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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR CINNARIZINE IN HUMAN PLASMA

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Abstract: The high performance liquid chromatography for the determination of cinnarizine in human plasma is described. The procedure involves liquid-liquid extraction followed by reversed phase high-performance chromatographic analysis with fluorometric detection. The method was validated for accuracy, precision, specificity, linearity, sensitivity, recovery, and stability. No endogenous compounds were found to interfere. The absolute extraction recovery of cinnarizine and clocinizine (internal standard) from plasma samples were 97% and 89%, respectively. The linearity was assessed in the range 1-100 ng/mL. The intra-day and inter-day relative standard deviations were less than 10%, and the accuracy of the assay expressed by bias was in the range 0.14-2.37%. The method was proved to be suitable for human pharmacokinetic studies following single oral dose.

Keywords: cinnarizine, Stugeron®, HPLC, human plasma, pharmacokinetics, fluorescence detection

Cinnarizine, (E)-1-(diphenylmethyl)-4-(3-phenyl-2-propenyl)piperazine (Fig.1), inhibits contractions of vascular smooth muscle cells by blocking calcium channels. It decreased entering into the cells of calcium ions and decreased their content in the depot. Cinnarizine is used in the treatment of cerebrovascular insufficiency (noise in the ears, reduction in memory), convalescence after craniocerebral injuries, migraine, in therapy for symptoms of peripherial arterial disease, equilibrium disruptions (Meniere's syndrome, motion sickness) and in food allergy (1).

After oral administration the drug is rapidly absorbed with maximum plasma concentration at 1-3 h. The elimination half-life is ranging from 3.4 to 60 h. It was dependend on age. The mean plasma elimination half-life for cinnarizine was reported 23.6 h in young volunteers (2, 3).

The determination of cinnarizine has been carried out in human plasma by analytical methods using high-performance liquid chromatography (HPLC) with UV and fluorometric detection (4, 5, 6), and gas chromatography with nitrogen-selective thermionic specific detection (7). High detection limits, or time consuming and using very toxic reagents for extraction were disadvantages of those methods.

The aim of present study was development of extraction method, simple and fast, and environmentally friendly. Chromatographic separation was conducted according to Rosseel and Lefebvre with a little modification of mobile phase composition (5).

EXPERIMENTAL

Chemicals

Cinnarizine (European Pharmacopeia, BP 907-F 67029, Strasbourg CEDEX 1) was obtained from Promochem, Poland. Clocinizine hydrochloride (internal standard) was Janssen Pharm. (N.V. USA) product. All chemicals used were of HPLC or analytical purity. Methanol, acetonitrile, chloroform and water were obtained from LabScan (Dublin, Ireland). Triethylamine and *o*-phosphoric acid conc. (85%) were purchased from Applichem (Darmstadt, Germany). Hydrochloric acid conc. (37.1 %),

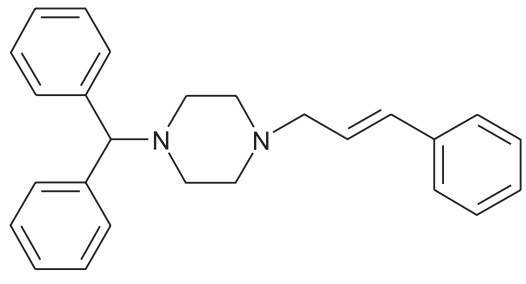

Figure 1. Structure of cinnarizine

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$NH_4H_2PO_4$, and n-hexane (99%) were supplied by POCH (Gliwice, Poland).

HPLC conditions

The chromatographic system (Perkin Elmer, Europe) consisted of a pump SERIES 200, a degaser SERIES 200, an autosampler SERIES 200 and fluorescence detector SERIES 200 operated at λ_{ex} = 245 nm and λ_{em} = 310 nm. The apparatus was connected to personal computer with TOTAL CHROM WORKSTATION 6.2.1. chromatography system software (Perkin Elmer).

The chromatographic separation was performed using the HPLC column Microsphere C_{18}, 100 × 4.6 mm I.D., 3 mm (Varian Inc., The Netherlands) preceded by a C_{18} guard column (4 × 3.0 mm, Phenomenex, Torrance, CA, U.S.A.). The column was heated at 40°C by using an oven SERIES 200. The mobile phase consisted of 0.01 M ammonium dihydrogen phosphate buffer, pH 4.2, which contained 0.038 % triethylamine and acetonitrile (25:75, v/v) and was delivered at a flow-rate of 1 mL/min. The phase was filtered (0.45 μm Nylon 66, Supelco) and ultrasonically degassed prior to use. A system suitability test was performed at the beginning of each working day: a mixture of cinnarizine (0.8 μg/mL) and clocinizine (internal standard, 1.25 mg/mL) in mobile phase was chromatographed before the sample analysis.

Extraction

The samples of plasma were stored in the freezer at – 70°C. The thawing was allowed at a room temperature. To 1 mL of plasma samples 100 mL of clocinizine working solution (1.25 mg/mL of methanol:water, 1:1 v/v) was added and the tubes were stirred vigorously on a vortex mixer. Then, 0.2 mL of 0.5 M HCl was added to each tube, mixed, and stirred vigorously again. Then, 5 mL of chloroform:n-hexane (2:3, v/v) was added to each tube. The solutions were shaken at 1000 rpm for 10 min on vibrax mixer (IKA-VIBRAX VXR, IKA-Labortechnik, Staufen, Germany), and centrifuged at 3500 rpm for 10 min. After freezing at – 70° C for 10 min, the organic layer was transferred to another glass tube and evaporated to dryness at 50° C under a gentle stream of nitrogen (TurboVap® LV, Zymark, Hopkinton, MA, USA). The sample residue was dissolved in 500 mL of methanol by shaking for 30 s at 2000 rpm, and evaporated to dryness. The residue was reconstituted in 100 mL of mobile phase and after mixing for 30 s on vibrax mixer, transferred to an autosampler vial. An aliquot of 30 mL was injected onto the HPLC system for determination.

Method validation

Calibration was performed by adding known amounts of cinnarizine to blank human plasma to yield concentrations over a range 1 – 100 ng/mL. These standards were then extracted according to the above procedure. The calibration curve was obtained by weighted linear regression (weighing factor $1/y^2$). The ratio of cinnarizine peak height to clocinizine peak height was plotted vs. cinnarizine concentration.

The absolute recoveries of cinnarizine and internal standard from plasma were calculated by comparing the peak heights obtained from extracts of spiked plasma samples and the peak heights from direct injections of known amounts of standard solutions of both compounds.

The precision and accuracy of the method were assessed by analyzing blank plasma samples spiked at concentrations of 3.125, 50.00 and 80 ng/mL.

The limit of detection (LOD) was defined at the sample concentration of cinnarizine resulting in peak height of 3 times of the noise level. The lower limit of quantification (LLOQ) was the lowest point on the calibration curve which can be detected with variation below 20 %.

The stability of cinnarizine in autosampler, during freeze-thaw cycles, short stability at room temperature and long stability in freezer (-70°C) were also checked.

Application

The method of analysis was applied to the plasma from 24 healthy volunteers (male), who participated in pharmacokinetic studies of cinnarizine. The study was performed according to the ethical guidelines of the revised Declaration of Helsinki. All subjects gave written informed consent and the study protocol was approved by the local Ethical Committee. The subjects received Stugeron® tablets (Jansen-Cilag) orally in 25 mg dose under fasting condition. Blood was sampled immediately before (time zero) and at 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12, 16, 24, 36, 48, and 72 h after drug administration.

Analysis of data

The pharmacokinetic parameters for cinnarizine were determined from plasma concentration-time data with the aid of the Summit PK Solutions 2.0 program (8). The maximum plasma concentration (C_{max}) and time to peak (t_{max}) for cinnarizine were taken directly from the experimental data. The elimination rate constant (k_{el}) was estimated by least-squares regression analysis from the data of the last 3-4 points of each plasma concentration-time

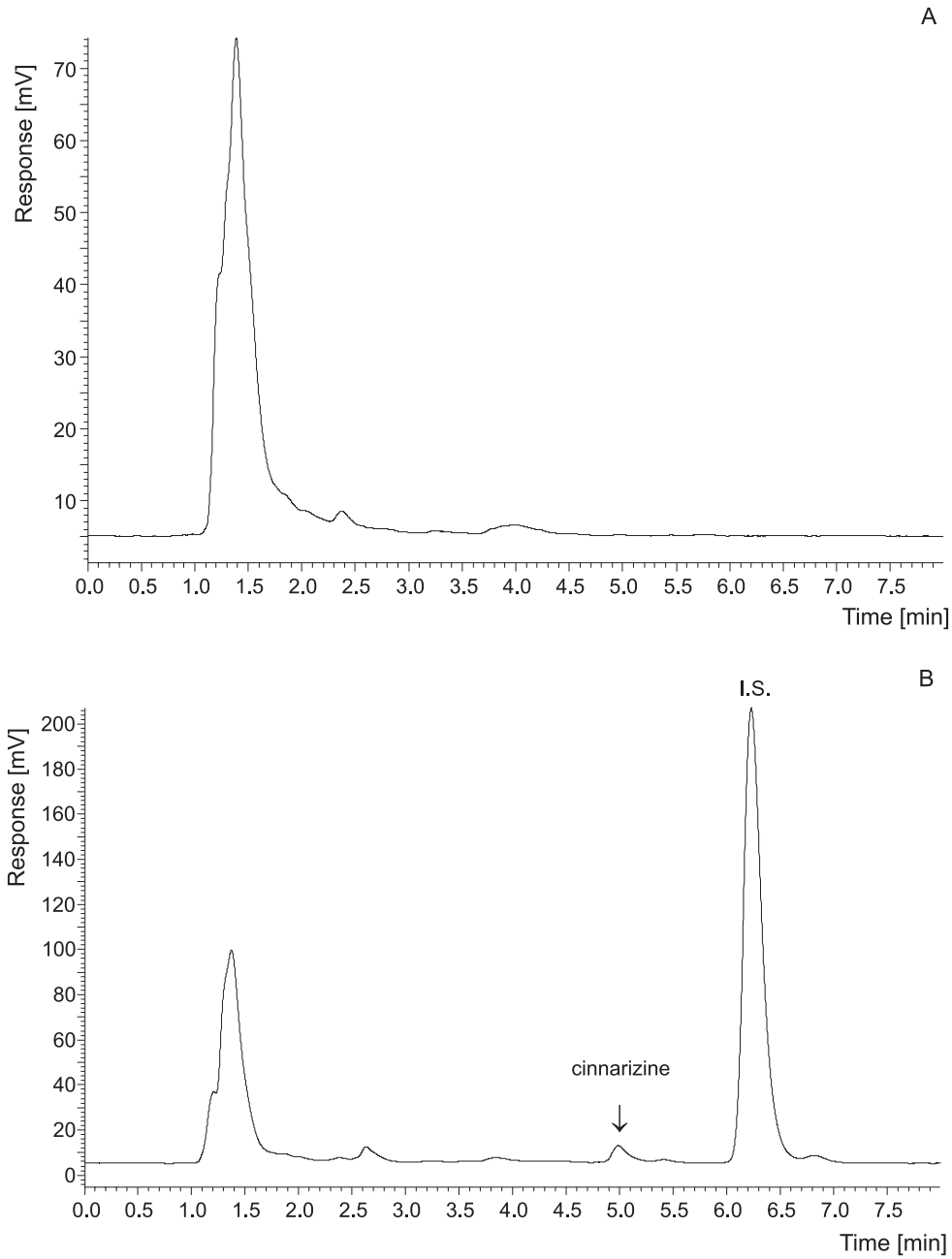

Figure 2. Chromatograms of (A) blank human plasma. (B) spiked human plasma with 3.125 ng/mL of cinnarizine and 125 ng/mL of internal standard. The retention times for cinnarizine and internal standard are 5.02 and 6.35 min, respectively.

curve. The terminal elimination half-life ($t_{1/2}$) was calculated as $\ln 2/k_{el}$. The area under the concentration versus time curve (AUC_t) was calculated using the trapezoidal rule and then extrapolated to infinity.

RESULTS AND DISCUSSION

The choice of organic solvent was the most important step during the extraction method development. In previously published methods carbon tetrachloride, a very toxic compound, was used (5). In other paper the extraction with mixture of n-heptane-isoamyl alcohol is described, but it led to low recovery and two-step extraction was necessary (7). The extraction solvent: mixture of chloroform and n-hexane, was chosen due to very height recovery (97% and 89% for cinnarizine and clocinizine, respectively) and simplicity. In addition, this mix-

Table 1. Intra-day and inter-day accuracy and precision of the method.

Reference value (ng/mL)	Intra-day (n = 6)			Inter-day (n = 6)		
	Mean ± SD	Precision % RSD	Accuracy % Bias	Mean ± SD	Precision % RSD	Accuracy % Bias
3.125	3.327 ± 0.03	1.02	6.46	3.160 ± 0.18	5.78	0.40
50.00	50.07 ± 0.95	1.90	0.14	51.01 ± 2.82	5.52	2.03
80.00	80.68 ± 0.82	1.02	0.85	81.90 ± 3.18	3.88	2.37

Table 2. Stability study.

Stability	Nominal concentration (ng/mL)	Time	Measured concentration (ng/mL)	% Bias	% RSD
Freeze and thaw stability	3.125	Cycle 3 72 h	2.822 ± 0.018	-9.69	0.64
	50.00		49.10 ± 3.51	-3.08	7.15
	80.00		75.99 ± 0.66	-5.01	0.87
Short stability at room temperature	50.00	4 h	52.92 ± 1.22	5.84	2.31
Long term stability at -70°C	3.125	3 months	3.224 ± 0.07	3.15	2.17
	50.00		47.16 ± 2.12	5.69	4.49
	80.00		71.28 ± 0.72	-9.87	1.03

Table 3. Pharmacokinetic parameters of cinnarizine following a single oral administration of 25 mg Stugeron tablets to 24 healthy volunteers.

Pharmacokinetic parameter	Mean value ± SD
k_{el} (h)	0.0374 ± 0.0286
$t_{1/2}$ (h)	29.48 ± 19.84
C_{max} (ng/mL)	35.39 ± 16.84
t_{max} (h)	2.2 ± 0.8
AUC_t (ng × h/mL)	289.75 ± 223.63
AUC_∞ (ng × h/mL)	370.10 ± 314.75
AUC_t/AUC_∞ (%)	82.6 ± 9.4

ture was more environmentally friendly than carbon tetrachloride. The method selectivity was demonstrated on six blank plasma samples obtained from healthy volunteers. The chromatograms were found to be free of interfering peaks. A typical chromatogram of a blank plasma is shown in Figure 2A. The peaks of cinnarizine and internal standard were well separated as is obvious from the chromatogram of a plasma sample spiked with the test mixture (Fig. 2B).

The calibration curves were linear over the range 1 – 100 ng/mL. The mean values of regression parameters for the curve consisting of eight points, described by the equation: $y = bx + a$, were calculated as follows: $a = 0.004209$, $b = 0.011733$ and $r^2 = 0.99$ (n = 6).

The minimum detectable concentration of cinnarizine (LOD) was determined as 1.25 ng/mL of the mobile phase (S/N = 3). The lowest limit of quantitation (LLOQ) was found to be 1 ng/mL of plasma and relative standard deviation (RSD) of replicate determinations was 7.4% (n = 6).

The intra-day and inter-day precision and accuracy are presented in Table 1. The intra-day precision and accuracy were determined for repeated analyses (n = 6) of the quality control samples (low, medium and high concentrations) on the same day, and the inter-day precision and accuracy on three consecutive days. Assay precision was assessed by percent relative standard deviation (%RSD). Accuracy is expressed by bias, which is the difference between the true and the measured value. The intra-day accuracy ranged from 0.14 – 6.46 % and precision from 1.02 -1.90%. The inter-day accuracy ranged from 0.40 – 2.37% and precision from 3.88 – 5.78%.

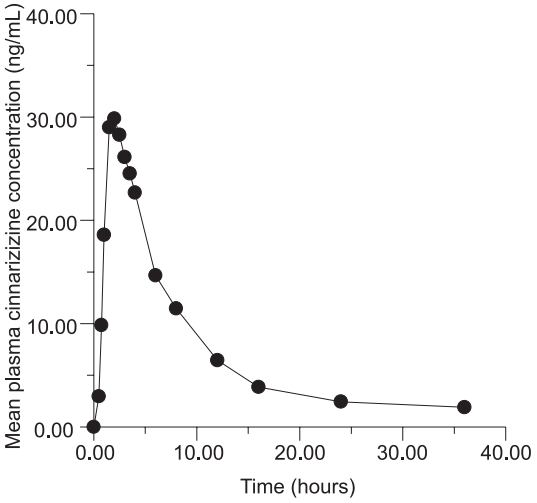

Figure 3. Mean plasma level of cinnarizine in 24 healthy volunteers following a single oral dose of 25 mg Stugeron tablets.

The stability of cinnarizine in autosampler during freeze-thaw cycles, short stability at room temperature and long stability in freezer (-70°C) were also checked.

The human plasma spiked with low, medium and high concentrations of cinnarizine (QC) were prepared. After 24 h of freezing, all samples are brought to room temperature without heating and next they were frozen again. The procedure was repeated in the further two cycles. After 72 h the concentrations of quality control samples were calculated from the daily calibration curve. The concentrations found were within ± 9% of nominal concentration, indicating good stability of the substance during repeated thawing and freezing.

Two sets of QC samples were prepared. One of them was analyzed after preparation and second was left in the autosampler at room temperature (21°C). Then that set was analyzed after 20 h. The mean recovery was 103%. The processed samples were stable at room temperature for at least 20 h. Plasma sample stability (short stability at room temperature) was tested at ambient temperature for 4 h. No significant decrease of the analyte concentration was observed (bias 5%). No tendency for decomposition was noticed in the frozen plasma samples during 3 months. Indeed, at -70°C, the accuracy presented for cinnarizine by bias percent was less than 10.

The proposed method has already been used in determination of cinnarizine in plasma samples from a pharmacokinetic study. Plasma samples were collected up to 72 h after oral administration of Stugeron® tablets (Janssen-Cilag) in 25 mg dose to 24 healthy male volunteers. The results are shown in Figure 3 and Table 3. The level of cinnarizine in plasma reached maximum at 2 h after the administration and decreased with the mean half-life of elimination of ca. 29 h.

CONCLUSION

The described method of the determination of cinnarizine in human plasma is sensitive and reproducible. The method was validated for accuracy, precision, specificity, linearity, sensitivity, recovery and stability. The applicability of the assay for pharmacokinetic study of cinnarizine in humans has been demonstrated.

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