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Automated high-performance liquid chromatographic assay for lesopitron, a novel anxiolytic, in human plasma using on-line solid-phase extraction

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Abstract

A totally automated liquid chromatographic assay method based on a Prospekt solid-phase extraction unit was developed for the analysis of lesopitron and its metabolite 5-hydroxylesopitron in human plasma. On-line solid-phase extraction of lesopitron, 5-hydroxylesopitron and its internal standard in human plasma was carried out using C_2 cartridges. After washing, the test substances were eluted with mobile phase onto an ODS-2 Inertsil column and measured by fluorescence detection. The total time for one analysis was 25 min.

The method developed was selective and linear in the concentration range from 1 to 40 ng/ml for both parent drug and metabolite. Recovery of lesopitron and 5-hydroxylesopitron were higher than 80% and the quantification limits were 1 ng/ml for both compounds. Coefficients of variation obtained for precision parameters were all below 14.5% and 13.9% for parent drug and metabolite, respectively. Good values of accuracy were also obtained.

1. Introduction

Lesopitron, (2- {4-[4-(4-chloro-1-pyrazolyl)-butyl] - 1 - piperazinyl}pyrimidine dihydrochloride) is a substance developed by Laboratorios Dr. Esteve (Barcelona, Spain), showing a high anxiolytic activity and a potency higher than buspirone [1–4]. Lesopitron acts as an agonist at 5-HT_{1A} receptors [3–6] and unlike other anxiolytics, lesopitron lacks sedative effects and does not potentiate alcohol or barbiturates effects [1,3,7].

Metabolism of lesopitron in humans takes place mainly through pyrimidine ring hydroxylation with 5-hydroxylesopitron as the chief me-

tabolite and with further formation of its conjugating derivatives. Another, less important, metabolic route in humans takes place through cleaving of the molecule with PmP formation [(2-(1-piperazinyl)pyrimidine)]. Though metabolism routes are highly conditioned by the animal species, in previous studies with rats it was demonstrated that PmP formation, when lesopitron is administered is lower than when ip-sapirone, buspirone or gepirone are administered [8].

The study and validation of an assay method suitable for simultaneous determination of unaltered drug and its main metabolite, in human plasma, is an essential step for further pharmacokinetic studies. Recent studies [9–11] have demonstrated the advantages of a Prospekt solid-

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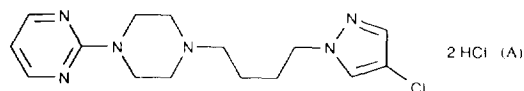
phase extraction system in the routine assays of active principles in biological samples.

The purpose of the present work was to validate the analytical method for simultaneous determination of lesopitron and 5-hydroxy-lesopitron (main metabolite) in human plasma. In this study we have used an on-line solid-phase extraction method with a high-performance liquid chromatographic system with fluorescence detection and automated data acquisition.

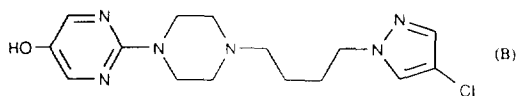
2. Experimental

2.1. Chemicals and reagents

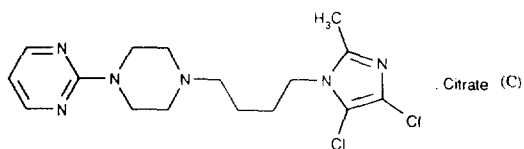
Lesopitron (E-4424). (Fig. 1A) was supplied by Esteve Química, and 5-hydroxy-lesopitron (2-[4-[4-(4-chloropyrazol-1-yl)butyl]-1-piperazinyl]-5-hydroxypyrimidine) (E-5043) (Fig. 1B) was supplied by the Synthesis Department of Laboratorios Dr. Esteve. The internal standard



LESOPITRON DIHYDROCHLORIDE



5-HYDROXYLESOPITRON



INTERNAL STANDARD

Fig. 1. Chemical structures of: (A) lesopitron; (B) 5-hydroxy-lesopitron; and (C) the internal standard.

used in liquid chromatography was (2-[4-[4-(4,5-dichloro-2-methylimidazole-1-yl)butyl]-1-piperazinyl]pyrimidine) (E-4804) (Fig. 1C), supplied by Esteve Química.

Drug-free human plasma used in this study was supplied by Hospital Clinic (Barcelona, Spain) and stored at -80°C until the moment of use.

Other reagents used were: monobasic potassium phosphate (Panreac, Barcelona, Spain), phosphoric acid (Carlo Erba, Milan, Italy), methanol and acetonitrile, HPLC grade (Scharlau, Barcelona, Spain). Water used in chromatography was purified through a Milli-Q system (Millipore, Barcelona, Spain).

2.2. Chromatographic system

The chromatographic system comprises a quaternary HP1050 pump (Hewlett-Packard, Barcelona, Spain), a Triathlon 1.0, refrigerated automatic injector, a Prospekt cartridge transportation system with three Rheodyne 6-way valves and a solvent pumping unit, all supplied by Spark Holland (Emmen, Netherlands). A detailed description of Prospekt solid-phase extraction system functions has been published by Nielen et al. [9]. The solutions used for cartridges conditioning, loading and washing were: 100% methanol, 100% water and acetonitrile-water (10:90, v/v).

Extraction of substances from plasma samples was carried out by using 10×2 mm I.D. Prospekt cartridges with 20 mg C_2 filling of Analytichem, also supplied by Spark Holland. For substances separation, an ODS-2 Inertsil $5\text{-}\mu\text{m}$ column was used; 250×4.6 mm I.D. in reversed-phase mode, supplied by GI Sciences (Tokyo, Japan). For protection of the chromatographic column, ODS-filled ($5\text{ }\mu\text{m}$) columns were used, supplied by Tecnokroma (Barcelona, Spain), which were replaced every week.

The mobile phase used was monobasic potassium phosphate 20 mM pH 3.0-acetonitrile (78:22, v/v). The flow-rate used was 0.5 ml/min and the eluent was monitored with a HP1046A fluorescence detector (Hewlett-Packard), at an

excitation wavelength of 235 nm and an emission wavelength of 385 nm.

Acquisition and plotting of chromatograms, area integrations and calculations, were carried out using a Nelson Series 3000 chromatographic data system, with the help of an interface Model 941 (PE Nelson, Cupertino, CA, USA).

2.3. Sample preparation procedure

Drug-free human samples, after being unfrozen, were centrifuged at 2000 *g* for 10 min. Aliquots of 200 μ l of plasma contained in 1.1-ml glass vials were spiked with 20 μ l of an aqueous solution of lesopitron, 5-hydroxylesopitron (at different concentrations) and the internal standard (50 ng/ml). Then, after being mixed, the samples were placed in the Triathlon autosampler. Finally, aliquots of 100 μ l were injected. All solutions were prepared daily.

2.4. Solid-phase extraction procedure

Before injecting the sample, the cartridge was activated by pumping methanol through it at a flow-rate of 1.5 ml/min for 1 min followed by water for 3 min. At this point the automatic

injector was set ready for injection and following a signal from the Prospekt microprocessor, the solvent delivery unit decreased the flow-rate from 1.5 to 1.0 ml/min, and a sample (100 μ l) was injected into the solid-phase extraction cartridge. Then, the cartridge was washed with water during 1.5 min at 1.0 ml/min and further on with acetonitrile–water (10:90, v/v) for 45 s at 1 ml/min. After finishing the washing process, the components retained were eluted in the forwardflush mode by the mobile phase from the extraction cartridge onto the chromatographic column for 2 min. At this point, data acquisition started. To end the process, the cartridge and the capillaries were rinsed with methanol and exchanged automatically for a new cartridge, starting a new cycle. The procedure described is represented in Table 1.

2.5. Assay validation

Drug-free human plasma spiked with a known amount of lesopitron, 5-hydroxylesopitron and the internal standard (50 ng/ml) working solution, were processed as described above. All chromatograms obtained were evaluated by peak-area measurement. Eight calibration curves

Table 1
Solid-phase extraction method

Time (min:s)	Switch valve No.	Solvent ^a	Flow (ml/min)	Comment
00:00		1	1.5	Change of cartridge
01:00		2	1.5	Activation of cartridge with 100% methanol
04:00		2	1.0	Activation of cartridge with water
				Adjusting flow-rate through cartridge
				Injection of sample
				Washing of sample with water
05:30		3	1.5	Washing of sample with acetonitrile–water (10:90, v/v)
06:15	1 (on)	3	0.1	Start of elution onto the analytical column
				Start of data collection
				Adjusting flow-rate through cartridge
08:15	1 (off)	3	1.5	End of elution
				Adjusting flow-rate
10:15		1	1.5	Washing of cartridge and capillaries with 100% methanol
19:25			0.0	End of washing
25:00				End of method

^a 1 = 100% methanol; 2 = 100% water; 3 = acetonitrile–water (10:90, v/v).

were prepared and run on eight different days in the concentration range 1–40 ng/ml in order to evaluate the linearity.

The percentage of substance recovery was calculated by comparison of the peak-area of the extracted plasma samples with the peak-area obtained from separate injection of the unextracted mobile phase solutions at three different concentrations (1, 10 and 40 ng/ml). These samples were made as described in Section 2.3, but using mobile phase instead of drug-free human plasma.

Precision was assessed by analyzing eight replicate samples prepared on the same day (intra-day precision) and one sample every day during eight days (inter-day precision). Accuracy was expressed as the mean value of the percentage ratio of found to added amounts, obtained at different concentration levels on the same day (intra-day accuracy) or on different days (inter-day accuracy).

The stability of lesopitron and 5-hydroxy-lesopitron under different storage conditions was studied as follows: stability in aqueous solutions of substances at 4°C for up to 1 week after preparation; stability in frozen plasma, at –80°C, for up to 2 weeks after preparation; stability in frozen plasma, at –30°C, for up to 2 weeks after preparation.

3. Results and discussion

3.1. Chromatographic system suitability test

The suitability of the chromatographic system was checked daily before the analysis by evaluation of the detection response of three replicate injections of a mobile phase standard solution of lesopitron, 5-hydroxy-lesopitron and the internal standard (10, 10 and 50 ng/ml, respectively). For both substances the experimental concentration obtained was found within the range 90–110% ($96.6 \pm 4.9\%$ for lesopitron and $99.7 \pm 5.5\%$ for 5-hydroxy-lesopitron), which demonstrates the good performance of the chromatographic system throughout the study. The retention times (mean \pm S.D.) obtained were: 12.0 ± 0.4 min for

5-hydroxy-lesopitron; 14.4 ± 0.5 min for lesopitron; and 16.4 ± 0.7 min for the internal standard.

3.2. Optimization of the chromatographic conditions

Fluorescence detection was used because it provides a greater specificity than UV absorbance. Several stationary and mobile phases were investigated to establish the optimum separation and the highest analytical sensitivity for lesopitron and 5-hydroxy-lesopitron. The best results were obtained with the conditions previously reported. The analysis time was fixed to 25 min. This time assured the elution of interfering peaks of plasma and permitted the Prospekt preparation and extraction of the next sample.

Different solid-phase extraction columns were tested. The best results with our conditions were obtained with C₂ cartridges. The use of more hydrophobic cartridges like C₈ or C₁₈ always caused loss of recovery because those phases produced a strong retention of substances in the cartridge.

The selection of the injection volume was performed by injecting into the system 100, 200 and 300 μ l of drug-free human plasma and human plasma spiked with the different substances. It was observed that an increase in injection volume did not improve the signal-to-noise ratio of substances, so it was decided to use the 100- μ l volume in order to extend the column life.

HPLC chromatograms for drug-free human plasma and the human plasma spiked with lesopitron, 5-hydroxy-lesopitron and the internal standard are shown in Figs. 2A and 2B, respectively. The chromatogram from drug-free plasma was free from interferences at the retention times corresponding to the drug peaks. As shown in Fig. 2B, the two peaks corresponding to lesopitron and 5-hydroxy-lesopitron and the peak corresponding to I.S. were separate baselines. Under the HPLC conditions employed, retention times for 5-hydroxy-lesopitron, lesopitron and the internal standard were approximately 12.0, 14.4 and 16.4 min, respectively.

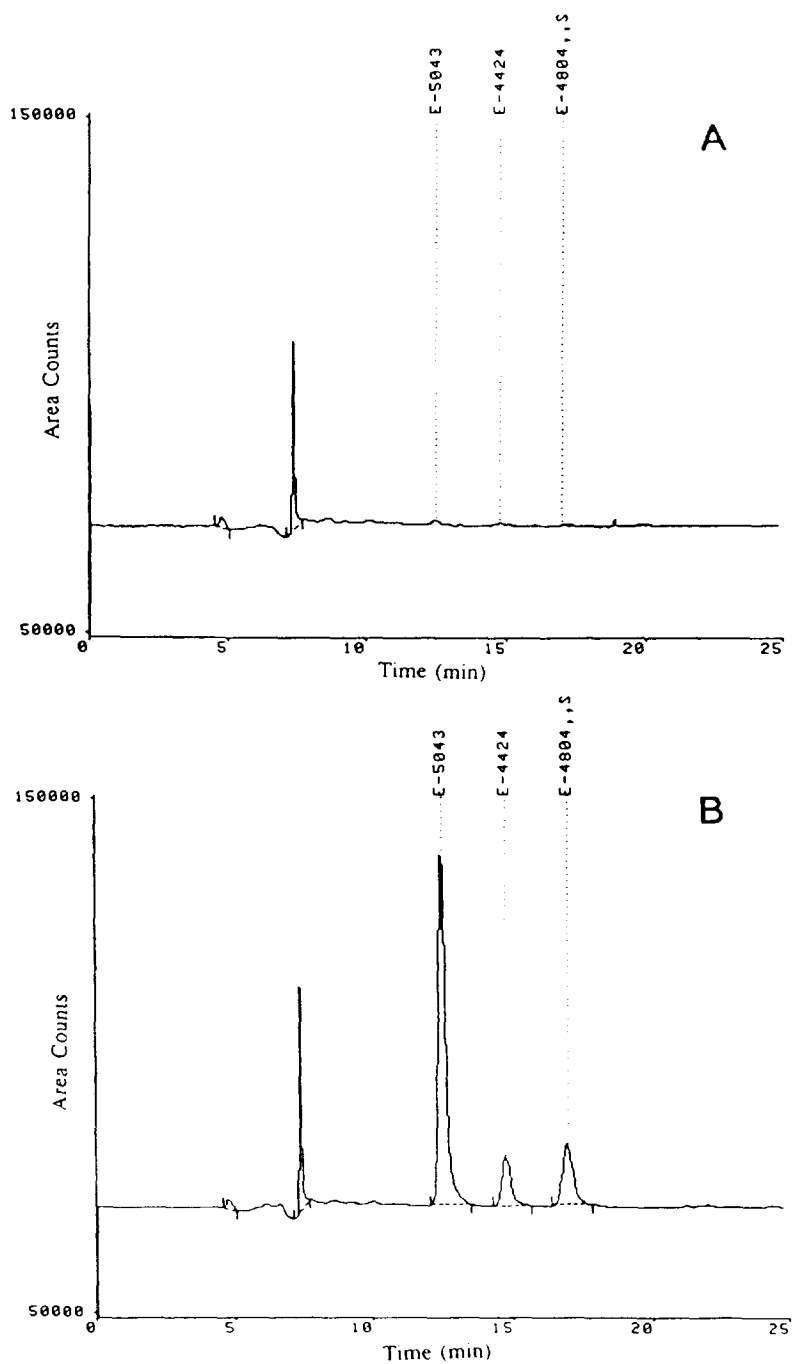


Fig. 2. Chromatograms of (A) a drug-free plasma and (B) a plasma containing lesopitron (E-4424; 10 ng/ml), 5-hydroxy-lesopitron (E-5043; 10 ng/ml) and 50 ng/ml of internal standard (E-4804).

Table 2
Calibration curve parameters for lesopitron and 5-hydroxylesopitron in human plasma ($n = 8$)

Product	Slope (mean \pm S.D.)	Mean intercept	Mean correlation coefficient (r^2)
Lesopitron ^a	1.87 \pm 0.09	-0.0239	0.9975
5-Hydroxylesopitron ^a	13.33 \pm 0.74	0.0507	0.9980

^a Range: 1–40 ng/ml.

3.3. Selectivity

Blank plasma samples from six different sources showed no endogenous peaks interfering with the assays of lesopitron, 5-hydroxylesopitron and internal standard.

3.4. Limit of detection

The limits of detection (signal-to-noise ratio > 2) for lesopitron and 5-hydroxylesopitron were ca. 1 ng/ml and 0.3 ng/ml, respectively. The limits of quantitation defined as the lowest concentration that can be determined from plasma with a coefficient of variation (C.V.) $< 15\%$ [12] were 1 ng/ml for both substances.

3.5. Validation

The calibration curves obtained on eight days showed a linear relationship between the peak-area ratio of products and the concentrations added. The intercepts of the regression lines

were close to zero and the correlation coefficients were more than 0.997 in the range of 1–40 ng/ml for both substances (Table 2).

The recovery of lesopitron and 5-hydroxylesopitron from plasma samples was determined by comparing the peak-area of the respective peaks from extracted standards in human plasma, to the peak-areas of unextracted standards. Peak-areas of lesopitron, 5-hydroxylesopitron and the internal standard were measured in chromatograms obtained after direct injection of the three compounds dissolved in mobile phase without extraction. The peak-areas were compared with those obtained when drug-free plasma spiked with known amounts of the three substances was assayed.

Table 3 shows the recovery values (mean \pm S.D.) of substances from human plasma. The performances obtained at the three concentration levels studied (1, 10 and 40 ng/ml) for both tests substances as well as for the internal standard, were in most cases higher than 80%.

The recovery at 1 ng/ml was found to be

Table 3
Recovery of lesopitron, 5-hydroxylesopitron and internal standard extracted from 100 μ l plasma on C_2 solid-phase extraction columns ($n = 8$)

Concentration (ng/ml)	Recovery (%)		
	Lesopitron	5-hydroxylesopitron	Internal standard
1	78.9 \pm 8.9	80.4 \pm 10.3	–
10	82.6 \pm 8.7	84.5 \pm 7.3	–
40	91.8 \pm 3.2	90.9 \pm 4.9	–
50	–	–	87.0 \pm 6.6

$78.9 \pm 8.9\%$ for lesopitron, $80.4 \pm 10.3\%$ for 5-hydroxylesopitron, and $87.0 \pm 6.6\%$ for internal standard (50 ng/ml).

The values obtained seem to confirm the independence of extraction performance from substance concentrations in samples. However, the small differences on recovery for the concentration levels studied could be due to the high protein-binding for those products ($>95\%$). This would be in disagreement with the results obtained by other authors [9,13], in whose papers the concentration effect on recovery of highly protein-bound drugs was not studied.

Intra-day precision of the method was determined by using the values obtained for three concentrations (1, 10 and 40 ng/ml) analyzed the same day. Inter-day precision was calculated for all plasma concentrations used in the daily calibration curves. In both cases the results were expressed as coefficient of variation (%). Table 4 shows the precision values obtained. Intra-day precision for lesopitron was 9.9, 4.3 and 3.0% at 1, 10 and 40 ng/ml, respectively. The inter-day precision at the seven points of the calibration curve for lesopitron were less than 14.5%; for

5-hydroxylesopitron the corresponding variations were less than 13.9%.

As expected, coefficients of variation increased as the level of substances decreased, but all coefficients were below 15%. Intra-day precision values as well as inter-day values were found to be within limits considered by Shah et al. as acceptable [14].

The accuracy of the HPLC method was evaluated at three different concentrations (2, 8 and 20 ng/ml) during eight days. The results are shown in Table 5 and expressed as percentage of analyte recovered by the assay (%). Intra-day accuracy for lesopitron ranged between 90.7% and 102.7% and intra-day accuracy for 5-hydroxylesopitron ranged between 87.2% and 110.8%. On the other hand, inter-day accuracy for lesopitron ranged between 93.4% and 103.8% and the inter-day accuracy for 5-hydroxylesopitron ranged between 90.0% and 102.4%.

The results from stability studies in aqueous solution (expressed as percentage of initial concentration $\% \pm$ S.D.; $n = 10$) showed that lesopitron and 5-hydroxylesopitron were stable for 1 week at 4°C ($96.4 \pm 1.8\%$ for lesopitron and $101.9 \pm 1.2\%$ for 5-hydroxylesopitron).

Stability studies in human plasma at -30° and -80°C ($n = 10$) showed that lesopitron and hydroxylesopitron remained stable for at least 2 weeks of storage and there was no effect of

Table 4
Precision of the determination of lesopitron and 5-hydroxylesopitron in spiked plasma ($n = 8$)

Concentration (ng/ml)	Intra-day C.V. (%)	Inter-day C.V. (%)
<i>Lesopitron</i>		
1	9.9	13.3
2		8.4
4		10.7
8		14.5
10	4.3	12.6
20		3.7
40	3.0	5.7
<i>5-Hydroxylesopitron</i>		
1	8.2	13.9
2		12.3
4		9.2
8		11.4
10	5.4	10.3
20		4.6
40	5.2	6.3

Table 5
Accuracy of the determination of lesopitron and 5-hydroxylesopitron in spiked plasma ($n = 8$)

Concentration (ng/ml)	Accuracy (mean \pm S.D., $n = 8$) (%)	
	Intra-day	Inter-day
<i>Lesopitron</i>		
2	90.7 ± 4.5	103.8 ± 10.0
8	97.4 ± 6.5	93.4 ± 5.5
20	102.7 ± 2.4	100.1 ± 4.5
<i>5-Hydroxylesopitron</i>		
2	87.2 ± 9.3	90.0 ± 10.4
8	110.8 ± 6.6	102.4 ± 7.4
20	106.9 ± 4.2	102.4 ± 4.4

storage temperature on stability of the substance ($101.8 \pm 3.2\%$ for lesopitron and $98.6 \pm 5.2\%$ for 5-hydroxylesopitron).

4. Conclusions

An analytical method has been developed and validated to determine lesopitron and 5-hydroxylesopitron in human plasma by means of liquid chromatography with fluorescence detection. The use of a solid-phase extraction process on-line with the chromatograph together with the use of a computerized data acquisition system provides the process with a complete automation. The sample preparation is therefore simplified by the mere manual addition of the internal standard.

The solid-phase extraction process of a sample in a C₂ cartridge is carried out while the chromatographic reading of the previous sample takes place, which brings a considerable saving time.

The method described in this study was selective, linear, precise and accurate within a concentration range between 1 and 40 ng/ml as much for lesopitron as for 5-hydroxylesopitron. All coefficients of variation obtained for this method were below 15%.

The process followed for loading, rinsing and eluting substances from the extraction cartridges provided an extraction performance around 80%, for both test substances and for the internal standard.

Lesopitron and 5-hydroxylesopitron are stable

in aqueous solution at 4°C and in plasma at -30°C and -80°C for a period of at least two weeks.

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