

HPLC method for simultaneous determination of impurities and degradation products in Cardiazol

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Abstract

Aim. The aim of study was to develop a simple and accurate procedure that could be applied for the determination of impurities and degradation products in cardiazol.

Materials and methods. Separation in samples was carried out with Acquity H-class UPLC system (Waters, Milford, USA) equipped with Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm) (Waters, Milford, USA). Xevo TQD triple quadrupole mass spectrometer detector (Waters Millford, USA) was used to obtain MS/MS data. Mobile phase A: 0.1% solution of trifluoroacetic acid R in water R; Mobile phase B: acetonitrile R. Samples were chromatographed in gradient mode (Table 1). Flow rate of the mobile phase: 1 ml / min. Column temperature: 30 °C. Detection: at 240 nm wavelength. Injection volume: 10 μl.

Results. The retention time of the main substance is about 18.5 minutes. The order of the peak, the retention times and relative retention times: impurity B (12.04; 0.65); impurity A (18.5; 0.98); Cardiazol (18.87; 1.00). The LOD and LOQ values obtained were in the range of 30 ng/mL to 100 ng/mL and 80 ng/mL to 310 ng/mL respectively (with respect to sample concentration of 2 mg/ml). Linearity was established in the range of LOQ level to 0.2% having regression coefficients in the range of 0.9996 to 0.9999. The change in the temperature of the column affects the degree of separation of cardiazol and the impurity A, and thus, with a decrease of 5 °C, the degree of separation is (1.06), while with increasing this index (3.43). When changing the flow rate of the mobile phase, the degree of separation changes in the following order, with a decrease to 0.9 ml / min separation (1.90), with an increase in speed to 1.1 ml / min (2.45). When the number of mobile phase B decreases by 5%, the degree of separation varies by (2.65), with an increase of 5% (1.82). In comparison with the chromatogram of the tested solution, the substance is not resistant to the action of peroxide, alkaline and acid decomposition.

Conclusion. 1) HPLC method was developed and validated for the simultaneous detection and quantitation of impurities formed during the synthesis of cardiazol. 2) The method proved to be sensitive, selective, precise, linear, accurate and stability-indicating.

Keywords

HPLC, Cardiazol, Impurities, Degradation Products

Introduction

Cardiazol (Fig. 1) (hydrobromide [3-Allyl-4-(4¹-methoxyphenyl)-3H-thiazole-2-ylidene] – (3²-trifluoromethylphenyl) amine) – the original newly synthesized cardioprotective, which, in addition to the cardioprotective effect, also exhibits hypolipidemic, anti-inflammatory, analgesic and antioxidant activity (Perekhoda et al. 2017). This indicates the promise of this substance for further preclinical studies.

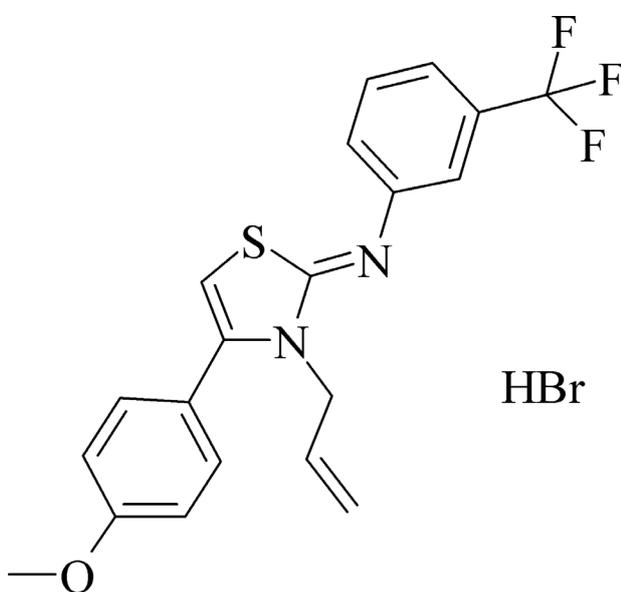


Figure 1. Chemical structure of cardiazol.

In MCQ of dosage forms the determination of the content of impurities in the preparation is proposed to be carried out by liquid chromatography. This test is controlled by intermediates, by-products, degradation products (Figs 2, 3). According to the method, the following impurities may be present in the preparation:

Therefore, it was thought desirable to develop a simple and accurate procedure that could be applied for the determination of impurities and degradation products in cardiazol. The method was validated according to

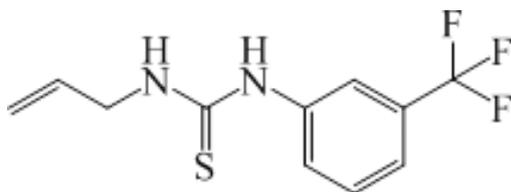


Figure 2. Chemical structure of N¹-Allyl-N²-(3¹-trifluoromethylphenyl)amine thiourea (impurity A).

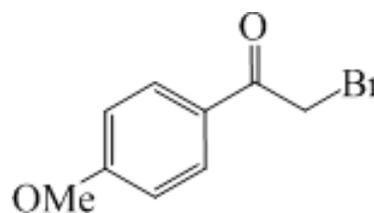


Figure 3. Chemical structure of α -bromo-4-methoxyacetophenone (impurity B).

ICH Q1A (R2), Q2A and Q2B guideline and also extended to separation of degradation products formed under various stress conditions. The results are reported in this paper.

Materials and methods

Chemicals and reagents

Cardiazol (purity 98.5%) and the impurities were synthesized and characterized in-house. Potassium dihydrogen phosphate (AR grade), methanol (HPLC grade), acetonitrile (HPLC grade), trifluoroacetic acid (HPLC grade), orthophosphoric acid, hydrochloric acid, sodium hydroxide and hydrogen peroxide were procured from Merck (Madrid, Spain).

Instrumentation and chromatographic conditions

Separation in samples was carried out with Acquity H-class UPLC system (Waters, Milford, USA) equipped with Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μ m) (Waters, Milford, USA).

Xevo TQD triple quadrupole mass spectrometer detector (Waters Millford, USA) was used to obtain MS/MS data. Positive electrospray ionization was applied with the following settings: capillary voltage – 1.5 kV, source temperature –150°C, desolvation temperature –350 °C, desolvation gas flow – 650 l/h, cone gas flow – 25 l/h. Collision energy and cone voltage was optimized for each compound separately. Collision energy varied in range from 6 eV to 20 eV and cone voltage was selected from 8 V to 38 V.

Mobile phase A: 0.1% solution of trifluoroacetic acid R in water R; Mobile phase B: acetonitrile R. Samples were chromatographed in gradient mode (Table 1). Flow rate of the mobile phase: 1 ml / min. Column temperature: 30 °C. Detection: at 240 nm wavelength. Injection volume: 10 μ l.

Chromatograph solvent (blank chromatogram), solution for checking the suitability of the chromatographic system.

Table 1. Gradient mode.

Chromatography time, min	Mobile phase A, %	Mobile phase B, %
0:00	75	25
1:00	75	25
17:00	40	60
21:00	40	60
22:00	75	25
25:00	75	25

The chromatographic system is considered suitable if:

- the degree of separation between the peaks of impurity A and the main substance, must be at least 2;
- the relative standard deviation (RSD,%) calculated for the base peak area of the reference solution (c) must not exceed the specified values [SPH U 2.0].

Normalization:

- impurity A: the peak area should not exceed 1.5 of the main peak area on the chromatogram of the reference solution (d) (0.15%);
- impurity B: the peak area should not exceed 1.5 of the main peak area on the chromatogram of the reference solution (d) (0.15%);
- any other impurity: the peak area should not exceed 1.0 of the main peak area on the chromatogram of the reference solution (d) (0.1%);
- amount of impurities: the sum of areas of all peaks should not exceed 1.5 areas of the main peak on the chromatogram of the reference solution (c) (1.5%);
- does not take into account: peaks whose area is less than 0.2 of the area of the main peak on the chromatogram of the reference solution (d). Also, peaks are not taken into account, which correspond to the corresponding peaks on the blank chromatogram in retention times.

Standard solutions and sample preparation

Test solution. 25 mg (exact weighting) of the Cardiazol substance is placed in a 25 ml volumetric flask, dissolved in 15 ml of *acetonitrile R*, and the volume of the solution is brought up to the mark with the same solvent, mixed thoroughly. Filter through a membrane filter with a pore diameter of no more than 0.45 μm .

Reference solution (a). 5.0 mg standard sample of N^1 -Allyl- N^2 -(3¹-trifluoromethylphenylamine) thiourea is placed in a 100 ml volumetric flask, dissolve in 50 ml of *acetonitrile R*, bring the volume of the solution to the mark with the same solvent and mixed.

Reference solution (b). 5.0 mg of α -bromo-4-methoxyacetophenone is placed in a 100 ml volumetric flask, dissolved in 50 ml of *acetonitrile R*, the volume of the solution is brought to the mark with the same solvent and mixed.

Reference solution (c). 1.0 ml of the test solution is placed in a 100 ml volumetric flask, the volume of the solution is adjusted to *acetonitrile R*, and mixed.

Reference solution (d). 5.0 ml of the comparison solution (a) is placed in a 50 ml volumetric flask, the volume of solution is adjusted to the mark by the *acetonitrile R* and mixed.

A solution for checking the suitability of the chromatographic system. 5.0 ml of the reference solution (a), 5.0 ml of the reference solution (c) and 5.0 ml of the solution of the reference solution (c) are placed in a 20 ml volumetric flask, the volume of the solution is adjusted to *acetonitrile R* to the mark and mixed.

All solutions are used freshly prepared.

On forced degradation, acid degradation. 25 mg (exact weighting) of the Cardiazol substance is placed in a 25 ml volumetric flask, dissolved in 15 ml of *acetonitrile R*, 2 ml of a 1 M solution of *hydrochloric acid* are added. Withstand the solution for two hours in a dark city. The acid was neutralized with 2 ml of a 1 M solution of *sodium hydroxide* and the volume was brought to the mark with the same solvent and mixed.

Alkaline degradation. 25 mg (exact weighting) of the substance Cardiazol is placed in a 25 ml volumetric flask, dissolved in 15 ml of *acetonitrile R*, 2 ml of a 1 M solution of *sodium hydroxide* are added. Withstand the solution for two hours in a dark city. The acid was neutralized with 2 ml of a 1 M solution of *hydrochloric acid* and the volume was brought to the mark with the same solvent and mixed.

Peroxidation degradation. 25 mg (exact weighting) of the substance Cardiazol is placed in a 25 ml volumetric flask, dissolved in 15 ml of *acetonitrile R*, 2 ml of a 3% solution of *hydrogen peroxide* are added. The solution was allowed to stand for two hours in a dark city and the volume was brought to the mark with the same solvent and mixed.

Temperature degradation. 25 mg (exact weighting) of the substance Cardiazol is placed in a 25 ml volumetric flask, dissolved in 15 ml of *acetonitrile R*, and the solution is heated at 100 °C for two hours. The solution was then cooled and brought to the mark with the solvent itself and mixed.

Method validation

Stock solutions of 0.002 mg/ml each of the impurities were prepared in water. Additionally, a solution containing a mixture of 0.002 mg/ml each was prepared for system suitability (Q1A (R2) 2003, Q2A 1994, Q2B 1996).

Specificity

A solution containing 0.002 mg/ml each was analyzed on HPLC using the above-given method. Further, individual solutions of 0.002 mg/ml each were analyzed to identify the individual peaks in the mixture.

Limit of Detection (LOD) / Limit of Quantitation (LOQ)

A series of solutions containing cardiazol and the impurities in the range of 30 ng/mL to 100 ng/mL were prepared for determining LOD and analyzed by using the above HPLC method. LODs were determined from visual observation of areas of each concentration in comparison

with background obtained by injecting a blank. LOQs were considered as 3 times of LODs. Six injections each of solutions containing concentrations equivalent to LODs and LOQs were performed to establish precision.

Linearity and range

The linearity of detector response at different concentrations was assessed covering an approximate range of 1.24–0.005 mg/mL (LOQ level to 200% of limit concentration) at six different concentration levels. Graphs of peak area against concentration were plotted for using a linear regression model.

Accuracy

The accuracy was established by recovery studies by spiking 0.05%, 0.1% and 0.15% (with respect to sample concentration of 2 mg/ml) with previously prepared stock solutions. The specified limit for each impurity was not more than 0.10%. The analysis for each level was carried out in triplicate.

Precision

The precision of the method was established by the study of repeatability (system precision), reproducibility (method precision) and intermediate precision. The repeatability was checked by making six injections of a solution containing 1 mg/ml each and % RSD was calculated for peak areas. For reproducibility, 2 mg/ml each of six different solutions of I were prepared and analyzed. Intermediate precision was performed by a second analyst on a different day using a different instrument.

Robustness

The robustness of the method was established by minor changes in chromatographic conditions by varying column temperature, correlation of the mobile phase and the flow of the mobile phase. The flow rates were changed from 1

ml/min to 0.9 ml/min and 1.1 ml/min, while temperature was changed from 25 °C to 35 °C. In all these experiments, concentration was 1 mg/ml each for the drug and cardiazol.

Forced degradation studies

Sample solutions at a concentration of 2 mg/ml each in 2M HCl, 2M NaOH and 10% H₂O₂ and were kept at room temperature. The drug was also exposed in solid and solution (2 mg/ml solution in water) to sunlight and UV rays. Solid samples were also kept at 105° and at melting point temperature of 158°. Additionally, 0.2% w/v solution of the drug was prepared in water as a control sample. All the samples were analyzed at 0 hours and after 5 days by HPLC.

Results and discussion

Cardiazol is original newly synthesized cardioprotective. The objective was to develop a single HPLC method for the simultaneous detection and quantitation of impurities in cardiazol. In the present study, optimization and critical evaluation of mobile phase composition (gradient), flow rate, and analytical column were important to obtain good resolution of peaks of interest from the endogenous components, which in turn affect reproducibility and sensitivity of the method. The effects of pH of mobile phase and column oven temperature on resolution between the components and tailing factors were also studied. Both column temperature and pH of the mobile phase were found to have a strong influence on the resolution and peak shapes (Kondratova et al. 2016, Logoyda et al. 2018, Logoyda 2018). A reasonably good separation between the components with good peak shapes was achieved on a Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm), 5 μ column using a gradient mode of mobile phase consisting of a mixture of mobile phase A: 0.1% solution of trifluoroacetic acid R in water R; mobile phase B: acetonitrile R., at a flow rate of 1.0 ml/min and column temperature at 30°. A representative chromatogram depicting resolution between all the components is shown in Fig. 4. The results

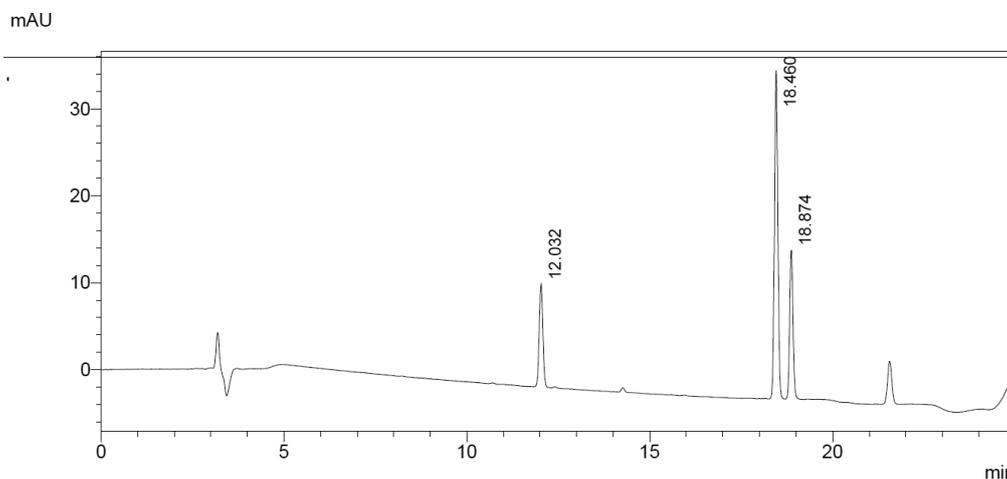


Figure 4. Chromatogram of a resolution mixture (impurity B (12.04; 0.65); impurity B (18.5; 0.98); Cardiazol (18.87; 1.00)).

indicated good resolution between the components with satisfactory peak shapes.

The retention time of the main substance is about 18.5 minutes. The order of the peak, the retention times and relative retention times: impurity B (12.04, 0.65); impurity A (18.5; 0.98); Cardiazol (18.87; 1.00).

The test requirements for checking the suitability of the chromatographic system are given in Table 2.

Table 2. Compliance of the test requirements to verify the suitability of the chromatographic system.

Parameter	Requirements	Found
The degree of separation between the peaks of Cardiazol and impurity B on the chromatogram of the suitability of the chromatographic system	Not less 2.0	2.2
Relative standard deviation calculated for the peak of Cardiazol with the comparison solution chromatogram (c)	Not more 1.0 %	0.09 %

The test requirements for checking the suitability of the chromatographic system are fulfilled, therefore the chromatographic system is considered suitable.

Blank-solution (solvent), test solution, solutions for checking the chromatographic system were prepared to determine the specificity (Figs 5, 6).

Comparison of chromatograms shows that in the conditions of the procedure for the determination of impurities, neither solvent, nor mobile phase, nor the main substance, interfere with the specificity of the method, do not interfere.

Table 3 summarizes the results obtained for LOD, LOQ and linearity for cardiazol and its impurities. The LOD and LOQ values obtained were in the range of 30 ng/mL to 100 ng/mL and 80 ng/mL to 310 ng/mL respectively (with respect to sample concentration of 2 mg/ml). Linearity was established in the range of LOQ level to 0.2%

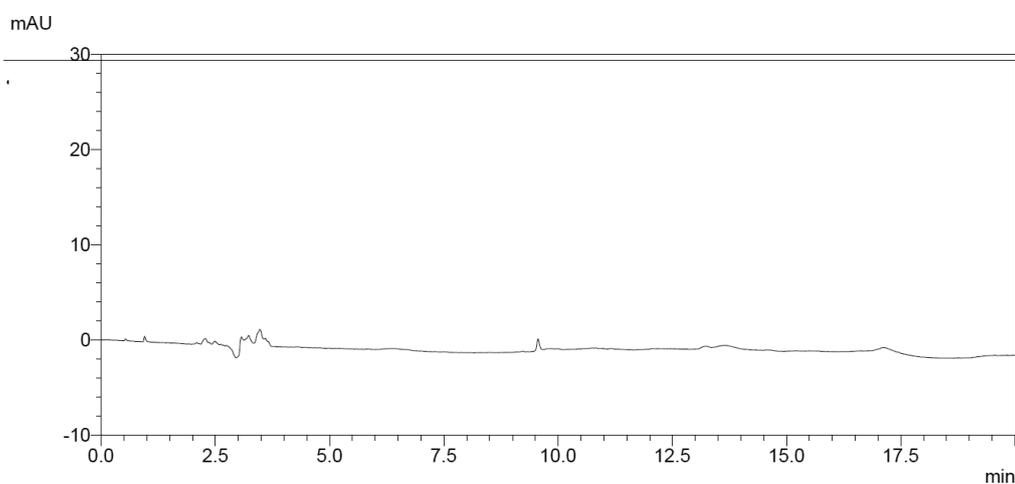


Figure 5. Chromatogram of blank solution.

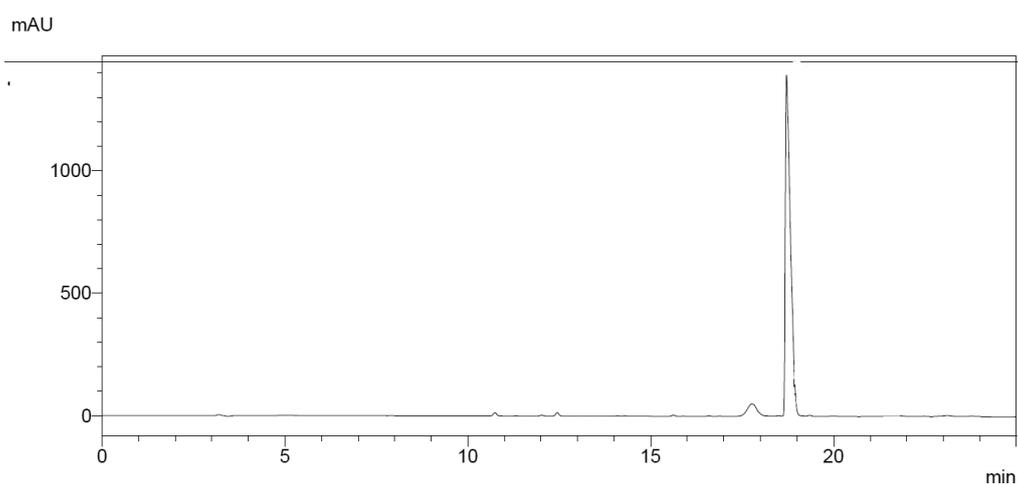


Figure 6. Chromatogram of the test solution.

Table 3. LOD, LOQ and linearity data for cardiazol and impurities.

Compound	Calibration curve	Correlation coefficient r^2 ($n=6$)	Linear range (mg/mL)	RSD, %	LOD (ng/mL)	LOQ (ng/mL)
Cardiazol	$f(x) = 7E + 06x + 63183$	0.9999	2.71–0.005	1.28	100	310
Impurity A	$f(x) = 3E + 07x + 98605$	0.9996	1.24–0.002	1.18	40	140
Impurity B	$f(x) = 8E + 06x + 3446$	0.9998	1.66–0.003	1.02	30	80

having regression coefficients in the range of 0.9996 to 0.9999. Calibration curve for cardiazol - $f(x) = 7E + 06x + 63183$, impurity A- $f(x) = 3E + 07x + 98605$, impurity B- $f(x) = 8E + 06x + 3446$.

The mean recoveries at each level for all the components, given in Table 4 ranged between 98.48–99.81% (Acceptance criteria: 90–110%) and % RSD was between 0.59% and 1.86% (Acceptance criteria: NMT 10.0%) establishing that the method was accurate for the quantitative determination of impurities.

Table 4. Recovery data for impurities.

Component	% Mean Recovery (% RSD)		
	Amount spiked = 0.05%	Amount spiked = 0.10%	Amount spiked = 0.15%
Impurity A	99.81 (0.72)	98.56 (0.72)	99.73 (0.59)
Impurity B	98.01 (1.86)	99.48 (0.20)	98.79 (1.73)

The following parameters were studied to determine robustness: column temperature; correlation of the mobile phase; the flow of the mobile phase.

To check the effect of these parameters, the comparison solution (b) was chromatographed at different temperatures: 25 °C, 30 °C and 35 °C; various flow rates of 0.9 ml / min, 1.0 ml / min and 1.1 ml / min and different ratios of mobile phases (the amount of mobile phase B was changed by 5%).

The change in the temperature of the column affects the degree of separation of cardiazol and the impurity A, and thus, with a decrease of 5 °C, the degree of separation is (1.06), while with increasing this index (3.43). When changing the flow rate of the mobile phase, the degree of separation changes in the following order, with a decrease

to 0.9 ml / min separation (1.90), with an increase in speed to 1.1 ml / min (2.45). When the number of mobile phase B decreases by 5%, the degree of separation varies by (2.65), with an increase of 5% (1.82).

The stability results are listed in Table 5.

Table 5. Stability data of cardiazol and its impurities.

Compound	Average value of S peak of freshly prepared solution	Average value of S peak of solution after 24 hours	Parameter change in percentages after 24 hours
Cardiazol	29309	29335	0.09
Impurity A	61456	61397	0.10
Impurity B	398920	399210	0.07

The results were found to be within the assay variability limits during the entire process.

To the substance Cardiazol accelerated degradation was applied in stressful conditions. The results obtained from degraded solutions were compared with the data obtained from the test. The degradation study results are presented in Table 6 and Figs 7–10.

Table 6. Degradation study of cardiazol.

	1 M NaOH	1M HCl	3% H2O2	100 °C
Impurity A	-	-	-	-
Impurity B	-	-	-	-
Cardiazol (in comparison with the test solution)	5.84%	1.84%	98.72%	99.90%
Impurity 1 (5.097)	1.27%	-	1.27%	-
Impurity 2 (5.77)	1.19%	0.069%	1.19%	-
Impurity 3 (6.30)	0.67%	0.02%	0.67%	-
Impurity 4 (7.69)	-	0.02%	-	-
Impurity 5 (8.91)	3.31%	0.063%	3.30%	-
Impurity 6 (9.75)	-	0.02%	-	-
Impurity 7 (15.03)	-	-	-	0.08%

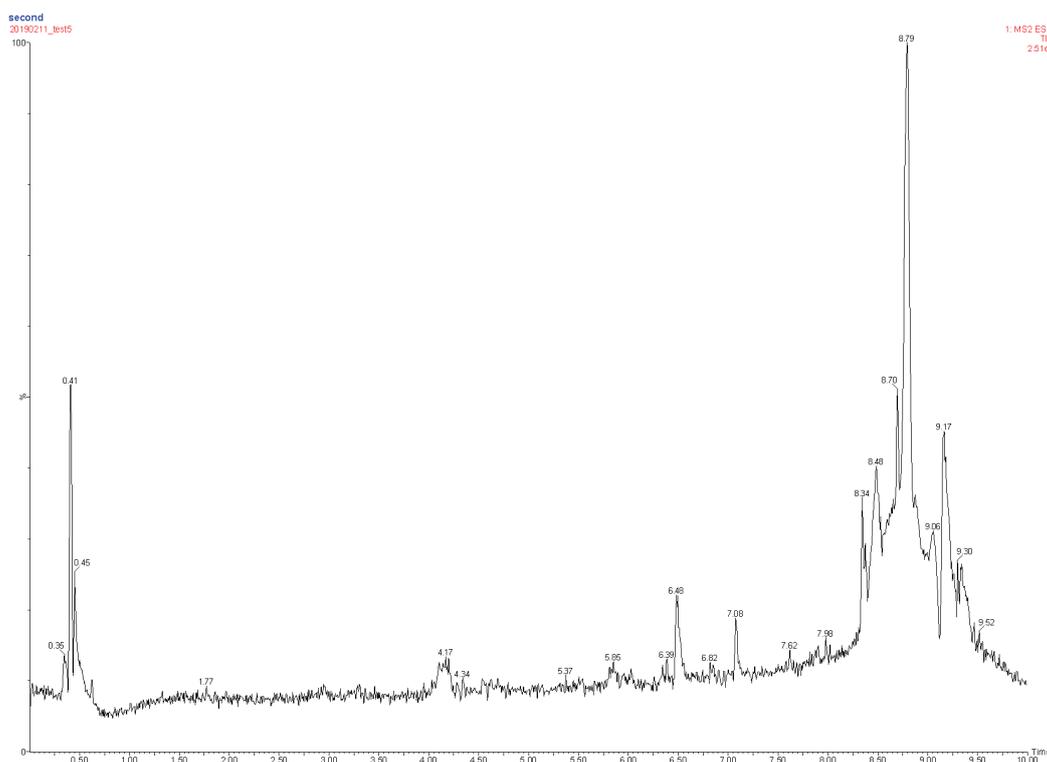


Figure 7. Chromatogram of cardiazol degraded under acidic conditions.

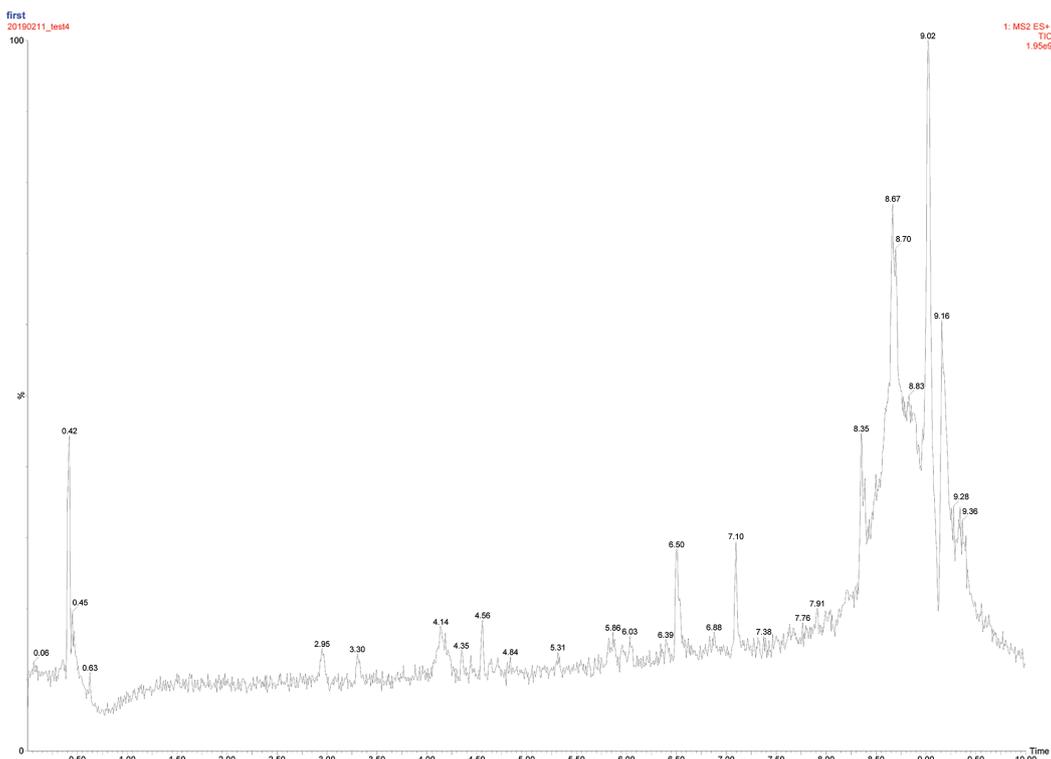


Figure 8. Chromatogram of cardiazol degraded under alkaline conditions.

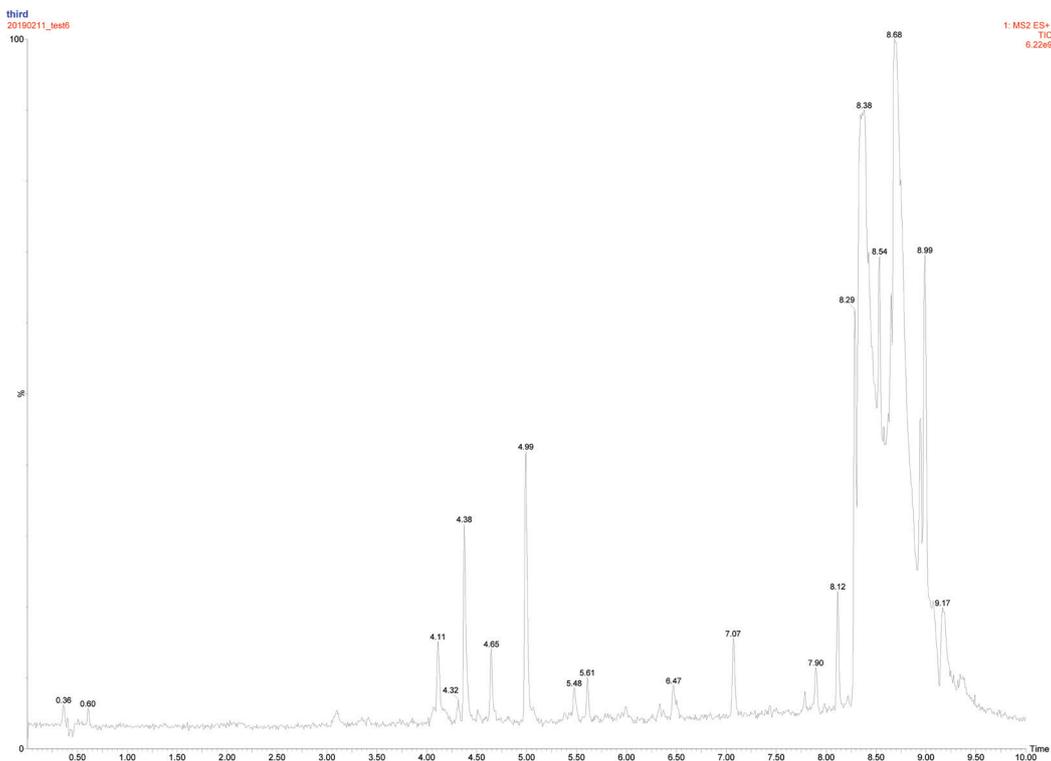


Figure 9. Chromatogram of cardiazol degraded under oxidative conditions.

At peroxidic degradation, the peak area of the main substance did not undergo significant changes; also unidentified impurities (1–3, 5) with retention times (5.09, 5.77, 6.30, 8.91 minutes, respectively) were found on the

chromatogram. Under conditions of temperature degradation, the area of cardiazol remained almost unchanged, the formation of an unidentified impurity (7) with the retention time (15.03 min) was observed. In the con-

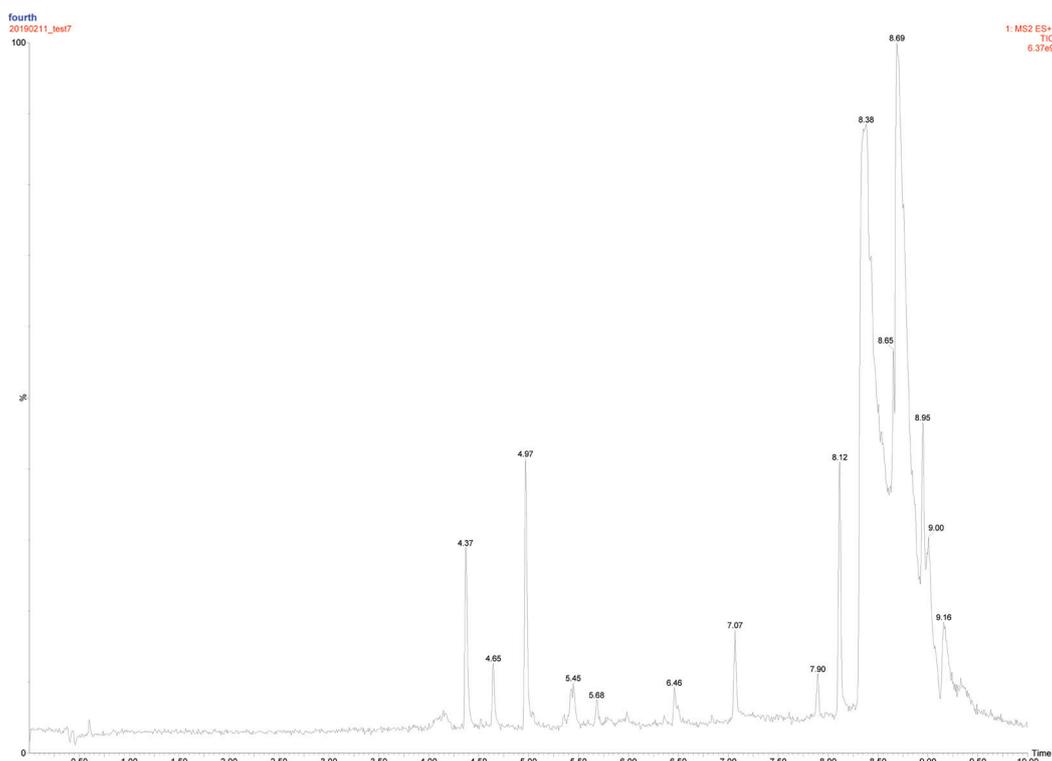


Figure 10. Chromatogram of cardiazol degraded under temperature conditions.

ditions of alkaline hydrolysis, the area of the main substance significantly decreased, there were unidentified impurities (1–3, 5) with retention times (5.09 minutes, 5.77 minutes, 6.30 minutes and 8.91 minutes, respectively). Acidic hydrolysis leads to a decrease in the area of cardiazol and the formation of unidentified impurities (2–6) with retention times (5.77 min, 6.30 min, 7.69 min, 8.91 min, 9.75 min).

In comparison with the chromatogram of the tested solution, the substance is not resistant to the action of peroxide, alkaline and acid degradation.

Conclusion

1. HPLC method was developed and validated for the simultaneous detection and quantitation of impurities formed during the synthesis of cardiazol.
2. The method proved to be sensitive, selective, precise, linear, accurate and stability-indicating.
3. The method was successfully applied to the analysis of demonstrating acceptable precision and adequate sensitivity for the detection and quantitation of the impurities. So it may be reasonable to claim that the method can be extended to the analysis of drug formulations and stability samples as well.

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