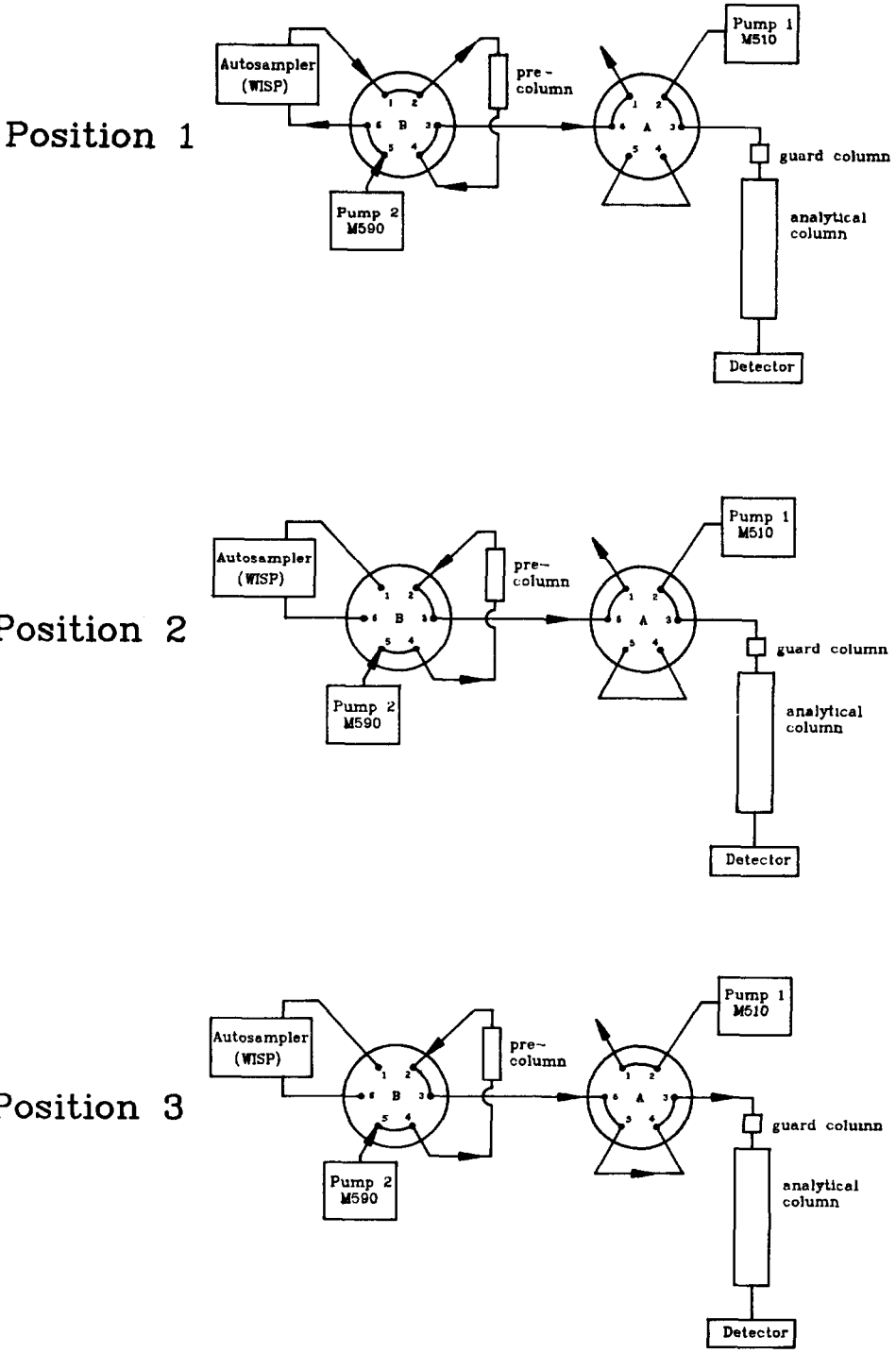

Fig. 2. Schematic diagram of the HPLC column-switching system.

TABLE I

TIME SEQUENCES STORED IN PROGRAMMABLE PUMP M590 FOR THE COLUMN-SWITCHING OPERATION

Cummulative time (min)	Position No.*	Solvent	Flow-rate (ml/min)	Event description
0 (initial)	1	A	1	Inject sample onto pre-column
5.0	1	A	2	Wash-off polar endogenous components
8.0	2	A	1	Valve B in back-flush mode
8.1	2	B	1	Wash line with solvent B
8.5	3	B	1	Elute sample from pre-column to analytical column; start computer
15.0	2	B	2	Disengage analytical column from pre-column and continue analysis; wash-off late eluting components from pre-column
17.0	1	A	1	Equilibrate pre-column for next injection

WISP analysis time: 28 min

*See Fig. 2 for schematic diagram.

tion curve was generated using linear regression analysis ($y=a+bx$). Concentrations of unknowns were determined by interpolation of their peak-height ratios into the calibration curve.

RESULTS AND DISCUSSION

On-line sample clean-up of medroxalol in plasma by HPLC column switching

The optimization of chromatographic conditions in HPLC column switching should include careful choice of the type of columns as well as the mobile phase compositions to obtain adequate on-line sample clean-up, good recoveries, and minimum band broadening of the compounds of interest. Corasil C_{18} (37–50 μm) was chosen to dry-pack the short pre-column. This pellicular packing provided good mass transfer and better recoveries for I and II than the microparticulate packing. The use of bigger-particle-size column packing and 10-μm frit on the pre-column eliminated clogging and subsequent pressure build-up. The initial solvent used in the pre-column should be mostly aqueous but not acidic to avoid partial elution of the compounds of interest and to avoid precipitation of proteins when the plasma sample is injected. In this study, (0.1 M) ammonium formate was a suitable initial solvent to wash off the endogenous biological materials and at the same time retained I and II.

Several reversed-phase analytical columns were investigated without use of ion-pair reagents in the mobile phase. A glass-lined column, SGE C_{18}, was chosen

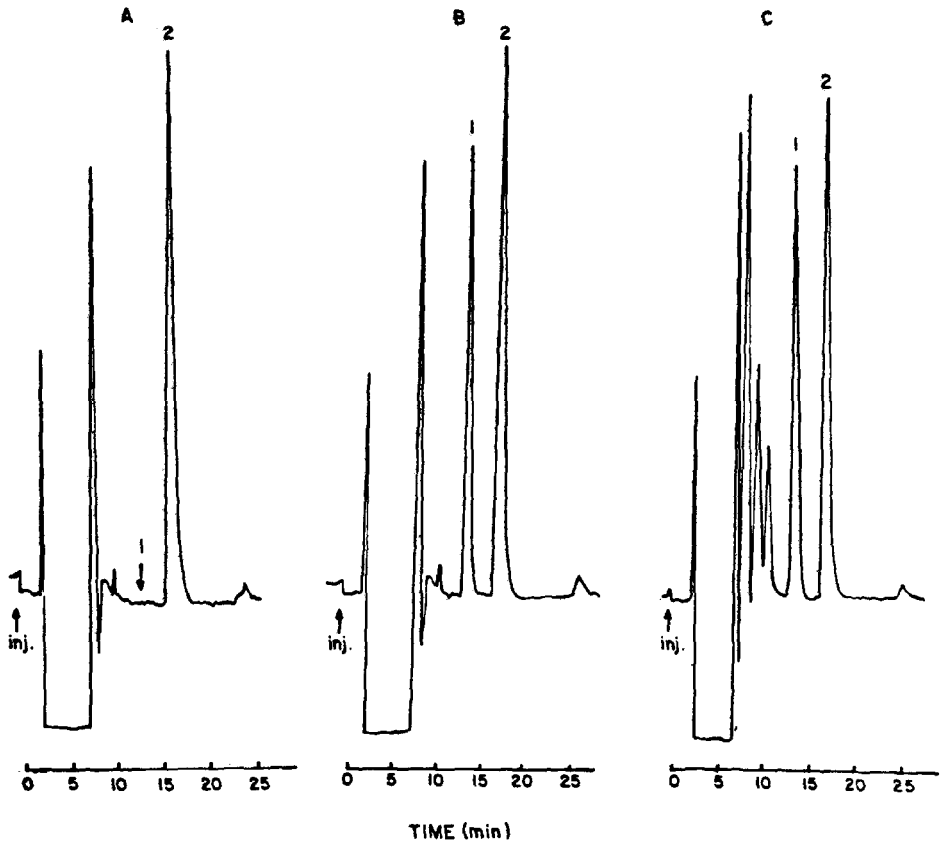

Fig. 3. Typical chromatograms of (A) human plasma spiked with internal standard, (B) human plasma spiked with medroxalol (400 ng/ml) and internal standard, and (C) 5-min post-dose (i.v.) plasma sample from a human subject. Peaks: 1 = medroxalol; 2 = internal standard.

for effective separation and peak symmetry of the analytes. Nova-Pak® C_{18} also afforded equivalent separation. Other reversed-phase columns investigated produced extensive tailing.

To establish the chromatographic conditions and time sequences necessary to automate the analysis, initial experiments were conducted by manual operation. After injection of the plasma sample (fortified with the analytes) onto the pre-column, the initial solvent was pumped at a flow-rate of 2 ml/min for 10 min. After the endogenous biological materials had cleared the pre-column, the pre-column was connected to the analytical column in the back-flush mode and the analytes were eluted with the mobile phase. To calculate the recovery, peak heights were compared to those obtained from the standards (in methanol) that were injected directly onto the analytical column. The integrated peak heights as well as the peak symmetry for the plasma sample and the standards were identical, thus indicating excellent recoveries and no additional band broadening. As illustrated in Fig. 3A, on-line sample clean-up was sufficient as shown by the absence of peak interferences. Fig. 3B and C shows typical chromatograms of plasma samples.

TABLE II

COMPARISON OF RESULTS OBTAINED BY THE EXTACTION PROCEDURE AND HPLC COLUMN SWITCHING

Plasma samples were obtained from human subject given a single 1 mg/kg i.v. dose of medroxalol. Duplicate analyses are shown.

Sample No.	Medroxalol concentration (ng/ml)	
	Extraction method	HPLC column switching
PI 302	745	833
	765	835
PI 303	225	235
	239	227
PI 304	145	137
	145	138
PI 305	104	115
	108	116
PI 306	96	98
	93	103
PI 307	81	84
	82	85
PI 308	79	84
	79	88

Validation study

The method was validated over the concentration range from 50 to 1000 ng/ml. Data obtained on three separate days showed good linearity and reproducibility with mean relative standard deviation (R.S.D.) of about 3%. Plots of peak-height ratio versus concentration had insignificant y-intercepts and average correlation coefficient was 0.999.

The detection limit was defined as 2.5 times the noise level at the sensitivity range for analysis (0.2 μA). Although a concentration of 10 ng/ml was still above the noise level, the lowest value could only be accurately quantitated with good precision and linearity at 50 ng/ml when the internal standard was kept at 500 ng/ml.

The precision and accuracy of the method was demonstrated by analyzing unknown samples. Two sets of unknown samples (five concentrations per set) ranging from 100 to 800 ng/ml in a randomly coded fashion were analyzed by three analysts. Analyst 1 validated the method for three days and analyzed the unknowns ($n=8$) for two different days. Recoveries ranged from 92 to 103% with mean R.S.D. values of 3%. Analysts 2 and 3 did not validate the method and each analyzed the unknowns ($n=4$) for only one day. Recoveries ranged from 101 to 113% with mean R.S.D. values of ca. 2%. Although some of the recoveries were high, the values obtained by the two analysts were in good agreement and good precision.

Application

The feasibility of the method was tested by analyzing some plasma samples from a previous single i.v. dose clinical study. As shown in Table II, the results are in good agreement to those obtained by the current extraction method.

CONCLUSIONS

HPLC column switching is an excellent technique for an on-line clean-up of physiological fluids in the analysis of trace substances. As demonstrated in this work, the pre-column was effectively flushed and regenerated, thus several hundred plasma samples could be injected directly on the system. The analytical column was well protected from the decomposition and the column life was improved. In addition, the total analysis time was short and the recoveries and the reproducibilities were excellent. The analytical procedure was very mild and therefore well suited to the analysis of sensitive compounds.

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