LC-MS/MS Bioanalytical Method for Simultaneous Quantification of Three H_1-Antihistaminic Drugs in Human Plasma

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The aim of the study was to validate a bioanalytical method, for quantifying desloratadine, loratadine and cetirizine in biological samples, useful in toxicological studies. The analytical performance was investigated using the following parameters: selectivity, limit of detection and quantification, linearity, precision, accuracy, matrix effect and analytical stability in the biological matrix. The method optimization was performed for the LC-MS/MS Ultimate 3000 instrument provided by Thermo Fisher Scientific.

Keywords: H_1-antihistaminic drugs, human plasma, LC-MS/MS

H_1-antihistamines (H_1 receptor antagonists) competitively block histamine at H_1 receptors and prevent the characteristic H_1-type effects of histamine released after antigen-antibody interaction. Currently, H_1-antihistamines represent the second most commonly used class of drugs, after antibiotics, with more than 40 varieties used in medical practice worldwide [1]. H_1-antihistamines, desloratadine (DSL), loratadine (LOR) and cetirizine (CTZ), are members of a very frequent used class of drugs, in children and adults, for the treatment of various allergic manifestations, even without a medical prescription.

The most liquid chromatographic methods for analysis of this compounds reported in the literature are focused on determining concentrations of H_1-antihistamines and their metabolites [2-11]. In many studies, only loratadine, desloratadine and cetirizine are determined from biological fluids or pharmaceuticals, but there are also methods for their simultaneous determination with other compounds found in combinations in certain drugs (pseudoephedrine) [12, 13] or compounds associated in the treatment of allergic manifestations (montelukast) [14, 15].

A few methods (capillary electrophoresys, liquid or gas chromatography) have been described in the literature for simultaneously cuantification of a wide range of H_1-antihistamines [16-21].

The aim of this study was the development and validation of a simple and sensitive LC-MS/MS method for the simultaneous quantitative determination of three commonly prescribed antihistamines (desloratadine, loratadine, cetirizine) in human plasma. The method can be used for the investigation of H_1-antihistamine related clinical cases, as well as for toxicokinetic and studies concerning the safety of antihistamines.

Experimental part

Materials and methods

The determinations were performed using a Ultimate 3000 high performance liquid chromatograph, supplied by Thermo Fisher Scientific equipped with Surveyor LC-Pump with a maximum pressure of 1000 barr, Open autosampler, thermostatted compartment for column.

Specific transitions of each compound were determined using a TSQ Quatum Access Max tripluquadrupol mass spectrometer detector, equipped with heated electrospray ionization source and capable of recording molecular ion spectra, as well as specific fragments.

The Aria MX Direct Control software, as well as the Thermo Excalibur Roadmap allowed the recording of each chromatographic test, the recording of all data for linearity evaluation and the simultaneous acquisition of the chromatographic signals and data interpretation.

Reagents

Loratadine and cetirizine (reference substance) were kindly donated by AC Helcor Research Center, Baia Mare, and desloratadine (reference substance) was donated by Gedeon Richter Research Center, Targu Mures; amitriptyline (HPLC purity) was purchased from European Pharmacopoeia. Solvents used, methanol, acetonitrile, formic acid (HPLC grade), were purchased from Merck-Germany. Trichloroacetic acid for protein precipitation (HPLC purity) was obtained from Merck-Germany, and human control plasma was supplied by Macopharma (Romania).

Chromatographic method

The compounds were separated using a Hypersil Gold C_{18} Chromatographic column (50x2.1 mm, 1.9 µm particle size). For the protection of the column, a pre-column with the same octadecylsilyl stationary phase was used.

The mobile phase consisted of a mixture of 0.1% formic acid solution as mobile phase A, and acetonitrile solution with 0.1% formic acid content as mobile phase B.

The elution was isocratic, the composition from the beginning to the end of the determinations remained constant and consisted in a mixture of mobile phase A and mobile phase B of 65:35 % (v/v). The flow rate was 0.25 mL/min and the analysis time was 5 minutes, sufficient to ensure the detection and separation of all compounds of interest. The temperature of the determination was 45°C and the injected volume was 10 µL.

The mass spectrometry system in the electrospray probe used a discharge potential of 3.5 kV, vaporization temperature of 200°C, 30 psi pressure for the ionizing gas, 350°C for the transfer capillary temperature of and the potential of 35V. For MS/MS determinations argon was used as collision gas at a pressure of 1 mTorr.

Specific transitions are presented in the Table 1.

Preparation of samples

Standard working solutions

For standard stock solutions, 10 mg of each substance were dissolved in methanol. The working standards for

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Table 1
SPECIFIC TRANSITIONS OF THE COMPOUNDS

Compound	Molecular Ion	Fragment	Scan width	Scan time	Collision energy	Q1 Potential	Q3 Potential	Tube lens
DSL	311.1	259.1	0.02	0.1	20	0.7	0.7	89
LOR	389.1	201	0.02	0.1	20	0.7	0.7	76
CTZ	383	337	0.02	0.1	22	0.7	0.7	94
AMT	278	233	0.02	0.1	22	0.7	0.7	67

the calibration curves were obtained by successive dilution of the standard stock mixture with mobile phase. Amitriptyline (AMT) was used as internal standard (IS). For superior stability, these solutions were maintained at -18°C for a maximum of 5 days.

Samples of desloratadine, loratadine, and cetirizine at a concentration of 1.0 µg/mL were obtained by diluting a stock solution to 1000 µg/mL with mobile phase. For each sample, a volume of 0.05 mL of internal standard (1000 µg/L amitriptyline solution in mobile phase) was added.

To verify the selectivity, the quality control (QC) solutions were prepared by adding 0.1 mL from each stock solution (1.0 µg/mL DSL, LOR, CTZ) to 0.7 mL human plasma. A volume of 0.5 mL was mixed with 0.05 mL IS stock solution, 0.25 mL of 20% trichloroacetic acid solution and 0.2 mL of water. The sample was centrifuged for 10 min at 6000 rpm, and a volume of 0.5 mL clear supernatant was subjected to analysis.

For estimating the sample stability, the analysis of the stock solutions and of the analytes from the matrix was performed by injecting individual samples. QC solutions consisted in the addition of 0.05 mL desloratadine and cetirizine stock solutions and 0.1 mL loratadine stock solution to 0.8 mL plasma; 0.5 mL of the mixture was mixed with 0.05 mL of internal standard stock solution, 0.25 mL of 20% trichloroacetic acid solution and 0.2 mL of water; the samples were centrifuged for 10 min at 6000 rpm and a volume of 0.5 mL clear supernatant was taken.

Results and discussions

Specificity

The specificity study was performed by monitoring the parameters regarding the separation efficiency on the chromatographic column. In Table 2 are described the parameters of chromatographic efficiency. The evaluation was performed in relation to a non-retained compound, with a retention time of 0.1 minute.

Table 2
PARAMETERS OF CHROMATOGRAPHIC EFFICIENCY

Chromatographic parameters	DSL	LOR	CTZ
Retention time (Tr)	0.82	1.97	1.38
Assymetry	0.91	1.01	1.00
Number of theoretical plates	10321	442345	122344
Rezolution factor (Rs)	6.86	5.33	5.62

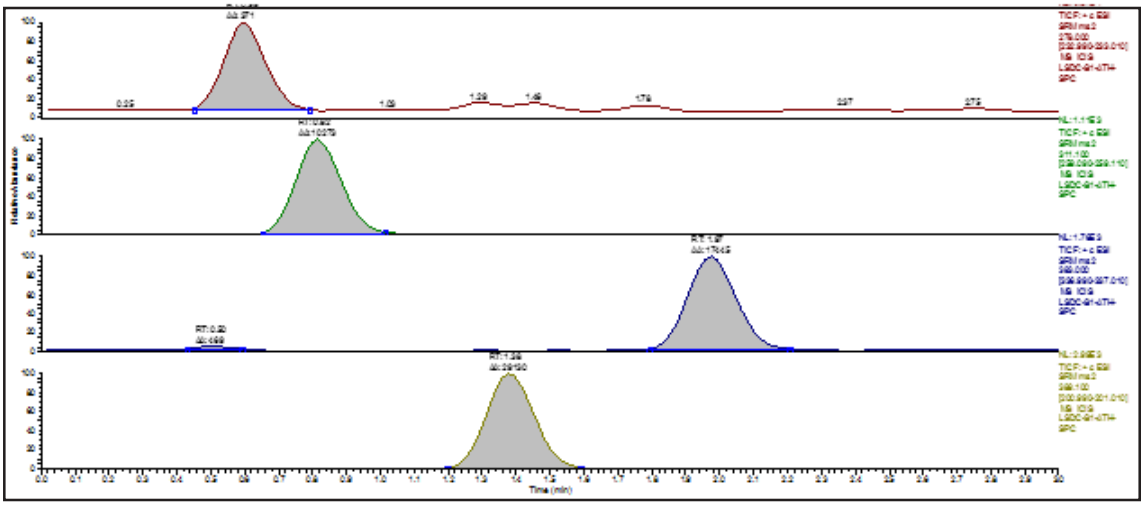

Fig. 1. Sample at the upper limit of quantification (DSL 311 –>259, LOR 383 –> 337, CTZ 389 –> 201)

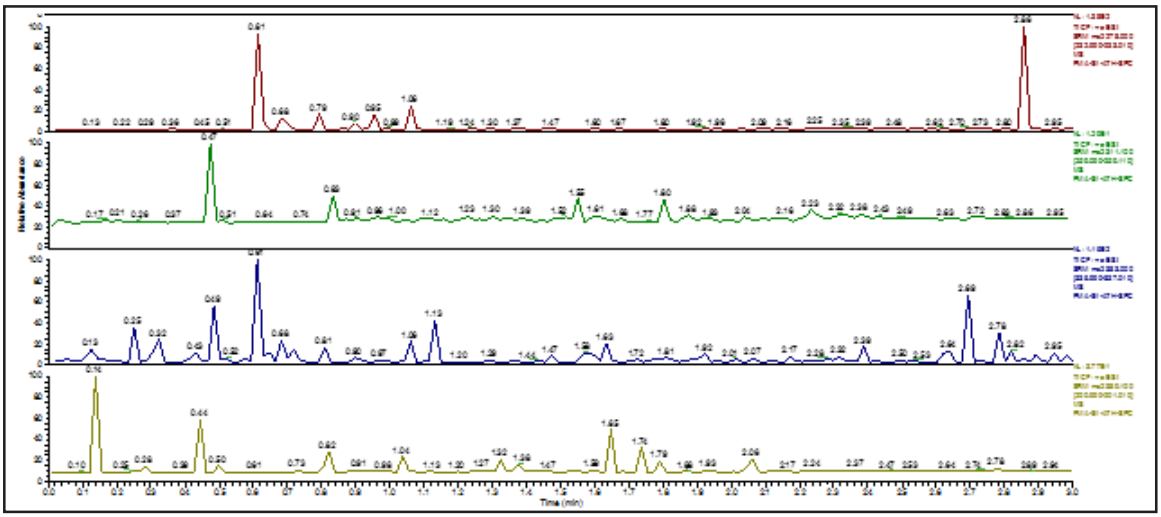

Fig. 2. Blank sample - source S1 (DSL 311 –> 259, LOR 383 –> 337, CTZ 389 –> 201)

Representative chromatograms are illustrated in Figures 1 and 2.

Selectivity

The selectivity was demonstrated for interferences using 6 individual sources of the control matrix.

The size of the interferences was determined for the metabolites of the compounds and also for the degradation products that can be formed during the preparation of the sample.

Because of the intense metabolism rate of loratadine and desloratadine, it was important to evaluate the interferences of metabolites. 3-hydroxidesloratadine was one of the interfering metabolite. The data from the literature showed a MS/MS method for determination of desloratadine and its metabolite, using positive ionization mode with a potential for the ionization source of 5.5 KV, the temperature of 500°C, the pressure of the nebulization gas of 50 psi and a collision gas of 3 psi [5]. Considering the similarity of the conditions of determination the specific transition for the analysis of 3-hydroxydesloratadine is 326.9 (*m/z*) -> 274.98 (*m/z*) with collision energy of 27 eV. Hydroxyzine may be a source of cetirizine, as a metabolite [22]. The specific transition used was 375 (*m/z*) -> 201 (*m/z*).

The analysis of individual samples for the determination of parallel interferences on concomitant analysis was performed by injection of the individual sample.

For the determination of concomitant interferences, the analysis of individual samples was performed by injecting of separated samples containing the 3 evaluated compounds.

In case of desloratadine, a signal for cetirizine was present and the concentration was 0.447 ppb corresponding to level of 17.88% from LLOD.

In case of injecting cetirizine, a chromatographic peak with a retention time of 1.4 min was registered and was associated with 3-hydroxidesloratadine. The relative concentration was of 5.2% in report with desloratadine.

For the study of loratadine, a signal corresponding to cetirizine was considered. The concentration was 0.37 ppb. Also it was present a metabolite of desloratadine with a retention time of 1.4 min and a concentration of 0.07 ppb.

In the analysis of selectivity, the interference resulted during the concomitant administration was also evaluated (Fig. 3).

The interferences concentrations determined are between 0.01 µg/L and 0.07 µg/L, and the degree of interference is between 1.44% and 0.33%. The limit is below 5%.

Residual effect

The residual effect was evaluated by injecting blank samples (BS) after injecting of sample with concentration of the compounds at the level of the upper limit of quantification. The residual effect of the blank sample after the high concentration standard should not exceed 20% of the LLOQ and 5% for the IS.

The residual effect is within the limits imposed. In the case of desloratadine the effect is maximum 11.81%, for loratadine 7.86 and 4.82% respectively for cetirizine.

In the case of the IS, the residual effect is 0.37%, a value that is below the 5% limit.

Linearity

Three independent series of concentrations between 2.5 and 100 µg/mL were determined. An 8 point calibration line was drawn for each compound, with 3 determinations for each concentration.

The results of the determinations were processed statistically and a graphical representation (correlation) for linear regression study of the data ($r \geq 0.990$) was drawn for the peak area in relation with the concentration of samples.

The parameters of the calibration curve (the slope and intercept in the case of a linear relationship) are determined, the concentrations of the calibration standards and the average values of the accuracy, expressed as a percentage of recovery over the nominal concentration, are recalculated.

The linearity was evaluated on the proposed field of work.

The correlation (calibration right) corresponding to each compound (Fig. 4-6) shows a linear dependency between the sample concentration and the peak area, therefore estimating the peak area will be done using the linear regression equation: Area-Intercept = Slope x Concentration.

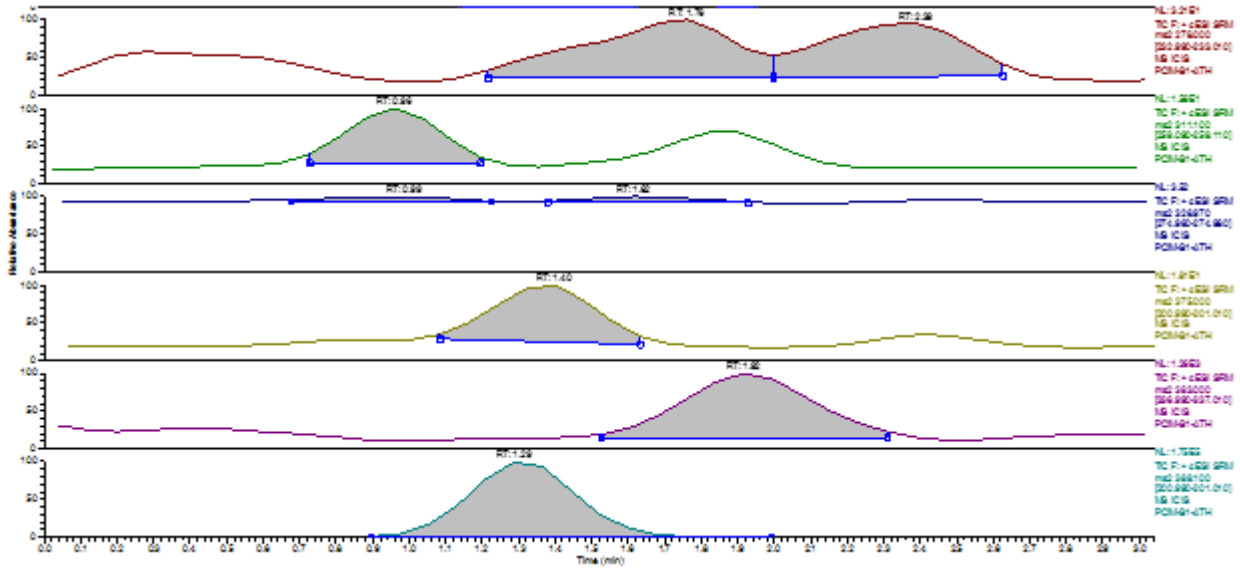

Fig. 3. Interferences due to concomitant administration DSL (311-259 *m/z*), 3-hydroxidesloratadine (326.9-275 *m/z*), LOR (383-337 m/z), CTZ (389-201 *m/z*), hydroxyzine (375-201 *m/z*)

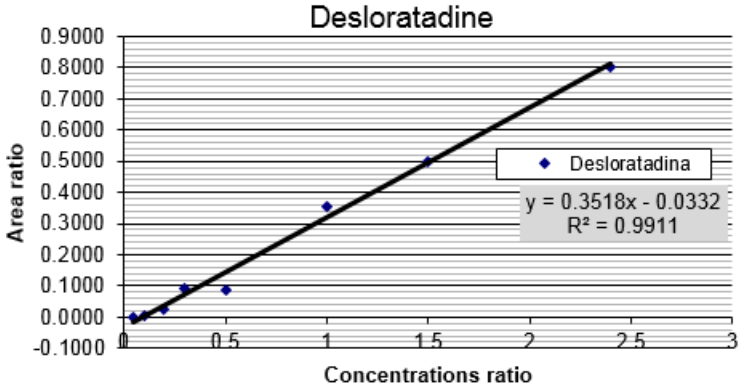

Fig. 4. Desloratadine correlogram

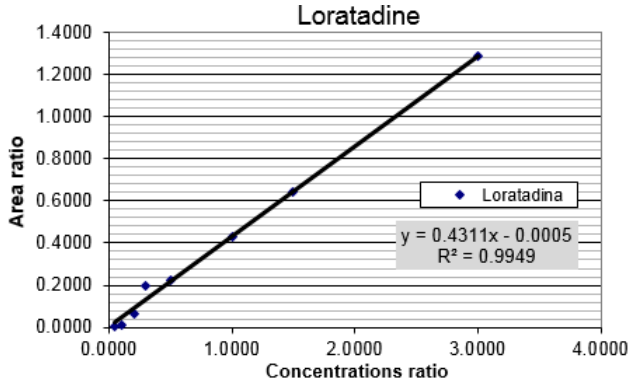

Fig. 5. Loratadine correlogram

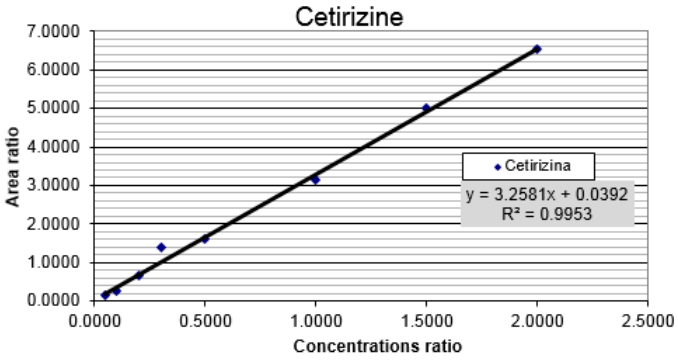

Fig. 6. Cetirizine correlogram

The regression coefficients determined has values of 0.9911, 0.9949 and 0.9953 for desloratadine, loratadine and cetirizine.

Analysis of variance

The value of the determined parameter *P* for the intercept and for the slope has values less than 0.05 ($P<0.05$), which indicates the correctness of the determined linear model.

The value for sum of squares of deviations (SS) and mean of squares (MS) have close values, the *ratio F* shows values *of* 665 for DSL, 1167.69 LOR and 1272.37 for CTZ, values greater than the relevant statistical threshold. The conclusion is that the independent variables significantly influence the behavior of the dependent variables, so the correlation ratio is statistically significant.

The deviations of the residual values and the average of the squares amounts have values close to 0, so the predicted values are similar to the values determined in the studied correlation.

Limits of quantification and detection

The supporting chromatogram is shown in Figure 7.

The minimum limit of corresponding quantification meets the acceptance criteria being at least 5 times the area of the signals corresponding to the analyzed control samples.

The lower limits of quantification (LLOQ) and detection (LLOD) were calculated taking into account the slope values as well as the standard errors. Thus, by applying the direct relation between slope and standard error, the values of the concentrations for the lowest quantification limits were established. By adding the theoretical concentration corresponding to the internal standard, it was possible to estimate the concentrations corresponding to the minimum quantification limits but also to the minimum detection limits (Table 3).

Accuracy and precision

Accuracy within an analytical determination

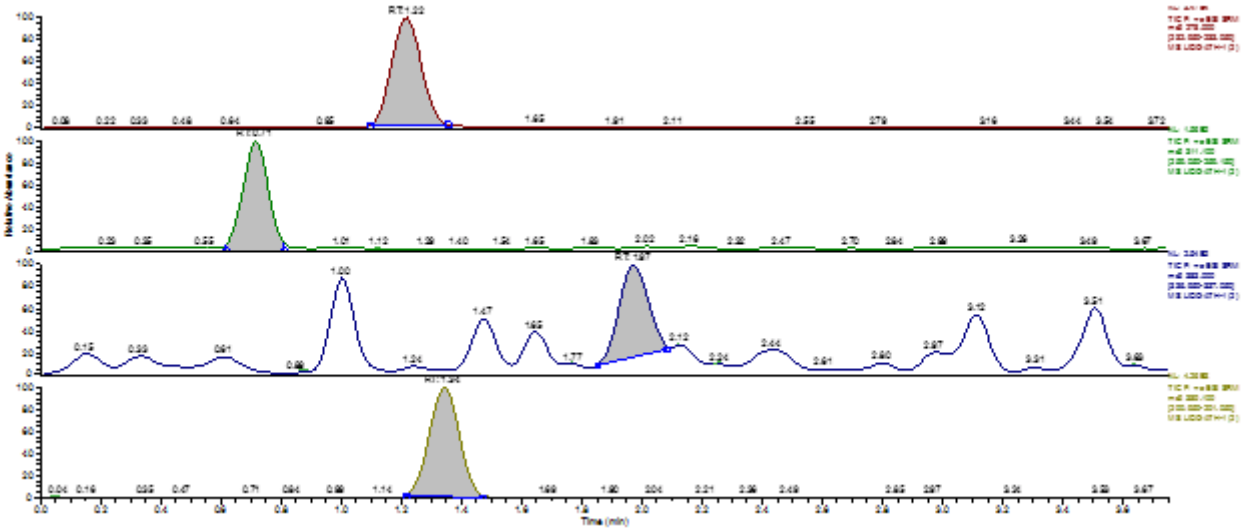

Fig. 7. Low limit of cuantification – 2.5 ppb

Compound	Slope	Standard error	LLOQ (µg/L)	LLOD (µg/L)
DSL	0.352	0.002	3.41	1.13
LOR	0.431	0.003	3.48	1.15
CTZ	3.258	0.030	4.60	1.52

Table 3
LOWEST LIMIT OF DETECTION (LLOD) AND CUANTIFICATION (LLOQ) VALUES

Compound	Parameter	Mean recovery		
DSL	Accuracy (procentual recovery) (day 1+2)	99.27	104.81	104.71
LOR		91.72	103.97	87.36
CTZ		93.71	103.35	90.76
Compound	**Parameter**	**Coefficient of variation (CV%)**		
DSL	Precision (general CV%) (day 1+2)	5.19	9.72	11.32
LOR		12.40	2.36	7.58
CTZ		9.50	7.42	8.78

Table 4
GLOBAL VALUES FOR THE STUDY OF ACCURACY AND PRECISION

Accuracy was evaluated on samples injected with known amounts of analytes (desloratadine, loratadine and cetirizine) as quality control samples (QC samples). QC samples were prepared independently of calibration standards, using different stock solutions. The QC samples were analyzed in comparison with the calibration curve, and the obtained concentrations are compared with the nominal values. Accuracy was reported as a percentage of the nominal value. Accuracy was evaluated for QC sample values obtained within a single determination (accuracy within a determination) and between different analytical determinations (accuracy between determinations) by reevaluating the samples on different days (Table 4).

Stability of samples

The long-term stability of the analytes in the matrix stored in the freezer at -20°C was studied, using QC samples, which were stored in the freezer under the same storage conditions and for the same time period as the samples in the study.

Stability was performed on an interval equal to the retention period of the biological samples under study. The stability followed the degree of recovery of the concentrations of active substance in biological samples. The substances were found to be stable for up to 1 year. For desloratadine, the variation of the signals was up to an interval of approximately 10 months. In the last time of the analysis, there was noticed a pronounced decrease up to 90.64%. For loratadine and cetirizine, the variations did not give information about certain sensitivities regarding the maintenance of the substances in the analyzed matrices at conditions of -20°C for one year.

Application of LC-MS/MS method to quantitative determination of desloratadine, loratadine and cetirizine in human plasma

The LC-MS/MS method developed and validated for the simultaneous determination of H_1-antihistamines from biological samples was tested in allergic patients undergoing treatment with these compounds.

The adult patients volunteers who participated in this study signed an Informed Consent form regarding the study protocol. The study was approved by the Research Ethics Committee of the Grigore T. Popa University of Medicine and Pharmacy, Iasi, no. 15474/27.07.2015 regarding good conduct in scientific research.

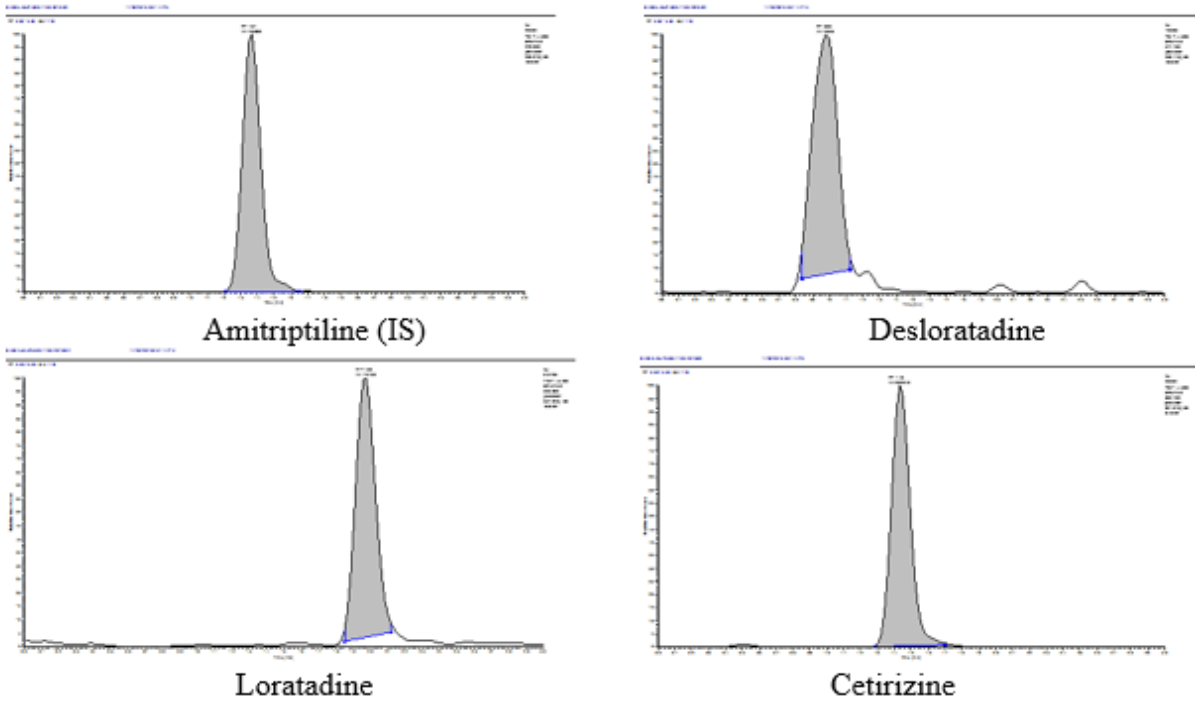

Fig. 8. Cromatograms of the compounds from human plasma

Biological samples were collected from patients undergoing treatment with H_1-antihistamines. Venous blood was collected by authorized personnel in Lithium-Heparin vacutainer, one hour after administration of the drug. The blood was allowed to coagulate, then centrifuged for 5 minutes at 3000 rpm, to obtain the plasma. The biological samples were frozen and kept at -20°C until the moment of processing and chromatographic analysis. Plasma samples were processed according to the extraction method described, by precipitating with trichloroacetic acid, using as internal standard, amitriptyline.

The concentrations of desloratadine, loratadine and cetirizine in human plasma were determined in patients who received treatment with H_1-antihistamines for various allergic manifestations by the validated LC-MS/MS method (Fig. 8).

The concentrations determined from human plasma, at one hour after the administration of therapeutic doses of the 3 antihistamines, ranged between: 4.72-6.66 µg/L for desloratadine, 3.84-6.09 µg/L for loratadine and 16.74-27.70 µg/L for cetirizine, with a medium concentration of 5.51 µg/L for desloratadine, 5.17 µg/L for loratadine and 23.05 µg/L for cetirizine. The results obtained in the quantitative determination of desloratadine, loratadine and cetirizine in human plasma by the LC-MS/MS method are comparable to the results reported in kinetic studies by similar methods in the literature [5, 12, 23].

Conclusions

The LC-MS/MS bioanalytical method developed and validated in the present study is fast, simple, precise and does not imply a high cost of analysis. Compared with other HPLC methods for the simultaneous determination of human plasma antihistamines H1, this method offers the advantage of being able to determine a more variable range of such compounds.

The main evaluated features of the LC-MS/MS method, which ensure the acceptability and veracity of the analytical results, were: selectivity, linearity of response, lower limit of quantification, matrix effect, accuracy, precision, stability of the analytes in the biological matrix.

The correlograms are linear in the studied area (2.5-120 µg/L), the regression coefficients determined were 0.9911 (DSL), 0.9949 (LOR), 0.9953 (CTZ). The lower quantification limits (3.41 µg/L - DSL; 3.48 - LOR; 4.6 µg/L - CTZ) and the lower limits of detection (1.13 µg/L - DSL; 1.15 - LOR; 1.52 µg/L - CTZ) were calculated, the estimation being based on the standard deviation and the slope of the regression line.

The LC-MS/MS method was applied for the determination of the compounds in human plasma, from patients undergoing treatment with H_1-antihistamines. The concentrations ranged from 4.72-6.66 µg/L for desloratadine, 3.84-6.09 µg/L for loratadine and 16.74-27.70 µg/L for cetirizine.

The results support the fact that the LC-MS/MS method can be applied to determine the plasma concentrations of the H_1-antihistamine compounds in therapeutic range or in case of overdose, and it can be applied for toxicological studies.

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Manuscript received: 4.11.2019