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Determination of mizolastine, a new antihistaminic drug, in human plasma by liquid–liquid extraction, solid-phase extraction and column-switching techniques in combination with high-performance liquid chromatography

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ABSTRACT

For the determination of mizolastine (2-[[[1-[(4-fluorophenyl)methyl]-1*H*-benzimidazol-2-yl]-4-piperidinyl]methylamino]-4(3*H*)-pyrimidinone, SL 85.0324), a new antihistaminic drug, in human plasma, three methods were developed based on liquid–liquid extraction, solid-phase extraction and column-switching in combination with high-performance liquid chromatography with ultraviolet detection. The liquid–liquid extraction method included a back-extraction step that preconcentrates the drug into a small aqueous volume, resulting in very high sensitivity (0.5 ng/ml of plasma); it can be used in conventional bioanalytical laboratories that do not have sophisticated automatic devices. The solid-phase extraction method is performed by using a robotic system (Benchmate). It is completely automated from the initial sampling to the final injection into the chromatograph. It has a good sensitivity (1 ng/ml of plasma), but requires an expensive apparatus and skilled analysts. The column-switching method is based on a solid-phase extraction performed on-line with chromatographic analysis; it is not completely automatic, because some operations are performed manually. The device required for valve switching is not expensive and can be managed by a simple integrator or a personal computer; it is very easy to use and affords a sensitivity (2.5 ng/ml of plasma) that generally satisfies the needs of pharmacokinetic investigations of mizolastine. The conditions were similar for all the three methods: a C_8 type column, an eluent of phosphate buffer and acetonitrile, and a spectrophotometric ultraviolet detector operated at 285 nm.

INTRODUCTION

Mizolastine (2-[[[1-[(4-fluorophenyl)methyl]-1*H*-benzimidazol-2-yl]-4-piperidinyl]methylamino]-4(3*H*)-pyrimidinone, SL 85.0324, Fig. 1), a new benzimidazole derivative, is a potent and selective antagonist of histamine H_1 receptors. It is completely free from the sedative effects which, in varying degrees, are associated with most of the currently marketed products [1]. The determination of this compound in human plasma, for pharmacokinetic studies in animals and humans, and for drug-level monitoring in clinical investigations, needs sensitive and specific chromatographic methods; bioanalytical laboratories in-

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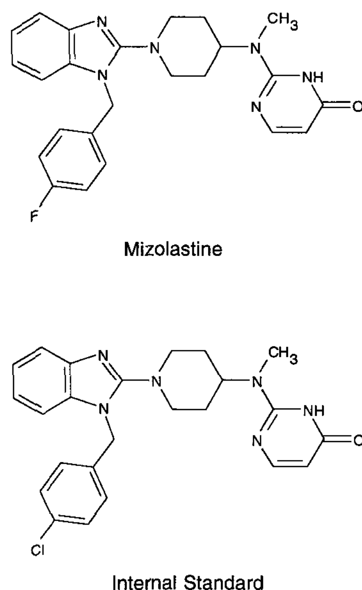

Fig. 1. Structure of mizolastine and the internal standard.

volved in the analysis of a small, medium or large number of samples generally use liquid–liquid extraction (LLE) or solid-phase extraction (SPE). LLE is a traditional approach for isolating non-polar xenobiotics from a biological matrix prior to quantitation [2–4]; the technique is very simple and requires just standard glassware. However, it is time-consuming, includes several handling steps and cannot be automated. SPE provides fast and efficient sample preparation, it reduces sample handling and eliminates the risk of emulsification [5]. It can be performed manually, and automatically (in a partial or total mode) by means of dedicated instruments. Therefore three different methods were developed, validated and compared for the determination of mizolastine in human plasma: a manual liquid–liquid extraction with a back-extraction enrichment which provides the highest sensitivity, a semi-automatic SPE method based on column-switching, a technique that has recently grown considerably as demonstrated by several reviews [6–9], and an automatic SPE method using a robot (Benchmate robot) programmed just to perform a task with human-like skill [10–13].

EXPERIMENTAL

Reagents, chemicals and standards

Methanol and acetonitrile were HPLC grade (Merck, Darmstadt, Germany), diethyl ether was analytical grade (Merck); Suprapur boric acid, analytical-grade sodium hydroxide pellets, analytical-grade potassium chloride, and analytical-grade potassium dihydrogenphosphate anhydrous (KH_2PO_4) were from Merck. Analytical-grade triethylamine was from C. Erba (Milan, Italy). Analytical-grade (85%) phosphoric acid and analytical-grade ammonium dihydrogenphosphate ($NH_4H_2PO_4$) were from Merck. Pure water was obtained from deionized water purified on a Milli-Q4 system (Millipore, Bedford, MA, USA). The extraction columns for SPE were Bakerbond CN 40 μm, 100 mg (J. T. Baker, Deventer, Netherlands).

0.7 *M* Borate buffer (pH 9) for extraction was prepared by dissolving 6.18 g of boric acid and 7.46 g of potassium chloride in 100 ml of pure water, then bringing to pH 9 with *ca.* 50 ml of 1 *M* sodium hydroxide. 0.025 *M* Phosphate buffer (pH 2.6) for back-extraction was prepared by dissolving 131.1 g of potassium dihydrogenphosphate in 1 l of pure water to obtain a 1 *M* solution, then diluting 25 ml of this solution to obtain a 0.025 *M* solution and adjusting at pH 2.6 with 1 *M* phosphoric acid solution. Methanol saturated with ammonium dihydrogenphosphate was used for conditioning the SPE column before use and for elution of analytes from the SPE column. 0.05 *M* Phosphate buffer (pH 4.6) to be added to the methanolic eluate after SPE was prepared from 1 *M* potassium dihydrogenphosphate by diluting 50 ml of this solution to obtain a 0.05 *M* solution.

Mizolastine and the internal standard (2-[[[1-[(4-chlorophenyl)methyl]-1*H*-benzimidazol-2-yl]-4-piperidinyl] methylamino]-4(3*H*)-pyrimidinone, SL 86.0116, Fig.1) were of pharmaceutical grade and obtained from Synthélabo Recherche (Bagneux, France).

Standard solutions

Stock solutions (1 mg/ml) of mizolastine and the internal standard (SL 86.0116) were prepared

TABLE I

STANDARD SOLUTIONS USED FOR THE DETERMINATION OF MIZOLASTINE IN HUMAN PLASMA

Standard solution	Mizolastine concentration (ng/20 μl)	I.S. concentration (ng/20 μl)
1	500	–
2	100	–
3	50	–
4	10	–
5	5	–
6	1	–
7	0.5	–
8	–	300

in methanol; standard solutions were prepared from stock solutions by suitable dilutions with methanol (Table I), and used for the preparation of plasma standards. Stock and standard solutions were stable for at least one month if stored at 0–5°C. The standard solutions were added to pre-dose human plasma for the preparation of the plasma standards used for daily calibration.

Basic chromatographic equipment

For all the proposed methods, the chromatographic equipment consisted of a Model 420 constant-flow double-piston pump (Kontron, Milan, Italy), an SPD-6A spectrophotometric LC detector (Shimadzu, Kyoto, Japan) with a standard HPLC cell (8 μl, 1 mm × 10 mm), operating at 285 nm, a Model 460 automatic sample injector (Kontron) with a six-port automatic valve and 200-μl external loop; the robotic method did not require the automatic sample injector.

Extended chromatographic equipment

Column-switching method. For the on-line SPE clean-up and pre-concentration of the plasma, the basic chromatographic apparatus was supplemented with a pre-column (7.5 cm × 0.21 cm I.D.), dry-filled with Perisorb C_{18}, 30–40 μm (E. Merck), a service pump, a Model 414 constant-flow pump (Kontron) for pumping the necessary solvents for the clean-up and pre-concentration on the pre-column, a Tracer MCS-670 compact column-switching device provided with six-way high-pressure valves and six-way solvent selector, and a Model 200 programmer (Kontron) for the complete automation of the switching operations and the control of the service pump (see Fig. 2).

Robotic method. The Benchmate robot (Zymark, Hopkinton, MA, USA) performed all the operations required in the analysis from weighing of plasma sample, addition of standard solutions, all SPE operations and injection into the chromatographic column. The HPLC robotic system imitates the analysts with a hand designed to weigh the samples in a test-tube, and finishes the analysis with a hand specifically designed to inject the concentrated purified sample into an HPLC injection port [13].

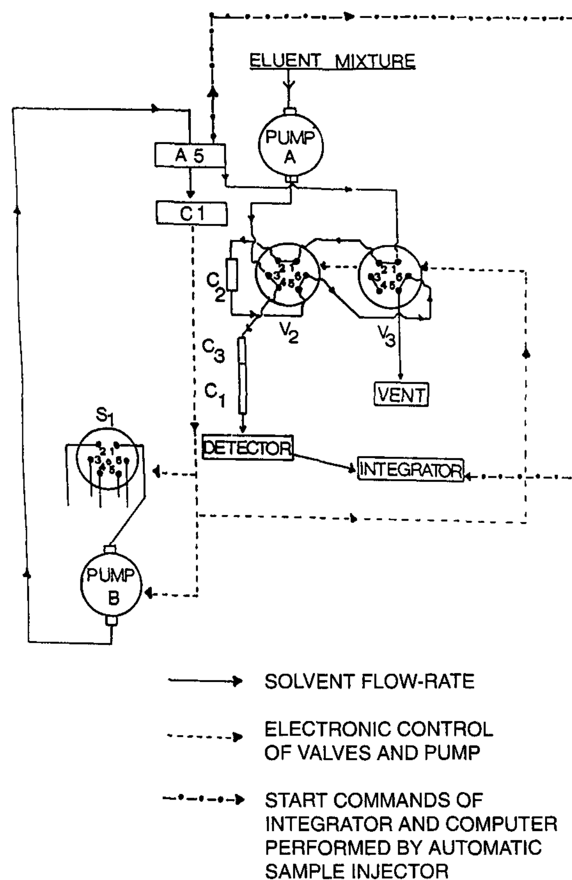

Fig. 2. Chromatographic apparatus used for automatic on-line clean-up of samples and HPLC with column-switching.

Chromatographic conditions

LLE method. The analytical column was C_8 type, Hypersil BDS, 5 μm (Shandon, Runcorn, UK), 15 cm × 0.46 cm I.D. or equivalent. Eventually a guard column (2 cm × 0.46 cm I.D.) can be used containing a Pellicular C_{18} material, Pelliguard, 40 μm (Supelchem, Milan, Italy). The mobile phase was 0.025 *M* phosphate buffer (pH 2.5)–acetonitrile (65:35, v/v) containing 0.1% triethylamine, supplied at a flow-rate of 1.0 ml/min.

Column-switching method. The analytical column and the guard column were similar to those described for the LLE method. The mobile phase was 0.025 *M* phosphate buffer (pH 4.5)–acetonitrile (60:40, v/v) supplied at a flow-rate of 1.0 ml/min.

Robotic method. The analytical column was C_8 type, Ultrabase, 5 μm (Shandon), 25 cm × 0.46 cm I.D. or equivalent; the mobile phase was 0.05 *M* phosphate buffer (pH 4.6)–acetonitrile (55:45, v/v) containing 0.1% triethylamine, supplied at a flow-rate of 1.0 ml/min.

Sample preparation

LLE method. The frozen plasma samples (pre-dose and unknowns) were thawed in a water-bath at 37°C then weighed at room temperature; then 1 g of pre-dose plasma (for each future plasma standard) and unknown samples were weighed. To the pre-dose plasma, 20 μl of standard solutions were added for each calibration point (Table I); 20 μl of internal standard solution were also added to all samples and homogenized. All the samples were made alkaline with 0.5 ml of 0.7 *M* borate buffer (pH 9); then the samples were extracted with 7 ml of diethyl ether by shaking at 40 rpm for 10 min (tumble extractor). After separation of the upper organic phase by centrifugation (2000 *g* for 5 min at 5°C), this was removed and then back-extracted with 0.25 ml of 0.025 *M* phosphate buffer (pH 2.6) on a tumble extractor (at 20 rpm for 10 min). The aqueous phase was separated from the organic layer (discarded) and, after evaporation of the solvent under a light stream of pure nitrogen at 40°C, it was transferred to conical vials for automatic sample injection. Finally, 150 μl of the solution were injected into the injection port of the HPLC system.

Column-switching method. The frozen plasma samples (pre-dose and unknowns) were thawed in a water-bath at 37°C and weighed at room temperature; then 1 g of pre-dose plasma (for each future plasma standard) and unknown samples were weighed in plastic Eppendorf vials. To the pre-dose plasma, 20 μl of standard solutions were added for each calibration point (Table I, No. 1 to No. 5); 20 μl of internal standard solution were also added to all samples and homogenized. A 200-μl volume of acetonitrile was added to all samples and vortex-mixed, then all vials were centrifuged in an Eppendorf centrifuge (11 000 *g* for 4 min). The clear plasma supernatants were transferred to conical vials for automatic injection into the injection port of the column-switching HPLC system (Fig. 2). The sample (200 μl) was loaded on the pre-column, where the clean-up and pre-concentration take place; the pre-column, after the sample injection, was flushed for 2 min with acetonitrile–water (10:90, v/v) in order to eliminate proteins and electrolytes. Then, after valve-switching, the pre-column was connected to the analytical column, where analytes were transferred by the HPLC mobile phase. The pre-column was disconnected after 1.5 min. While chromatography took place on the analytical column, the pre-column was back-flushed with different solvents such as acetonitrile–water (50:50,v/v), acetonitrile and methanol–water (50:50, v/v) and finally re-equilibrated with acetonitrile–water (10:90, v/v). All these latter operations were performed automatically during the chromatographic analysis.

Robotic method. The frozen plasma samples (pre-dose and unknowns) were thawed in a water-bath at 37°C, left at room temperature and centrifuged at 19 120 *g* for 10 min at *ca.* 4°C; then the supernatant plasma samples were transferred to test-tubes suitable for the Benchmate rack. The operations of weighing samples (as described for the other methods) were automatically performed by the hand of the robot. The other robot operations were: (1) the SPE columns were con-

ditioned first by 1 ml of methanol saturated with ammonium dihydrogenphosphate, then by 2 ml of water; (2) 1 ml of plasma sample (pre-dose and unknowns) was mixed with 0.5 ml of acetonitrile–water (50:50, v/v) containing 100 ng of internal standard, and homogenized; (3) 1.4 ml of the plasma solution were loaded on the SPE column, and this was flushed twice with 2 ml of water, then twice with 1 ml of water–methanol (70:30, v/v); (4) the column was dried; (5) the column was eluted with 0.6 ml of methanol saturated with ammonium dihydrogenphosphate (the eluted volume was *ca.* 0.3 ml); (6) to the eluted fraction, 0.4 ml of 0.05 *M* phosphate buffer (pH 4.6) was added and homogenized; (7) 650 μl were injected, automatically, onto the HPLC system.

Quantitation

Peak-height ratios of mizolastine to the internal standard, obtained from human plasma standards, were plotted *versus* the nominal concentration of mizolastine, to generate the linear least-squares regression lines (calibration equations). The concentrations of mizolastine in the unknown specimens were obtained by interpolation from the calibration equations using peak-height ratios of mizolastine to the internal standard, obtained from unknown specimens. All the calculations concerning the quantitative analysis and regressions were automatically performed on an SP 4270 calculator integrator (Spectra-Physics, San José, CA, USA).

RESULTS

Stability

Both mizolastine and the internal standard are stable in methanol, in the stock and standard solutions for at least one month at 0–5°C in the refrigerator.

In the LLE method, both mizolastine and the internal standard are stable in alkaline human plasma, at room conditions for at least 5 h; the compounds are stable, after extraction from plasma, in the HPLC injection solvent for at least 24 h on the autosampler rack (pre-injection conditions).

In the column-switching method, both mizolastine and the internal standard are stable in human samples, after the addition of acetonitrile and centrifugation at room temperature, for at least 24 h on the autosampler rack (pre-injection conditions).

In the robotic method, both mizolastine and the internal standard are stable in human plasma, for at least 29 h at room temperature, on the Benchmate rack (pre-processing conditions); the compounds are also stable in 0.05 *M* phosphate buffer (pH 4.6)–methanol saturated with ammonium dihydrogenphosphate (57:43, v/v) for at least 22 min (pre-injection conditions during sequential analysis).

Linearity

Standard curves were constructed by plotting the peak-height ratio of mizolastine and the internal standard *versus* the concentration of mizolastine. For the LLE method, the calibration curve was linear from 0.5 to 500 ng/ml mizolastine in human plasma [plot of nominal concentrations (x) *versus* calculated concentrations (y): $n = 6$, $r = 1.000$, slope = 1.001, y-intercept = -0.073]; for the column-switching method, the calibration was linear from 2.5 to 500 ng/ml [plot of nominal concentrations (x) *versus* calculated concentrations (y): $n = 6$, $r = 0.9997$, slope = 0.981, y-intercept = 0.897]; finally for the robotic method, the calibration was linear from 1 to 400 ng/ml [plot of nominal concentrations (x) *versus* calculated concentrations (y): $n = 8$, $r = 0.9996$, slope = 1.012, y-intercept = -2.68].

Recovery

The absolute recovery for the LLE method was determined by calculating the ratio of the amount of the extracted compound from plasma to the amount of the compound added to 0.25 ml of 0.025 *M* phosphate buffer (pH 2.6) just before HPLC injection; the recovery was investigated at 10 and 100 ng/ml, giving 75.8 ± 5.7% (C.V.) and 72.7 ± 3.6% (C.V.), respectively ($n = 5$ for both the concentrations).

The absolute recovery for the column-switching method was determined by calculating the ra-

tio of the amount of the compound extracted from plasma on the pre-column (on-line SPE) to the amount of the compound added to 1 ml of acetonitrile–water (10:90, v/v) mixture, and directly injecting 0.2 ml of this solution into the HPLC column (just by-passing the pre-column). The recovery was investigated at 20 and 200 ng/ml, giving 62.3 ± 9.6% (C.V.) and 72.6 ± 2.7% (C.V.), respectively (n = 3 for both the concentrations).

The absolute recovery for the robot method was determined by calculating the ratio of the amount of the extracted compound from plasma, on the SPE column, to the amount of compound added to 0.7 ml of aqueous 0.05 *M* phosphate buffer (pH 4.6)–methanol saturated with ammonium dihydrogenphosphate (57:43, v/v) just before HPLC injection. The recovery was investigated at 100 ng/ml, giving 63.4 ± 3.0% (C.V.) (n = 5).

Selectivity

Several pre-dose plasma samples from different subjects were tested for the absence of interfering compounds. In no case was any chromatographic interference found at the retention times of mizolastine or the internal standard for the LLE method, for the column-switching method or for the robotic method (see Figs. 3A, 4A and 5A).

Limit of quantitation

The limit of quantitation for the LLE method is 0.5 ng/ml in human plasma (Fig. 3B), for the column-switching method it is 2.5 ng/ml (Fig. 4B), and for the robotic method it is 1 ng/ml (Fig. 5B). The limit was taken as a chromatographic peak three times higher than baseline noise; it is always the lowest point of the calibration curve.

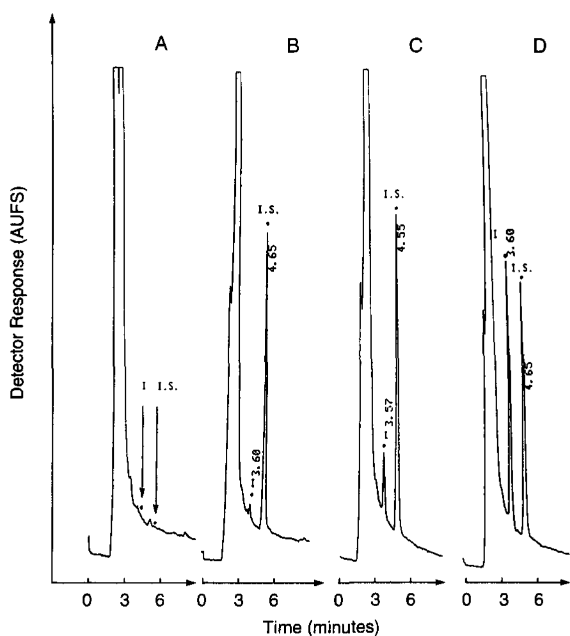

Fig. 3. Chromatograms of (A) a pre-dose human plasma sample, (B) a human plasma sample, showing the limit of quantitation (0.5 ng/ml), (C) a QC sample (3 ng/ml) and (D) a plasma sample from a subject treated orally with a single dose (10 mg) of mizolastine; sample taken 12 h after administration, mizolastine level found 15 ng/ml. Peaks: I = mizolastine; I.S. = internal standard. LLE method.

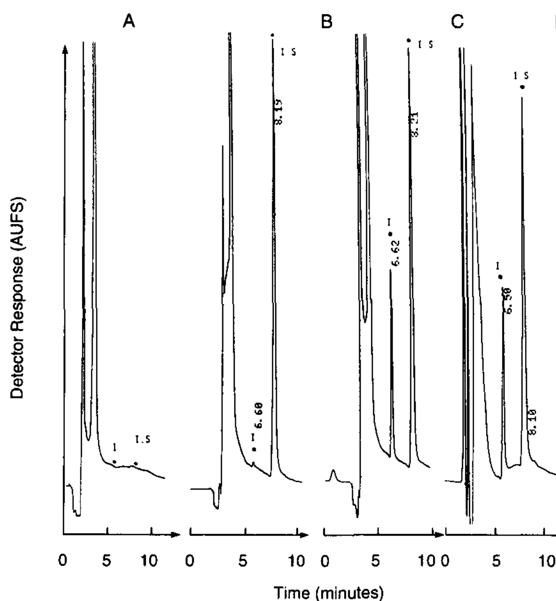

Fig. 4. Chromatograms of (A) a pre-dose human plasma sample, (B) a human plasma sample, showing the limit of quantitation (2.5 ng/ml), (C) a QC sample (100 ng/ml) and (D) a plasma sample from a subject treated orally with a single dose (10 mg) of mizolastine; sample taken 2 h after administration, mizolastine level found 112 ng/ml. Peaks: I = mizolastine; I.S. = internal standard. Column-switching method.

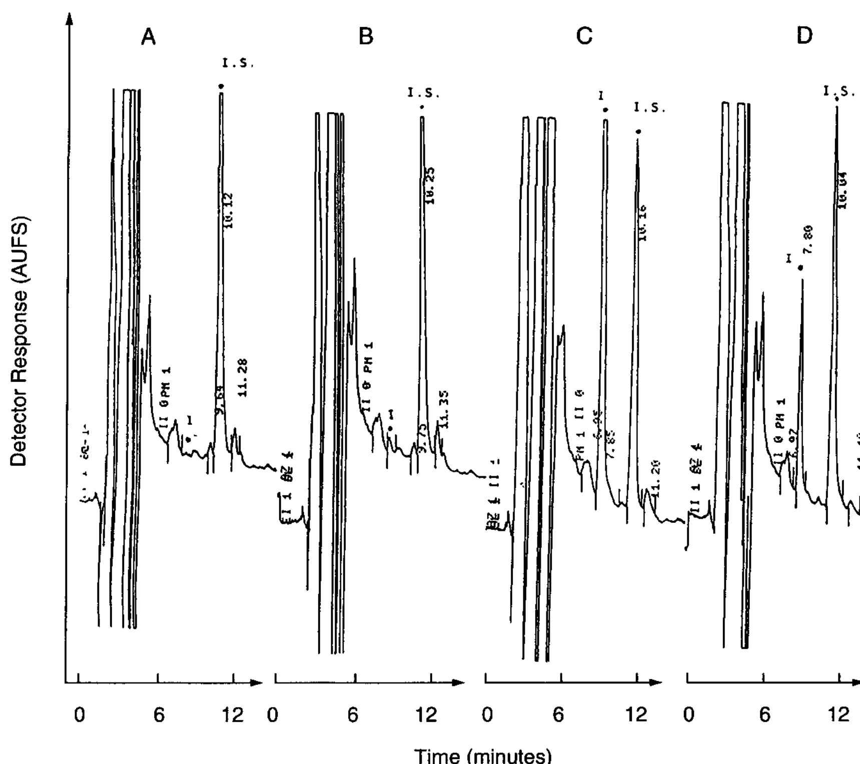

Fig. 5. Chromatograms of (A) a pre-dose human plasma sample, spiked with 100 ng of internal standard, (B) a human plasma sample, showing the limit of quantitation (1 ng/ml), (C) a QC sample (125 ng/ml) and (D) a plasma sample from a subject treated orally with a repeated dose of 10 mg of mizolastine once a day; sample taken 6 h after administration on the 8th day of treatment, mizolastine level found 75 ng/ml. Peaks: I = mizolastine; I.S. = internal standard. Robotic method.

Precision and accuracy

The precision and accuracy of the methods were evaluated by analysing quality control (QC) samples in human plasma at different concentrations on different days by two analysts; each analyst, after the daily calibration (performed in quadruplicate), analysed a low and a medium QC sample (in quintuplicate or less) over a two-day period. The results for the LLE method, the column-switching method and for the robot method are reported in Tables II, III and IV, respectively.

TABLE II

PRECISION AND ACCURACY FOR MIZOLASTINE IN QC HUMAN PLASMA SAMPLES OBTAINED WITH THE LLE METHOD (n = 12)

	1.48 ng/ml	29.5 ng/ml	297.4 ng/ml
Precision			
Intra-day R.S.D. (%)	6.1	3.4	3.3
Inter-day R.S.D. (%)	0	2.6	0
Total R.S.D. (%)	6.1	4.3	3.3
95% Upper confidence limit for total R.S.D. (%)	10.4	7.5	5.6
Accuracy			
Recovery (mean ± R.S.D.) (%)	97.0 ± 5.5	99.1 ± 3.3	98.0 ± 2.6

TABLE III

PRECISION AND ACCURACY FOR MIZOLASTINE IN QC HUMAN PLASMA SAMPLES OBTAINED WITH THE COLUMN-SWITCHING METHOD (n = 20)

	15 ng/ml	300 ng/ml
Precision		
Intra-day R.S.D. (%)	2.5	1.5
Inter-day R.S.D. (%)	2.1	0.9
Total R.S.D. (%)	3.3	1.8
95% Upper confidence limit for total R.S.D. (%)	5.4	2.7
Accuracy		
Recovery (mean ± R.S.D.) (%)	99.8 ± 2.4	98.2 ± 1.1

Figs. 3C, 4C, and 5C represent chromatograms from QC samples.

Comparison of LLE, column-switching and robot methods

In order to evaluate the performance of the proposed methods, several plasma samples, obtained from *in vivo* studies, were analysed using all three methods: the LLE method *versus* column-switching, the LLE method *versus* robotic, the column-switching *versus* robotic. The correlation lines obtained from these comparisons were, respectively, $y = 1.05x + 1.97$, $y = 1.08x - 0.13$ and $y = 1.02x + 2.8$, with an even distribution of the points about these lines.

DISCUSSION

The method based on LLE gave the highest sensitivity (0.5 ng/ml) in human plasma; it can be very useful to have such high sensitivity in pharmacokinetic studies in humans when sampling is provided up to 60 h after single-dose administration (10 mg) of mizolastine. The method involves several handling steps that could not be automated; however, it can be used by conventional bioanalytical laboratories without sophisticated automatic devices. The LLE method takes less time than the conventional liquid extraction method with a back-extraction step, because it does not necessitate the evaporation of the organic phase or the redissolution of residue prior to chromatography. The column-switching method is partially automatic and has a sensitivity of 2.5 ng/ml in human plasma, which generally satisfies pharmacokinetic needs. It is rapid: during the chromatographic run it performs the automatic clean-up and extraction of the plasma sample in only 2 min; it is not expensive: the column-switching apparatus to be coupled to the basic chromatographic system needs basically two six-port high-pressure valves and one solvent selector, which can be managed by a conventional integrator (in the event time program section) and uses a laboratory-prepared pre-column that can be used for more than 200 samples. The Benchmate robot method is completely automat-

TABLE IV

PRECISION AND ACCURACY FOR MIZOLASTINE IN QC HUMAN PLASMA SAMPLES OBTAINED WITH THE ROBOT METHOD

	4 ng/ml (n = 18)	125 ng/ml (n = 20)	250 ng/ml (n = 20)
Precision			
Intra-day R.S.D. (%)	6.0	3.7	4.6
Inter-day R.S.D. (%)	0	4.6	2.7
Total R.S.D. (%)	6.0	5.9	5.8
95% Upper confidence limit for total RSD (%)	8.8	11.1	7.9
Accuracy			
Recovery (mean ± R.S.D.) (%)	94.4 ± 2.9	96.3 ± 7.4	99.2 ± 5.4

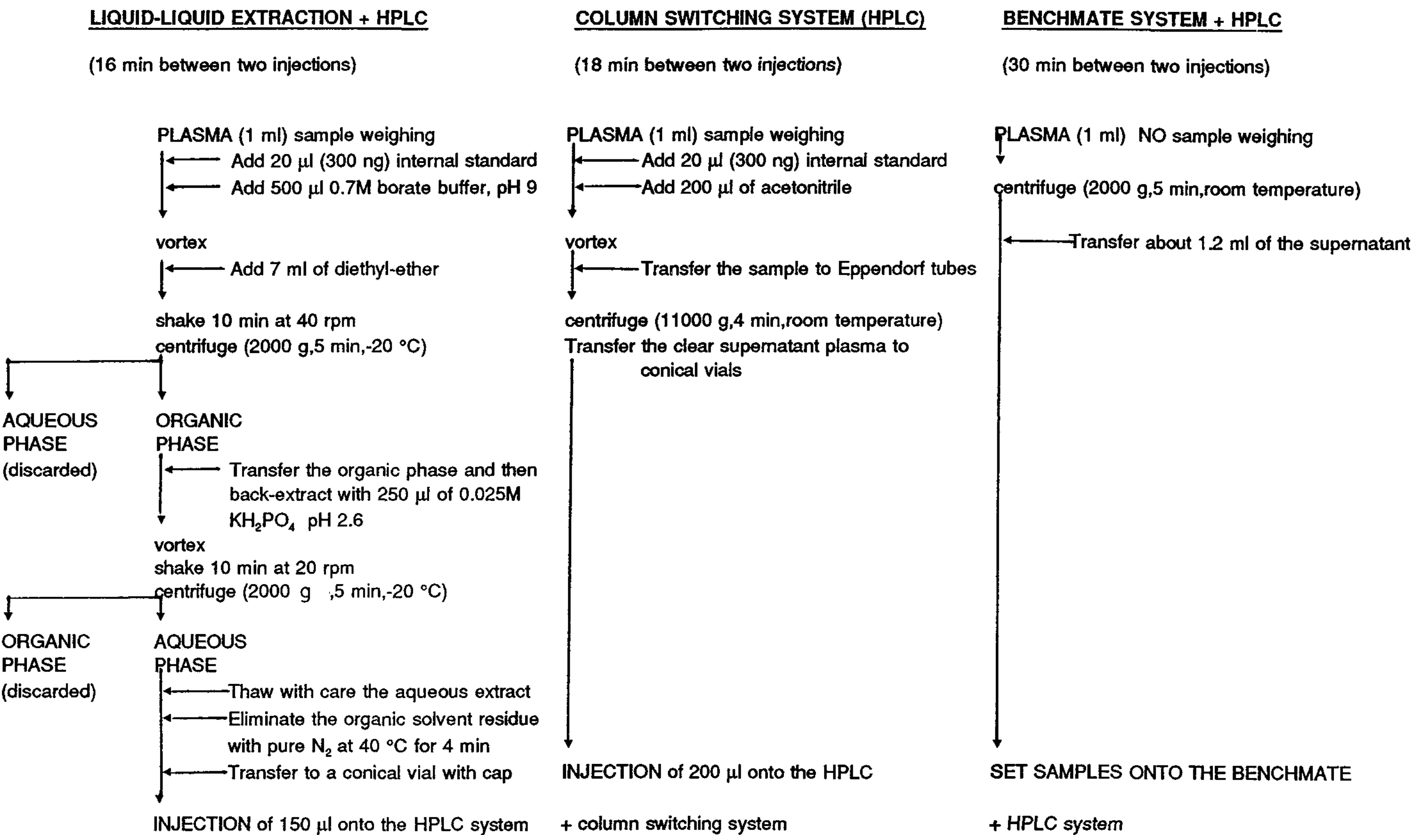

Fig. 6. Comparison of three ways for determining mizolastine levels in plasma.

ic and has a very good sensitivity (1 ng/ml) in human plasma, but it requires an expensive apparatus (the robot) and uses disposable SPE columns that make the cost per analysis higher than that of the other methods.

A schematic comparison of the three methods is shown in Fig. 6. It is important to point out that both the column-switching and robot methods are based on the SPE technique (on-line and off-line, respectively, in regard to chromatographic process), that needs the previous addition of acetonitrile to plasma to release the binding of mizolastine to plasma proteins. The presence of acetonitrile, in a content not less than and not exceeding 17% of the total volume, guarantees a high and reproducible recovery of mizolastine from human plasma. An acetonitrile content above *ca.* 17% causes protein precipitation and clogging of the SPE column in both methods.

In conclusion, these three methods show good correlation and can be used by bioanalytical laboratories with different analytical backgrounds and equipped with conventional or sophisticated automatic devices. These methods have been used successfully during phase I to phase III clinical studies for the determination of mizolastine.

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