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Robotic sample preparation and high-performance liquid chromatographic analysis of verlukast in human plasma

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Abstract

A fully automated HPLC assay has been developed and validated for the quantitation of verlukast, a leukotriene D_4 antagonist, in human plasma. An upgraded Zymate I robotic system was utilized to perform protein precipitation and on-line injection followed by reversed-phase HPLC with fluorescence detection. Inter-day accuracy and precision were 100.8 and 4.6%, respectively, for the low quality control standards (0.125 μg/ml). The automated robotic method was shown to be more efficient and accurate than the manual method.

1. Introduction

The introduction of laboratory automation via robotics has created a viable alternative to manual sample preparation and assay procedures by freeing a significant amount of analyst time. The need for analyzing large numbers of biological samples is addressed by creating a 24-h work day around the robotics system. The automated system can work "around-the-clock" without constant intervening and monitoring by the analyst. Laboratory robotics have made significant advances in recent years [1–4] among which are the ability to rapidly and reliably add and mix numerous solvents, evaporate, centrifuge, inject on-line, and perform liquid–liquid and solid-phase extractions. By adapting the robotics system to perform previously developed and established manual procedures, a significant amount of time can be saved by the analyst to do more challenging tasks.

As an ongoing effort to demonstrate and evaluate the utility of laboratory robotics for quantifying drug concentrations in biological fluids, a high-performance liquid chromatographic (HPLC) assay for verlukast (**1**, Fig. 1), a potent leukotriene D_4 (LTD_4) receptor antagonist [5–7], has been developed and automated. The method involves reversed-phase HPLC with fluorescence detection after protein precipitation and extraction of the drug from plasma.

In the present paper, an automated method for determination of verlukast in the concentration range of 0.1 to 5 μg/ml in human plasma using an upgraded Zymate I system, is described. The method has been used successfully to support several clinical studies in which normal and asthmatic subjects received aerosol, oral or intravenous doses of verlukast. The system also provides unattended sample preparation and analysis, as well as automated data acquisition,

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integration, and report generation. This automated method is capable of analyzing 120 plasma samples in a period of 15 h.

2. Experimental

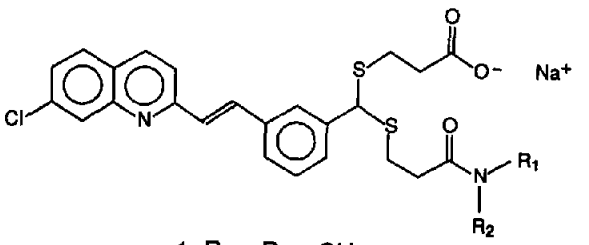
2.1. Materials and chemicals

Verlukast and the internal standard, 3-diethylamino analog (**2**, I.S., Fig. 1) were obtained from Merck Frosst Laboratories (Montreal, Canada). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Springfield, NJ, USA). Analytical-grade phosphoric acid (85%) and ammonium phosphate (dibasic) were from Mallinckrodt (Paris, KY, USA). Triethylamine (gold label) was from Aldrich (Milwaukee, WI, USA). Heparinized human control plasma was supplied by Sera-Tech Biologicals (New Brunswick, NJ, USA). All other reagents were of ACS grade and were used as received.

2.2. Apparatus and chromatographic conditions

A Zymark Zymate I robotic system (Hopkinton, MA, USA) was upgraded to Zymate II robotic system. System peripherals included a System II controller, a general purpose hand, two 50-tube racks, a tube dispenser, a tube disposal station, a dilute and dissolve station, a centrifuge station, a liquid–liquid extraction station, an LC sipping injector, a master laboratory station and a power and event controller.

The chromatographic system consisted of a Perkin-Elmer (Norwalk, CT, USA) Series 10 pump and a McPherson (Boston, MA, USA) Model FL-750 fluorescence detector equipped with a high sensitivity accessory and a Xe–Hg lamp. The detector output was interfaced to Hewlett-Packard (Palo Alto, CA, USA) Model 3357 laboratory automation system via a Hewlett-Packard 1865A analog-to-digital interface. An excitation wavelength of 365 nm and an emission wavelength of 418 nm (band filter) were used for fluorescence detection. The analytical column (15 cm × 4.6 mm I.D.) was packed with Chemcosorb 5-ODS-UH (5 μm, DyChrom, Sunnyvale, CA, USA) and protected by a SSI column inlet filter (1.5 mm × 0.5 μm, State College, PA, USA) and a Supelco LC-18 guard column (2 cm × 4.6 mm I.D., 5 μm, Bellefonte, PA, USA). Fig. 2 shows the layout of the Zymate II robotic system used in the study.



1; $R_1 = R_2 = CH_3$
2; $R_1 = R_2 = CH_2CH_3$

Fig. 1. Chemical structures of verlukast (**1**) and internal standard (**2**).

The mobile phase was prepared by mixing 86% (by volume) of methanol and 14% ammonium phosphate buffer (0.01 *M* phosphoric acid and 0.05 *M* ammonium phosphate, adjusted to pH 4.5 with phosphoric acid) and filtering through a Nylon filter (0.20 μm) prior to use. The HPLC column was operated at ambient temperature with a flow-rate of 1.2 ml/min.

2.3. Clinical samples

Plasma samples were collected from healthy and asthmatic males who received oral, aerosol, or intravenous doses of verlukast. Blood samples were collected at time intervals as specified in the protocol. After separation, plasma samples were stored at −15°C until the time of analysis. At all times during sample preparation, precautions were taken to minimize the exposure of the specimen to light.

2.4. Sample preparation and extraction of plasma

Working standards were prepared at concentrations of 1.0, 2.0, 5.0, 10.0, 25.0 and 50.0 μg/ml verlukast in plasma. Samples for constructing standard curves were prepared by add-

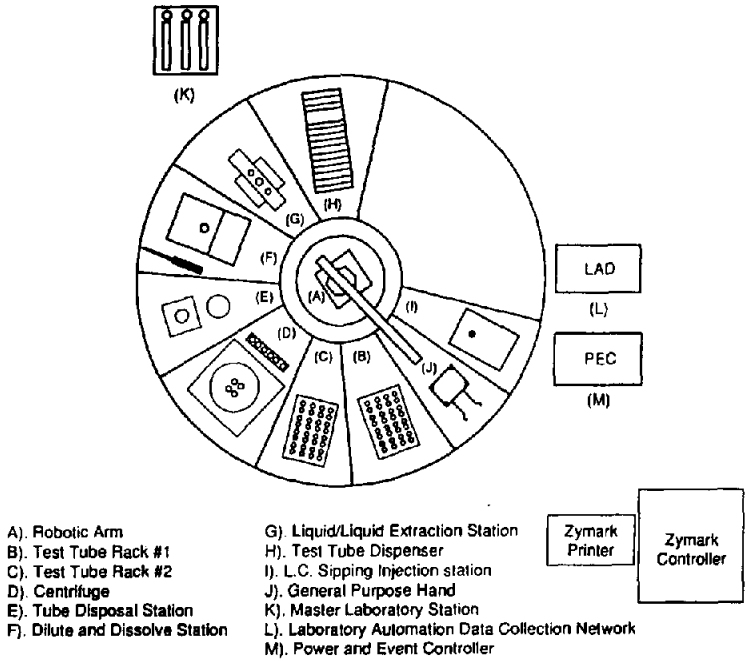


Fig. 2. Schematic of the robot configuration used in the study.

ing 0.1 ml of the appropriate working standard to 0.9 ml of human control plasma. The resulting standard concentrations ranged from 0.1 to 5 μg/ml.

Quality control (QC) samples were prepared at high and low ends of the standard curve range. Plasma was spiked with verlukast at 0.125 and 2.5 μg/ml and 1-ml aliquots were stored at −15°C. The QC samples were assayed on each day of analysis to monitor and ensure the system performance.

Plasma specimens were prepared for extraction by placing 0.2 ml of plasma (sample or standard) and 0.05 ml of working I.S. (10 μg/ml) in a 16 × 100 mm borosilicate culture tube.

In the manual assay, plasma proteins were precipitated with 0.5 ml of acetonitrile, and after vortex-mixing and centrifugation (7 min, 2000 *g*), 400 μl of supernatant was aspirated and transferred to the HPLC sample vial for injection (50 μl) onto the HPLC system. The manual assay was later automated, and all steps required for analysis including vortex-mixing, protein precipitation, and on-line HPLC injection were executed by the robot as outlined in Fig. 3.

2.5. Data acquisition and analysis

Peak-height data for verlukast and I.S. were acquired and automatically processed using a Hewlett-Packard Model 3357 laboratory automation system. Verlukast concentrations in study samples were calculated from the daily standard curve obtained by least-squares linear regression of peak-height ratios of the drug to the I.S. vs. concentration. When a calculated amount exceeded the standard curve range, the clinical sample was diluted with control plasma and reanalyzed.

3. Results and discussion

3.1. System productivity

To increase the throughput of plasma samples, the robot was programmed through the use of Easylab™ software in a scheduled fashion. Each sample was moved through the stations in a serial fashion rather than as a batch of samples

200 µl of human plasma + 50 µl I.S. (2, 0.5 µg)

↓

Vortex-mix (15 s, 50 cps) *

↓

Add 500 µl of acetonitrile *

↓

Vortex-mix (15 s, 50 cps) *

↓

Centrifuge (7 min, 2000 g) *

↓

Aspirate 400 µl supernatant *

↓

Dispense to clean tube *

↓

Sip 250 µl and fill the 50-µl sample loop *

↓

HPLC

Fig. 3. Chart showing all steps required for analysis. I.S. = internal standard; cps = cycles per second; * = performed by the robotic system.

being processed together from step to step as in the manual method. To utilize all of the robot's time, several of these serial samples were interleaved as they were processed such that while the robot was waiting for one sample to complete, e.g. a long non-robotic step (such as centrifugation), the robot can perform other tasks involving another sample. Typically, it took the robot 15 h to analyze 120 plasma samples. The advantages of using a robotic sample preparation are illustrated by comparing the efficiency, precision and time saved by a chemist during the analyses. Manual preparation, for example, of 50 plasma samples required eight working hours and a full commitment of an analyst to perform repetitive steps. By using a robotic method, this time could be saved by a chemist to do other laboratory tasks.

3.2. Assay specificity

Fig. 4 shows representative chromatograms of human control plasma, control plasma spiked with verlukast and I.S. (0.2 and 2.5 µg/ml, respectively) and a subject plasma after receiving an intravenous dose of verlukast and spiked with 2.5 µg/ml of I.S.. The retention times for verlukast and I.S. were 4.2 and 5.8 min, respectively, and the total run time was 7.5 min. Assay

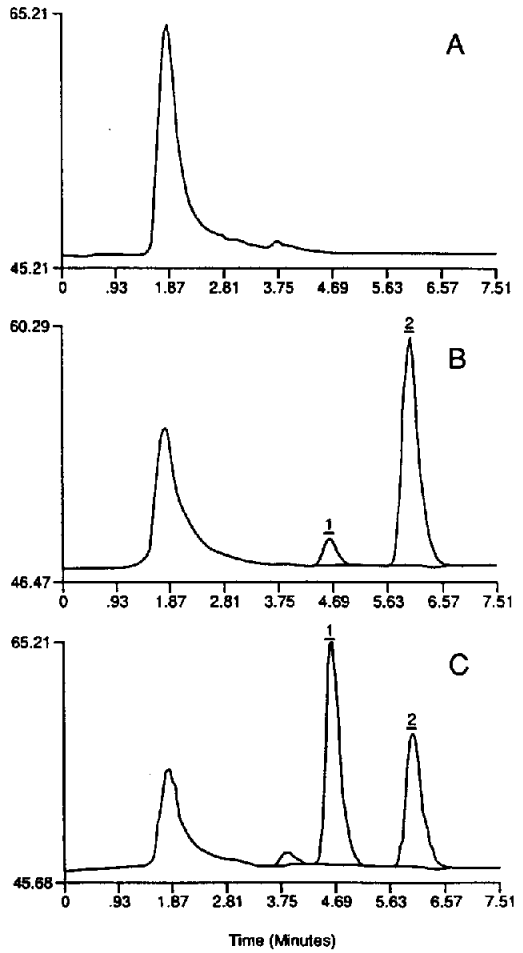


Fig. 4. Representative chromatograms of verlukast and internal standard in human plasma. (A) control plasma; (B) control plasma spiked with 0.2 µg/ml verlukast and 2.5 µg/ml I.S.; (C) plasma sample from a subject one hour after receiving an intravenous dose of verlukast (500 mg) and spiked with 2.5 µg/ml of I.S.; concentration of verlukast was 3.1 µg/ml.

specificity was confirmed by the lack of interferences observed at the retention times of verlukast and I.S. in any of the pre-dose plasma samples from subjects participating in clinical trials.

3.3. Linearity, limit of quantitation and recovery

Plots of the peak-height ratios of verlukast to I.S. vs. drug concentrations were linear, and the typical equation describing the standard line was $y = 0.54x \pm 0.39$ ($r^2 = 0.999$). The limit of quantitation (LOQ), defined as the lowest concentration on the standard line for which acceptable accuracy [100 ± 10%, expressed as (mean observed concentration)/(found concentration)· 100] and precision (expressed as the coefficient of variation, C.V. ≤ 10%) were obtained, was 0.1 μg/ml. The recovery of verlukast over the entire concentration range studied was higher than 95%.

3.4. Assay precision and accuracy

Replicate plasma standards ($n = 6$) spiked with verlukast were analyzed to assess the intra-day variability of the assay. Mean accuracy and precision data for the assay are shown in Table 1. Quality control samples (0.125 and 2.5 μg/ml) were analyzed daily in eight separate runs over a period of three months. The results in Table 1 indicate that the intra-day and inter-day variability of the assay was less than 5% C.V.. Accuracy and precision of the robotic method (Table 1) was excellent and was better or similar than those of the manual method (Table 2).

3.5. Stability

Verlukast has been shown to be stable in human plasma stored at −15°C for up to 12 months. Photochemical stability has been verified by analyzing the spiked and subject plasma samples during robotic sample preparation procedure in the dark and under yellow fluorescence light. No difference in the recovery was observed.

3.6. Application

The applicability of the assay was illustrated by analyzing more than 1000 plasma samples from several clinical studies. Fig. 5 shows the

Table 1
Validation data for the determination of verlukast in plasma

Concentration (μg/ml)	Precision[a] (%)		Accuracy[b] (%)	
	Intra-day[c]	Inter-day[d]	Intra-day[c]	Inter-day[d]
0.1	0.8	2.6	102.0	102.0
0.2	1.2	2.9	96.0	99.5
0.5	0.8	2.3	99.4	100.0
1.0	0.9	1.8	97.2	99.4
2.0	0.6	1.5	100.0	100.5
5.0	1.9	1.1	98.4	100.6
QC[e] (0.125 μg/ml)	1.0	4.6	97.6	100.8
QC (2.5 μg/ml)	2.0	1.8	101.2	99.9

[a] Coefficient of variation.
[b] Expressed as [(mean observed concentration/spiked concentration)· 100].
[c] $n = 6$.
[d] $n = 8$; obtained from the analyses of quality control samples over a period of three months.
[e] QC = quality control. Quality control samples were analyzed daily with the study samples and concentrations calculated from daily standard curves.

Table 2
Comparison of precision[a] and accuracy[b] of the robotic vs. manual methods for the determination of verlukast in human plasma based on the analyses of quality control samples

Analysis No.	0.125 μg/ml		2.5 μg/ml	
	Robotic	Manual	Robotic	Manual
1	0.122	0.127	2.51	2.61
2	0.122	0.127	2.53	2.53
3	0.122	0.129	2.48	2.56
4	0.119	0.124	2.64	2.57
5	0.123	0.126	2.51	2.63
6	0.122	0.122	2.51	2.69
Mean	0.122	0.126	2.53	2.60
S.D.	0.001	0.002	0.056	0.057
C.V. (%)	1.0	1.8	2.2	2.2
Accuracy (%)	97.6	100.8	101.2	104.0

[a] Expressed as coefficient of variation (C.V., %); $n = 6$.
[b] Expressed as (mean observed concentration/spiked concentration) · 100%; $n = 6$.

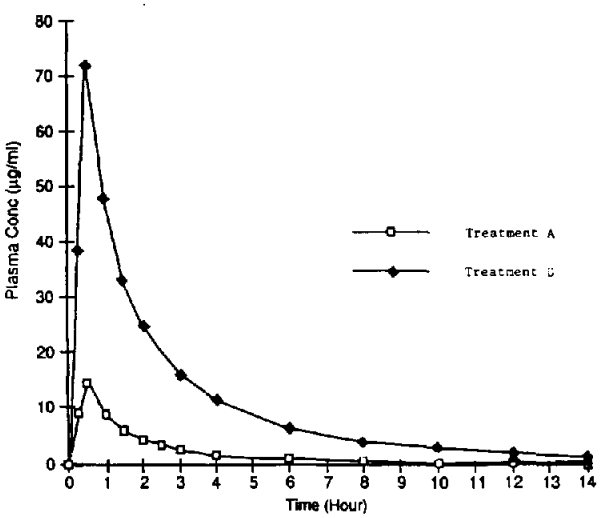


Fig. 5. Mean plasma concentration (μg/ml) of verlukast vs. time following intravenous infusion of verlukast. Treatment A: 100 mg; treatment B: 500 mg.

mean plasma concentration vs. time profile obtained from normal subjects receiving single intravenous doses of verlukast.

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