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RESEARCH ARTICLE

Development and validation of a stability-indicating HPLC assay method for determination of ethacrynic acid in solution formulation. HPLC-MS identification of hydrolytic and oxidative degradation products

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Abstract: An isocratic stability-indicating HPLC assay method was developed to quantitate ethacrynic acid in solution formulations. Utilizing the method, the main thermal, hydrolytic, and one of the oxidative degradation products could be separated. The structure of the separated degradation products was confirmed by negative ion-mode HPLC-MS. The hydrolytic results confirmed the previously reported sequence of the formation of the dimeric Michael adduct. At the lower temperature, hydrogen peroxide can react with ethacrynic acid to form the epoxide derivative (**7**). At the higher temperature, several other oxidation products were also formed. The validated analytical method can be used to quantitate ethacrynic acid in a solution formulation in the 0.5-500 μg/mL concentration range.

Keywords: forced degradation, hydrolytic degradation products, oxidative degradation products, Michael addition reaction, stability-indicating HPLC method, HPLC-MS

1 Introduction

Ethacrynic acid (EA) (**1**) (Figure 1), a sulfhydryl-reactive diuretic, is primarily used to reduce body water and treat high blood pressure[1]. EA inhibits the activity of the electroneutral Na^+-K^+-$2Cl^-$ (NKCC) symporters, primarily in the thick ascending limb of Henle's loop (TALH), but also in the proximal and distal tubules. This pharmacological action results in excretion of these ions increased urinary output and reduction in extracellular fluid[2,3]. Examples of the most used loop diuretics include ethacrynic acid, furosemide, bumetanide, and torsemide[4].

Na^+/K^+/$2Cl^-$ symporters are membrane-bound channels that play a prominent role in a variety of epithelial absorptive and secretory processes and a direct role in cell volume regulation. As they use the energy of the ion gradients generated by the Na^+/K^+-ATPase to transport Na^+, K^+, and Cl^- from the outside to the inside of a cell, they are considered secondary active transport mechanisms. Ethacrynic acid inhibits this sodium/potassium-transporting ATPase, through interaction with the alpha-1 subunit of the protein[5]. This protein is the catalytic component of the active enzyme, which catalyzes the hydrolysis of ATP coupled with the exchange of Na^+ and K^+ across the plasma membrane[6].

Ethacrynic acid is most commonly used in solid formulation (as a tablet); however, its sodium salt can be administered in solution (as an injection). The onset of action is usually within 30 minutes after an oral dose and within 5 minutes after an intravenous injection. The bioavailability of EA approximates 100%, with maximal blood level between 40 and 92 minutes. Most side effects of EA can be attributed to its effectiveness (volume depletion); however, it may cause metabolic alkalosis that is preventable by KCl replacement[7]. Within 4 hours after *i.v.* administration of ^{14}C-ethacrynic acid, 60 to 70% was excreted into the bile of rats. Less than 25% was EA, and the remainder was biotransformation products. The two major metabolites in bile were identified; one was the glutathione adduct of EA (EA-GSH), and the other was EA-mercapturate[8].

It has been observed to increase the conventional outflow facility in both enucleated calf eyes, human eyes, as

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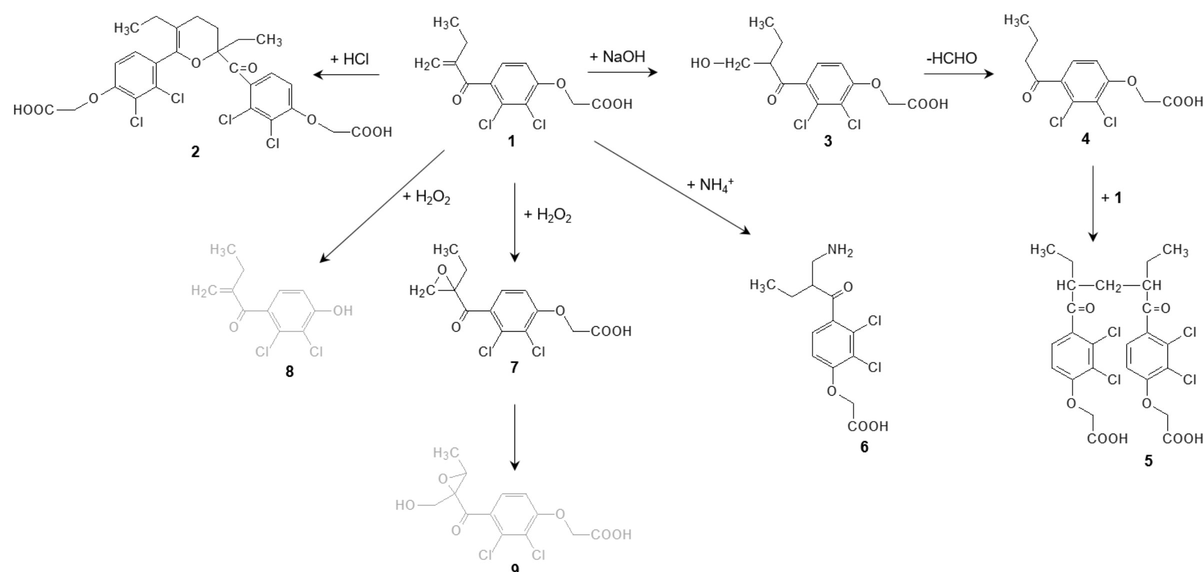

Figure 1. Degradation products of ethacrynic acid based on[27]

well as monkey eyes, following anterior-chamber perfusion of ethacrynic acid[9–11]. A pilot study of intracameral ethacrynic acid injection in humans with chronic open-angle glaucoma demonstrated remarkable degrees of both efficacy and safety. No acute corneal or anterior chamber side effects were observed, and results of corneal endothelial cell counts were essentially unchanged two months after treatment[5]. Findings in several physiological and biochemical investigations had indicated that the outflow pathway function was extremely reactive to SH-modification[10]. Thus, ethacrynic acid, being an SH-reactive α,β-unsaturated ketone derivative[1], seemed to have potential as an ocular hypotensive agent.

Based on the clinical observations, a drug development program was launched at the University of Pécs to develop an ethacrynic acid-containing external formulation to increase the eye's conventional outflow of open-angle glaucoma patients[13]. As a part of the program, forced degradation studies of ethacrynic acid test sample was performed. Forced degradation studies help in generating degradants in a much shorter time. The samples generated from forced degradation can be used to develop the stability-indicating method (SIM), which can be applied later for the analysis of samples generated from accelerated and long term stability studies[14].

Traditionally, forced degradation studies are performed in solution, using concentrations of the target compounds ranging between 1 and 10 mg/mL, aiming for a degradation level of approximately 5-20%[14–20]. For small organic molecules, the analytical methods of choice are HPLC-UV-Vis and HPLC–MS analysis. The SIM is defined as a validated analytical procedure that accurately and precisely measures active ingredients (drug substance or drug product) free from process impurities, excipients, and degradation products[21]. The ability to differentiate the active ingredients from the closely related process and degradative impurities is usually the single most important requirement for stability-indicating methodology[22].

Earlier, several analytical methods have been applied to investigate the kinetics and mechanism of decomposition of ethacrynic acid[23–28]. The early work by Cohen[23] identified the principal route of degradation in tablets and lyophilized injections as a Diels-Alder type of condensation, leading to the formation of a dimer (**2**) (Figure 1). Goerlitzer and Hoebbel, on the other hand, reported on sodium-hydroxide-initiated decomposition of ethacrynic acid to form formaldehyde and compound **5**, the dimeric derivative of ethacrynic acid. The authors proposed a reaction scheme involving [2,3-dichloro-4-(2-hydroxymethyl-1-oxobutyl)phenoxylacetic acid (**3**) as an unstable intermediate[24]. Compound **5** was identified almost simultaneously by Auterhoff and Thinnes[25].

Yarwood *et al.* reported the first HPLC-based in-solution stability study of ethacrynic acid[26]. The authors investigated the kinetics of degradation of ethacrynic acid in neutralized aqueous solution as a function of temperature and concentration. Based on the previous work of Cohen, the authors supposed to form the Diels-Alder adduct (**2**) of ethacrynic acid under the experimental conditions[26]. Later, Yarwood *et al.* reported a gradient HPLC method that could be successfully applied to separate each of the known degradation products of ethacrynic acid[27]. The authors summarized the previously published results and published the chart of degradation pathways, as shown in Figure 1.

Accordingly, in dilute aqueous solutions, addition of water onto the polarized carbon-carbon double bond takes place in a pH- and temperature-dependent manner to give

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3. Under basic conditions, the water adduct is supposed to lose formaldehyde to give **4**, which can react with the parent compound (**1**) to form the Michael adduct (**5**). On the contrary, in aqueous slurries and the solid-state, a Diels-Alder type of condensation takes place, generating **2**[27]. In the presence of ammonium salts, addition of ammonia onto the polarized carbon-carbon double bond can take place to form the N-Michael-adduct (**6**) (Figure 1). The extent of degradation was influenced both by the species and the concentration of the cation[28].

In the present contribution, we report on the development of a stability-indicating HPLC-UV-Vis method, which can be used to quantitate ethacrynic acid in solution. The method was validated for specificity, linearity, precision, accuracy, and solution stability. EA acid was subject to acid and base hydrolysis, and oxidation to apply stress conditions. According to the previous results, our SIM aimed to separate decomposition products **3**, **4**, and **5**, of which formation can be expected in dilute aqueous solutions[27]. Furthermore, as a possible product of the oxidative degradation, the epoxy-derivative of EA (**7**) (Figure 2) has also been involved in the method development. Synthesis of this derivative was reported by Koechel *et al.*[29]. The authors obtained the derivative by treating EA with hydrogen peroxide under strongly basic conditions. The epoxide did not have diuretic effect; however, it showed to possess SH-reactivity[29]. This latter could be one of the molecular bases of the reported hepatotoxicity of EA when administered in high dosages[30].

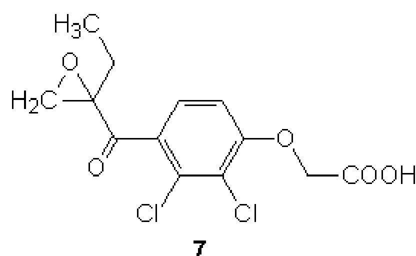

Figure 2. The epoxy-derivative (**7**) of ethacrynic acid

2 Experimental

2.1 Chemicals and reagents

Ethacrynic acid (**1**), the ethacrynic acid Diels-Alder adduct (**2**), and the epoxy-derivative of ethacrynic acid (**7**) were generous gifts of Pannonpharma Kft (Pécsvárad, Hungary) and Egis Pharmaceuticals (Budapest, Hungary). Their purity was checked by thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC). Formic acid, acetic acid, ethyl acetate, chloroform, methanol (MeOH), and acetonitrile (ACN) were obtained from Spektrum-3D Kft (Debrecen, Hungary). HPLC-MS grade formic acid, methanol, and acetonitrile were purchased from Merck Kft (Budapest, Hungary). Distilled water was purified in the Institute of Pharmaceutical Chemistry, the University of Pécs, by use of a Purelab Option Q7 Water System. A Mettler Toledo MP 220 pH meter and a Mettler Toledo Inlab 413 electrode were used to adjusting the pH.

2.2 Preparation of standard solutions

Stock solution of **1** was prepared by dissolving 100 mg of ethacrynic acid in 5.0 mL of acetonitrile (concentration: 20 mg/mL). The solution was stored at 2-7°C. The stock solutions were stable for three days at room temperature.

Working standard solutions for the validation and the forced degradation studies were prepared by diluting the appropriate volume of stock solutions to the proper volumes and treated them as samples.

2.3 TLC analysis

Normal-phase TLC investigation of ethacrynic acid (**1**), ethacrynic acid Diels-Alder adduct (**2**), and epoxy-derivative of ethacrynic acid (**7**) (20 mg/mL solution in ACN each) were performed on 20 × 20-cm silica gel 60 F_{254} aluminum sheets (Merck, Darmstadt Germany, No. 5554), using acetic acid - ethyl acetate - chloroform (15.5:38.5:46, v/v), as developing solvent. For visualization, the chromatograms were illuminated by λ = 254 nm UV light and subsequently subjected to iodine vapor. The average R_f values of three parallel runs as follows. R_f (**1**) = 0.48; R_f (**2**) = 0.18; R_f (**7**) = 0.35.

2.4 HPLC-UV-Vis analysis

HPLC analyses were performed using an Agilent 1100 (Agilent Technologies, Waldbronn, Germany) integrated HPLC system equipped with a quaternary pump, a degasser, an autosampler, and a variable wavelength UV-Vis detector. Separation of compounds was performed on a LiChroCART® (125 mm × 4.0 mm, particle size 5 μm) HPLC column. Data were recorded and evaluated by the use of using Agilent ChemStation (Rev.A.10.02) software.

Mobile phases used for HPLC were degassed in an ultrasonic bath (Realsonic cleaner) and filtered through ROBU glass filter (Porosity 4) before use. A Mettler Toledo MP 220 pH meter and a Mettler Toledo Inlab 413 electrode were used to adjusting the pH of the electrolyte solutions.

Isocratic analysis using a mobile phase consisting of formic acid (0.1%, v/v, pH 2.70 ± 0.02) - methanol - acetonitrile (45:8:47, v/v) was used. Chromatography was performed at 40°C, and the mobile phase flow rate was 0.8 mL/min. The volume of injection was 10 μL.

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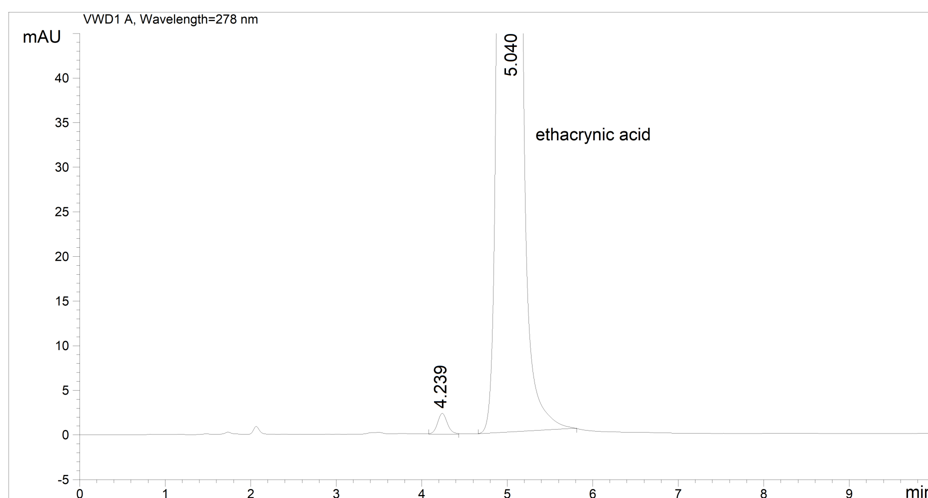

Figure 3. System suitability chromatogram of the ethacrynic acid (**1**) standard solution (c = 500 μg/mL)

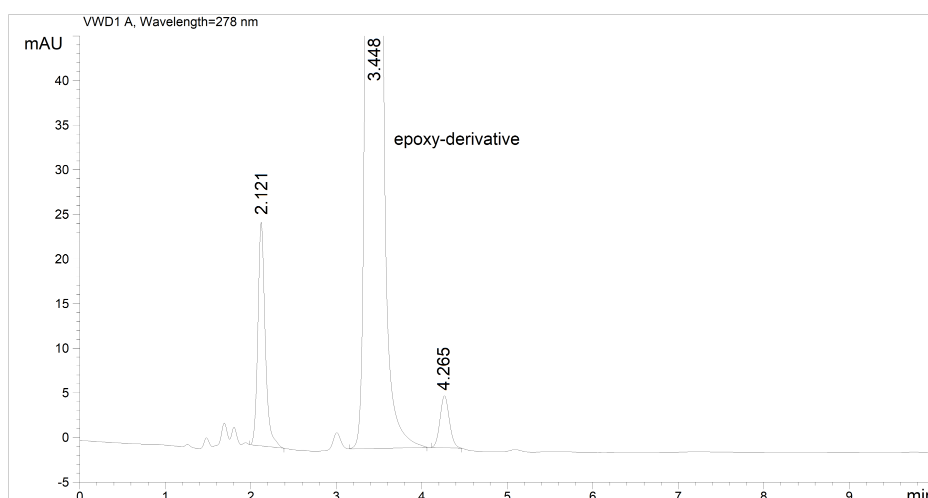

Figure 4. System suitability chromatogram of the epoxy-derivative (**7**) standard solution (c = 1 mg/mL)

Detection was performed at λ = 278 nm.

2.5 Validation parameters

2.5.1 System suitability

System suitability data and system precision were evaluated based on the chromatograms of the solutions containing ethacrynic acid (**1**) (c = 500 μg/mL) in acetonitrile, and on the other hand, based on the chromatograms of the solutions containing epoxy-derivative of ethacrynic acid (**7**) (c = 1 mg/mL) in acetonitrile. Results were obtained from 6 parallel injections. Data were evaluated by the relative standard deviation (RSD%), which was lower than 1.5% for retention time values and lower than 0.07% related to the peak areas.

The average t_R (retention time) values of six parallel runs as follows: t_R (**1**) = 5.06 min; t_R (**7**) = 3.42 min. Figure 3 and Figure 4 show the representative HPLC chromatograms of system suitability.

2.5.2 Linearity

Linearity was studied by preparing standard solution of ethacrynic acid at seven different concentrations from 0.5 to 500 μg/mL (0.5; 1; 10; 50; 100; 250; 500 μg/mL) in acetonitrile. Data were obtained from three parallel injections of two parallel dilutions of two independent weighings applied at each concentration level. Calibration curves were generated by plotting the theoretical concentrations (μg/mL) against the peak areas. Linearity was determined by the least-squares regression. The method was linear in the examined range. The equation of the regression line is y = 8.9523x + 3.1103, where y is peak area, and x is concentration, r^2 = 1.0000.

2.5.3 Precision

Precision was calculated from the data of repeatability and intermediate precision. Repeatability was determined

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by measuring intra-day data of three parallel injections of two parallel dilutions from two independent weighings of ethacrynic acid (dissolved in acetonitrile) at two different concentration levels (50 μg/mL; 500 μg/mL).

Intermediate precision was based on inter-day data (by injection of the samples over three consecutive days) of three parallel injections of two parallel dilutions from two independent weighings of ethacrynic acid (dissolved in acetonitrile) at two different concentration levels (50 μg/mL; 500 μg/mL). The evaluation was performed by calculating the relative standard deviation (RSD%). Data for precision were summarized in Table 1 and Table 2.

Table 1. Data for repeatability

Concentration (μg/mL)	Weighings	Area (ethacrynic acid)
500	1	4497.38
	2	4474.92
*average		4486.15
**RSD%		0.35
50	1	450.68
	2	463.96
*average		457.32
**RSD%		2.05

Notes: *calculated from six injections; **calculated from six injections

Table 2. Data for intermediate precision

Concentration (μg/mL)	Days	Area (ethacrynic acid)
	1	4489.17
500	2	4490.37
	3	4461.43
*average		4480.32
**RSD%		0.37
	1	451.43
50	2	457.13
	3	460.06
*average		456.21
**RSD%		0.96

Notes: *calculated from nine injections; **calculated from nine injections

2.5.4 Determination of LOD and LOQ

Limit of detection (LOD) was determined experimentally, and taken as the concentration producing a detector signal that could be clearly distinguished from the baseline noise (3 times the baseline noise). The limit of quantification (LOQ) was taken as the concentration that produced a detector signal ten times greater than the baseline noise[31]. The LOD and LOQ values of ethacrynic acid were found to be 0.2 μg/mL and 0.5 μg/mL, respectively.

2.6 HPLC-MS analysis

The integrated HPLC system (Jasco) - qualified and verified according to the pharmaceutical requirements - was equipped with an intelligent HPLC pump (PU-980), a degasser, a manual injector (RHEODYNE 7725i) with a 5 μL loop, a column oven and a diode-array detector (MD-2010). Data were recorded and evaluated by ChromNav software (ver. No. 1.21). Jasco LC-system is connected with a Waters 3100 Mass Detector. MS data were recorded and evaluated by Empower 2 software.

Separation of compounds was performed on a LiChroCART® (125 mm × 4.0 mm, particle size 5 μm) HPLC column. The mobile phase was a mixture of 0.1% formic acid solution (pH 2.70 ± 0.02), methanol, and acetonitrile (45:8:47, v/v). Chromatography was performed at 40°C, and the mobile phase flow rate was 0.8 mL/min. Detection was performed at 278 nm. The injection volume was 5 μL for all sample solutions.

The MS measurement was performed in negative ion mode. The electrospray ionization (ESI) source was operated with a spray voltage of 3000 V. Desolvation gas was delivered at 250 L/hour and temperatured at 250°C. Cone gas was also set at 250 L/hour. Cone voltage ramp was applied (from 30 V at 150 Da to 100 V at 650 Da). The capillary temperature was set at 100°C. Full-range mass spectra (m/z 70-700) were collected under the optimal conditions. Mass accuracy of the detector was 0.01 Da in the examined range.

2.7 Forced degradation studies

Forced degradation studies were performed in 50% acetonitrile solutions. The concentration of ethacrynic acid was the same (1 mg/mL) in each incubation. The degradation studies were performed at room temperature and 70°C. In this latter case, the samples were stored in a controlled temperature shaking waterbath (Memmert, Schwabach, Germany). HPLC analysis of the samples was performed at the beginning (t = 0) and after 24-hour incubation. The specific conditions were as follows.

2.7.1 Solvent stability

50% v/v ACN/H_2O solution of ethacrynic acid solution (c = 1 mg/mL) was incubated at room temperature and 70°C.

2.7.2 Acid degradation

50% v/v ACN/H_2O solution of ethacrynic acid solution (c = 1 mg/mL), containing 0.1 M hydrogen chloride (HCl) was incubated at room temperature and 70°C.

2.7.3 Alkali degradation

50% v/v ACN/H_2O solution of ethacrynic acid solution (c = 1 mg/mL), containing 0.1 M sodium hydroxide (NaOH) was incubated at room temperature and 70°C.

2.7.4 Oxidative degradation

50% v/v ACN/H_2O solution of ethacrynic acid solution (c = 1 mg/mL), containing 5% hydrogen peroxide (H_2O_2) was incubated at room temperature and 70°C.

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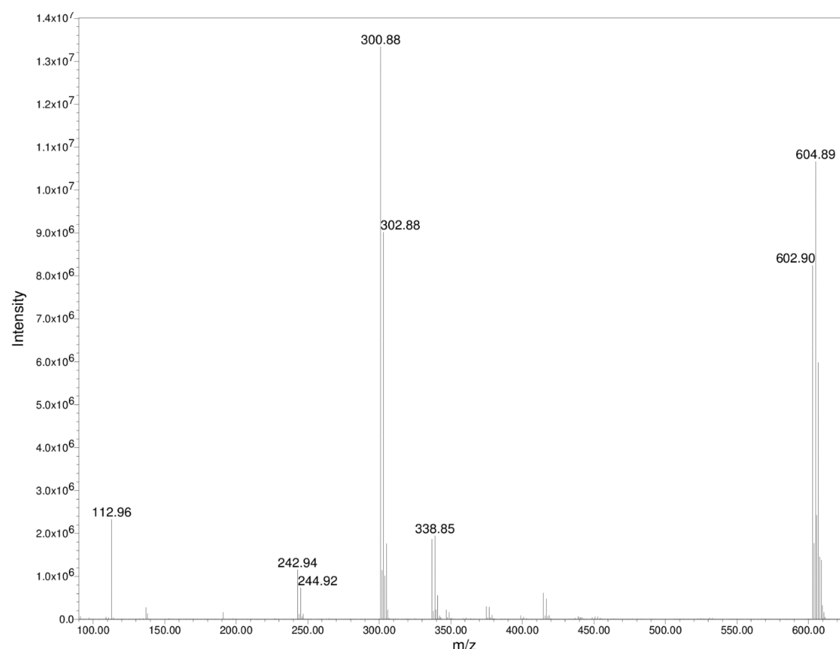

Figure 5. Negative ion mode ESI mass spectrum of the ethacrynic acid standard (**1**) ($[M_1 - H^+]^-$ and $[2M_1 - H^+]^-$)

3 Results and discussion

HPLC analysis of ethacrynic acid in pharmaceutical formulations or biological samples is a frequently used analytical method to quantitate the active ingredient and its thiol (cysteine, N-acetylcysteine, or glutathione) conjugates[32–37]. There have been, however, only a limited number of SIMs published in the literature[27]. The previously published method took into consideration of the thermal and the hydrolytic degradation products of ethacrynic acid. That method, using gradient elution, MeOH: 0.05 M phosphoric acid buffer (pH 5.6), was optimized for separation and quantitation of only EA (**1**) and its degradation products **2-5**[27]. Accordingly, a new stability-indicating method was aimed to develop involving the oxidative degradation product **7** of ethacrynic acid. The present method, having very similar (0.5 μg/mL) LOQ values, has the advantages of being isocratic and applicable to detect and quantitate not only the thermal and the hydrolytic degradation products **2-5** but the potentially toxic oxidative degradation product **7** as well.

The initial HPLC development aimed at the separation of the parent compound (**1**) from the available degradation products **2** and **7**. Since compound **2** has reported having a substantially longer relative retention time (RRT) than any other previously reported degradation product of **1** for HPLC analysis for them[27], gradient elution was needed. Since degradation product **2** was reported to be formed only in aqueous slurries of ethacrynic acid and in the solid-state, our final HPLC development aimed at separation of only the rest of the previously reported degradation products, plus the oxidation product **7**. For this purpose, an isocratic HPLC-UV-Vis method using a mobile phase consisting of formic acid (0.1%, v/v, pH 2.70 $\pm$ 0.02) - methanol-acetonitrile (45:8:47, v/v) was used.

The HPLC-UV-Vis chromatogram of the reference ethacrynic acid standard (**1**) is shown in Figure 3. Figure 5 depicts the negative ion mode ESI mass spectrum of the ethacrynic acid standard. As the figure shows, the dimer of the compound ($[2M_1 - H^+]^-$) also appears in the spectrum. Being the single chromatographic peak of the pure standard analyzed, dimerization of the compound occurred in the ionization chamber under the used conditions.

As shown in Figure 3, the sample (**1**) has got contamination (t_R = 4.24 min) with RRT of 0.84, which is very close to the previously reported value (0.85) of **4**[27]. The exact mass of **4** ($C_{12}H_{12}O_4Cl_2$) is 290.01 (49.73%), and the negative ion mode mass spectrum ($[M_4 - H^+]^-$) of the component (Figure 6) is in accordance with the structure of the compound. This contamination takes 0.16% of the total AUC of the sample, which corresponds to 0.09% (m/m) contamination.

Incubation of 50% ACN/50% H_2O solution (c = 1.0 mg/mL) of ethacrynic acid for 24 hours at room temperature or 70°C resulted in no change in the initial chromatograms.

Incubation of ethacrynic acid in an acidic solution (50% ACN/50% 0.2 M HCl; c = 1.0 mg/mL) at 70°C resulted in the appearance of one new peak in the chromatograms. The new peak appears with lower AUC in the incubations kept at room temperature. As shown (Figure 7), the new

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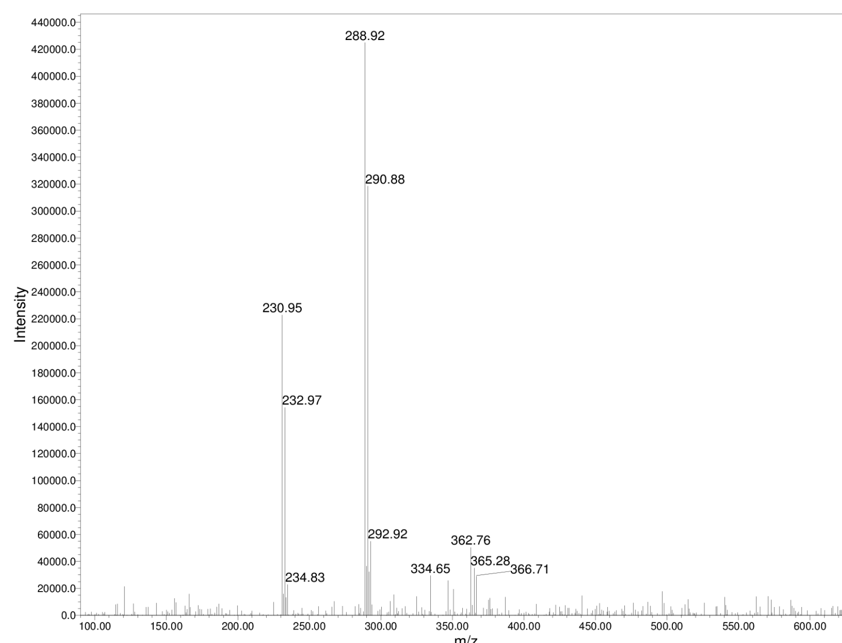

Figure 6. Negative ion mode ESI mass spectrum of contaminant 4 (t_R = 4.24 min) of the ethacrynic acid investigational sample

peak (t_R = 2.08 min) has got an RRT of 0.41, which is rather close to the previously reported value (0.53) of **3**[27]. The exact mass of **3** ($C_{13}H_{14}O_5Cl_2$) is 320.02 (49.06%), and the negative ion mode mass spectrum ($[M_3 - H^+]^-$) of the component (Figure 8) is in accordance with the structure of the compound **3**.

Incubation of ethacrynic acid in a basic solution (50% ACN/50% 0.2 M NaOH; c = 1.0 mg/mL) at 70°C resulted in the appearance of several new peaks in the chromatograms of the neutralized incubation (Figure 9). The RRT (2.10) of the new peak with t_R = 10.66 min. corresponds to one of the most apolar known degradation product **5**. Besides this peak, some other short retention time peaks appeared in the chromatogram. One of them (t_R = 2.11 min) was identified as degradation product **3**. Furthermore, the AUC of the peak with t_R = 4.26 min (compound **4**) substantially increased through the incubation. This observation is in accord with the previous findings that elimination of formaldehyde from the water adduct 3 to form 4 is much faster under basic conditions[24,25,27]. The exact mass of **5** ($C_{25}H_{24}O_8Cl_4$) is 592.02 (24.46%), and the negative ion mode mass spectrum ($[M_5 - H^+]^-$) - including the isotopic mass intensities - of the component (Figure 10) is in accordance with the structure of the compound **5**.

Incubation of ethacrynic acid with hydrogen peroxide solution (50% ACN/50% 10% H_2O_2; c = 1.0 mg/mL) at 70°C resulted in formation of several new decomposition products in the chromatograms (Figure 11). The peak with RRT = 0.67 is different from any of the previously investigated decomposition products[27], and its retention time (t_R = 3.40 min) is the same as that of the epoxide derivative of ethacrynic acid (**7**). The mass spectrum of the reference compound **7** is shown in Figure 12. The exact mass of **7** ($C_{13}H_{12}O_5Cl_2$) is 318.01 (49.07%), and the negative ion mode mass spectrum ($[M_7 - H^+]^-$) - including the isotopic mass intensities - of the component (Figure 13) is in accordance with the structure of the compound **7**.

Analyzing the mass spectra related to the (not separated) oxidized products with short retention times, two previously not disclosed structures could be taken into considerations. The exact mass of the component corresponding to the HPLC peak with RRT = 1.35 (t_R = 6.80 min) is 244.01 (50.54%). Based on the negative ion mode mass spectrum (Figure 14) and the previously published path of oxidation of phenoxyacetic acids with hydrogen peroxide[38], the structure of the new compound can be considered to be **8** ($C_{11}H_{10}O_2Cl_2$; $[M_8 - H^+]^-$ = 242.97) (Figure 15). The other proposed structure corresponding to the HPLC peak with RRT = 0.46 (t_R = 2.30 min) can be considered to the **9** epoxide (Figure 15). Formation of the compound can be rationalized as an oxidation product of the water adduct of the **7** epoxide, which can undergo water elimination to result in formation of a new enone derivative. The formed enone can form the epoxide **9**. The exact mass of the formed oxidation product is 334.00 (48.95%), which corresponds to the $C_{13}H_{12}O_6Cl_2$ molecular formula. Indirect proofs of the proposed structure are the M_9+18 isotopic peaks (Figure 16), which can be considered as the signals of the water adduct of the epoxide. Verification of the two proposed structures, as well as

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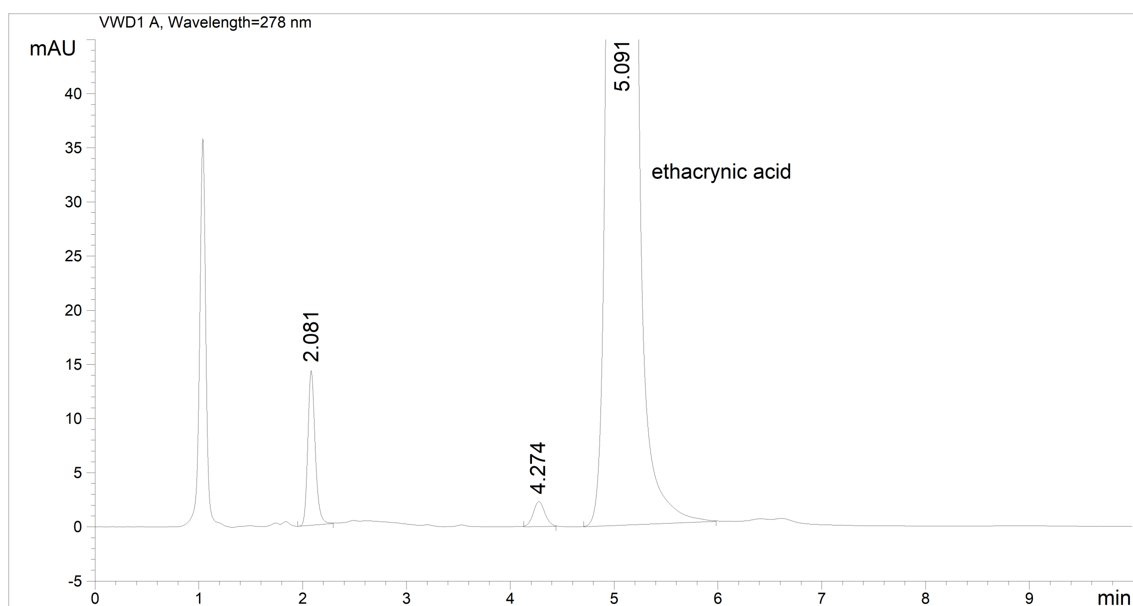

Figure 7. HPLC-UV-Vis chromatogram of the acidic incubation (50% ACN/50% 0.2 M HCl; c = 1.0 mg/mL; t = 70°C) of the ethacrynic acid (**1**) investigational sample

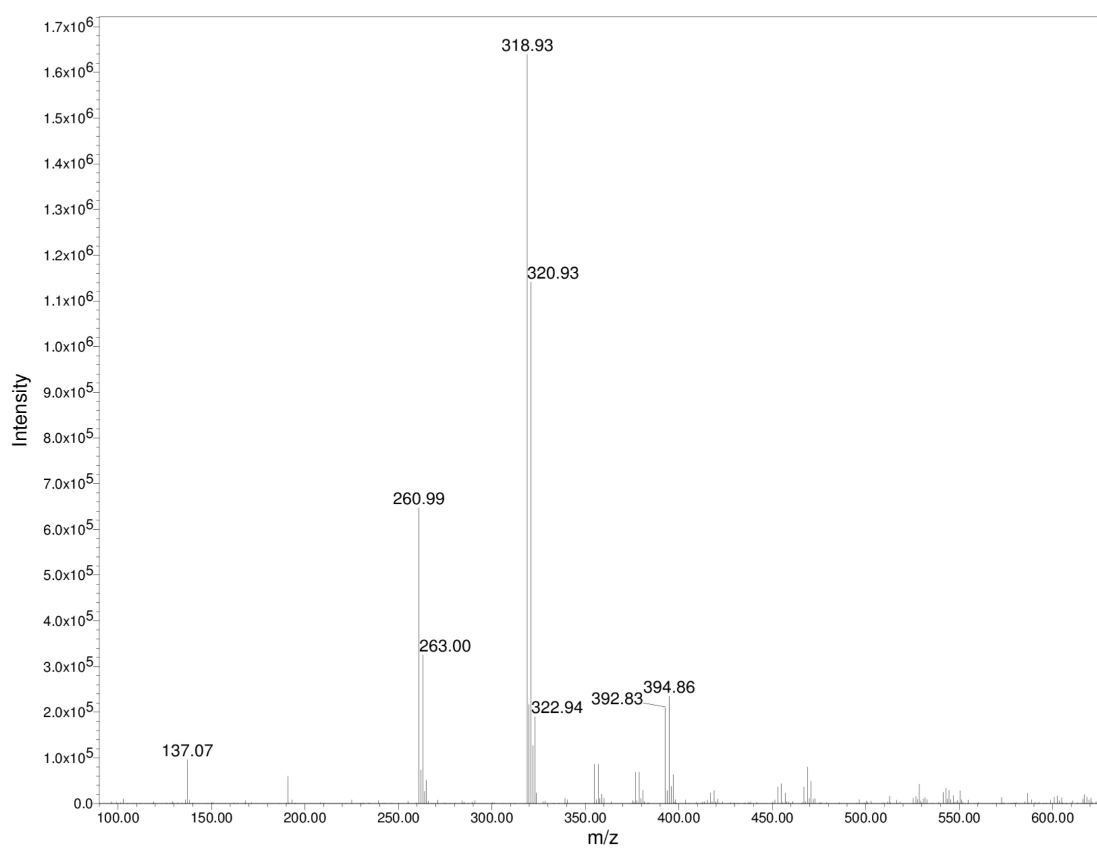

Figure 8. Negative ion mode ESI mass spectrum of ethacrynic acid derivative **3** (t_R = 2.08 min) formed in acidic incubation of the ethacrynic acid investigational sample

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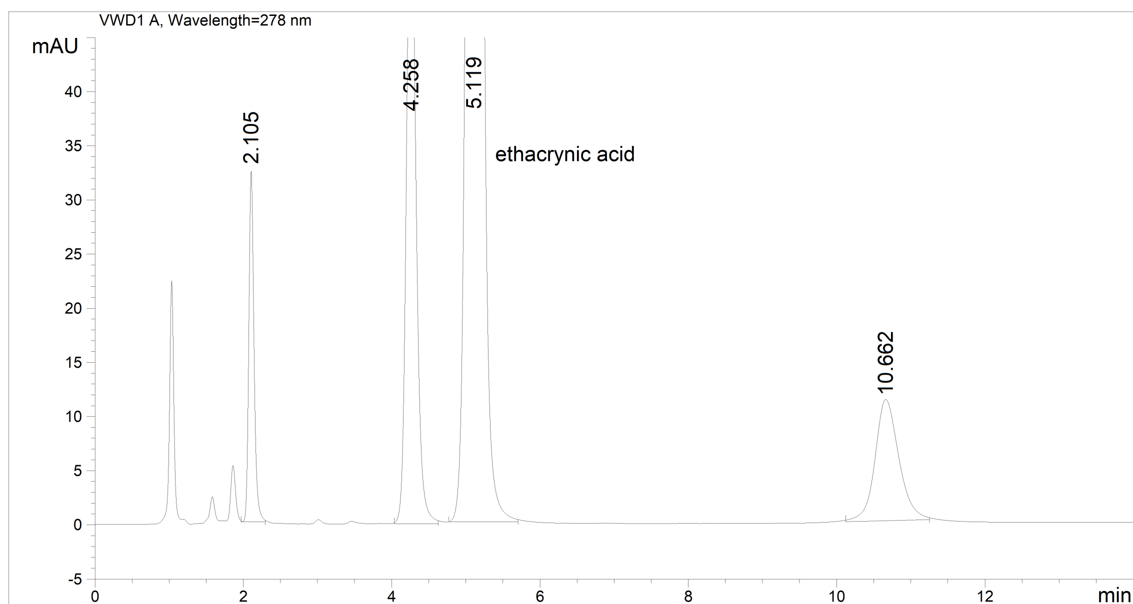

Figure 9. HPLC UV-Vis chromatogram of the basic incubation (50% ACN/50% 0.2 M NaOH; c = 1.0 mg/mL; t = 70°C) of the ethacrynic acid (**1**) investigational sample

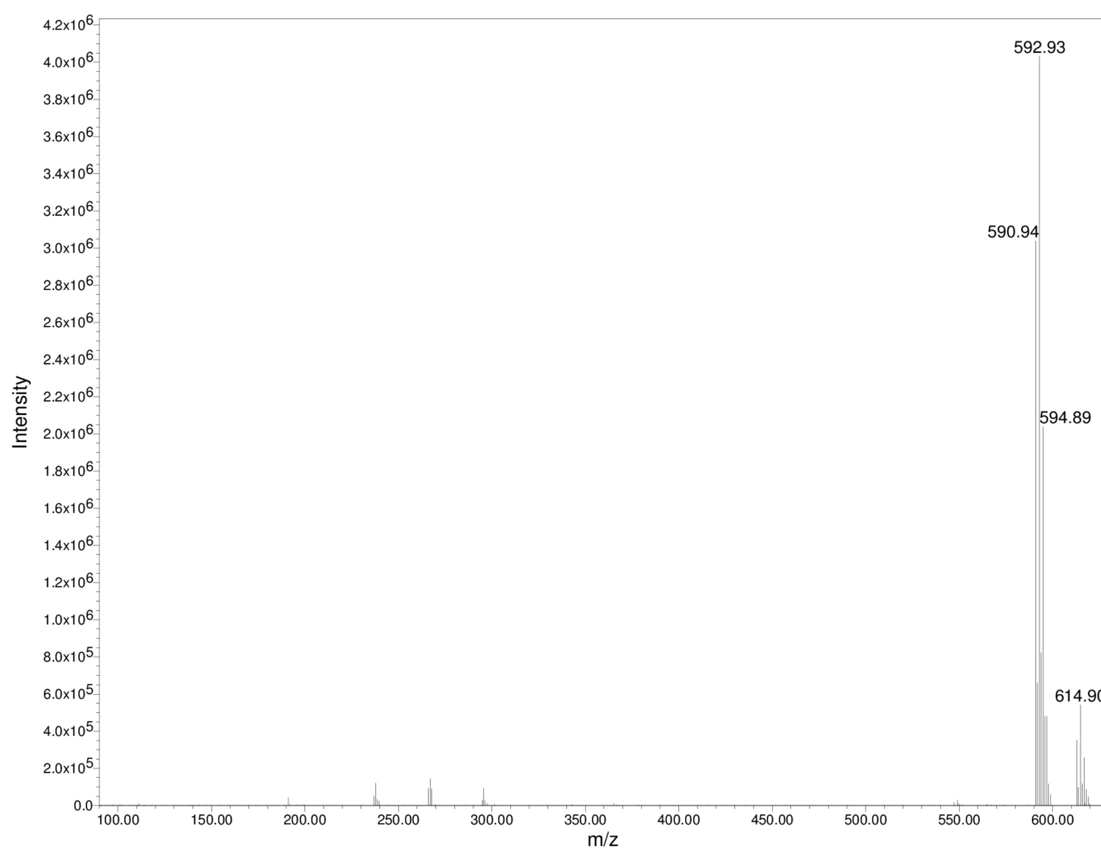

Figure 10. Negative ion mode ESI mass spectrum of ethacrynic acid derivative 5 (t_R = 10.66 min) formed in alkali incubation of the ethacrynic acid investigational sample

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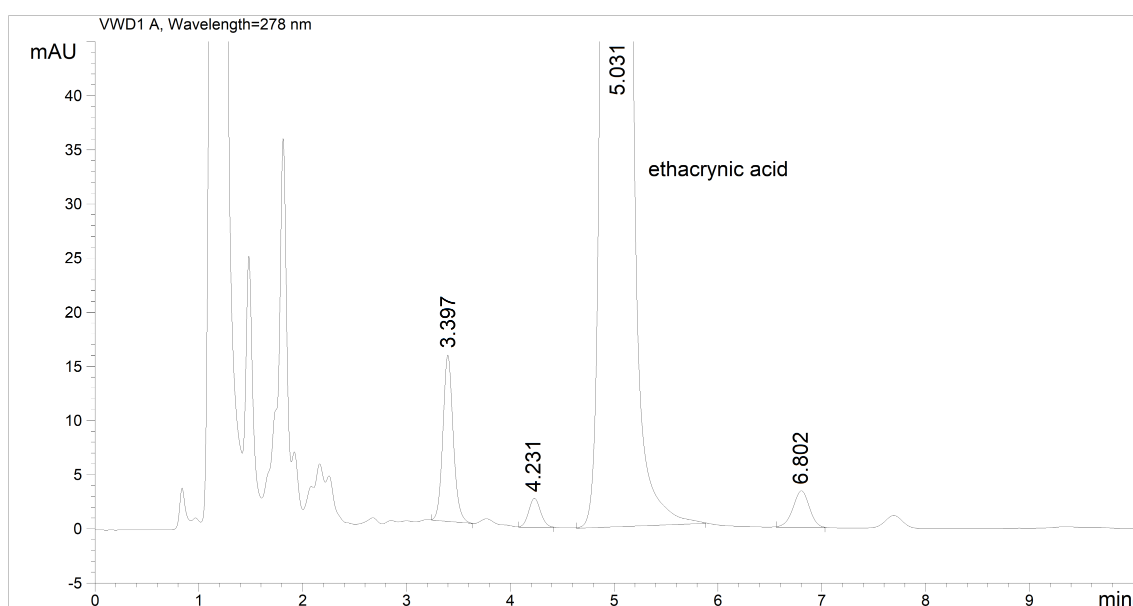

Figure 11. HPLC-UV-Vis chromatogram of the hydrogen peroxide incubation (50% ACN/50% 10% H_2O_2; c = 1.0 mg/mL; t = 70°C) of the ethacrynic acid (**1**) investigational sample

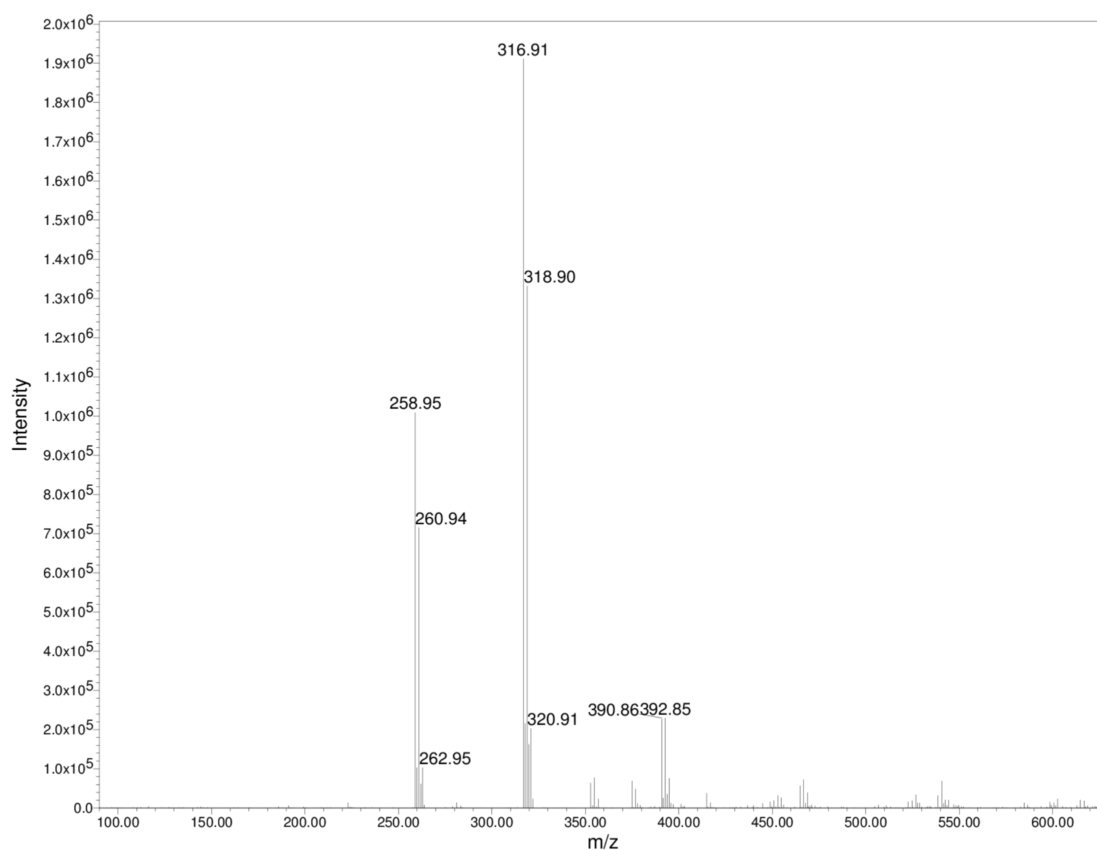

Figure 12. Negative ion mode ESI mass spectrum of epoxy-derivative standard (**7**) ($[M_{7-}H^{+}]^{-}$)

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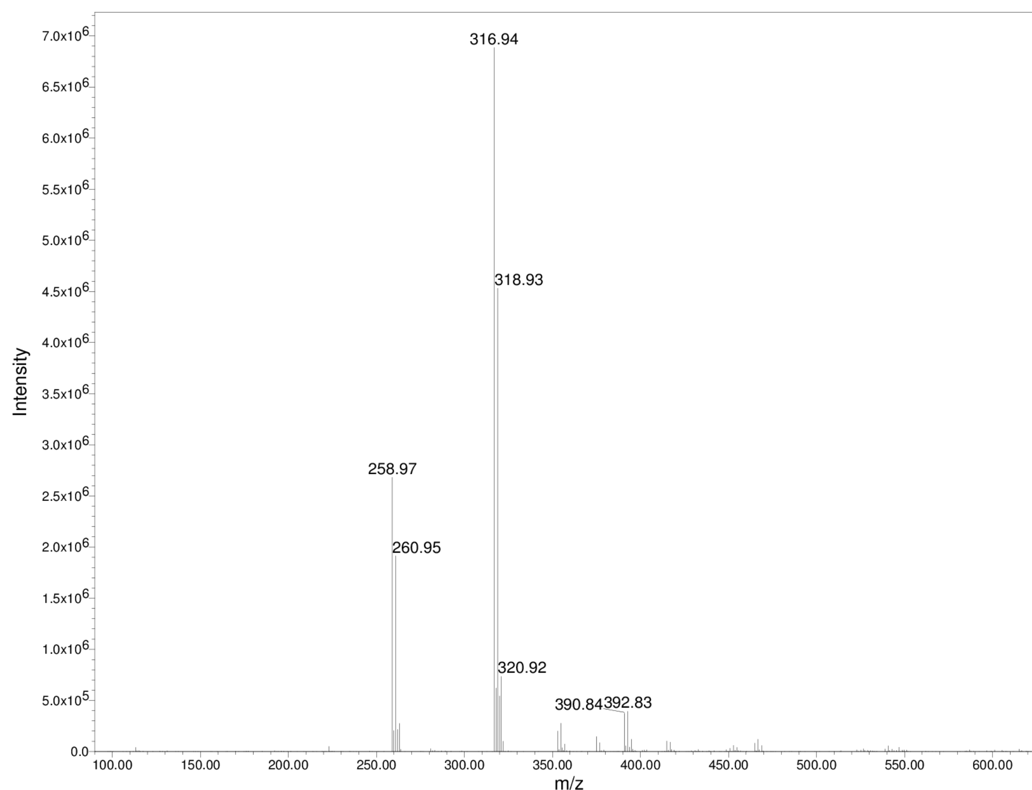

Figure 13. Negative ion mode ESI mass spectrum of ethacrynic acid derivative **7** (t_R = 3.40 min) formed in incubation of the ethacrynic acid investigational sample with hydrogen peroxide

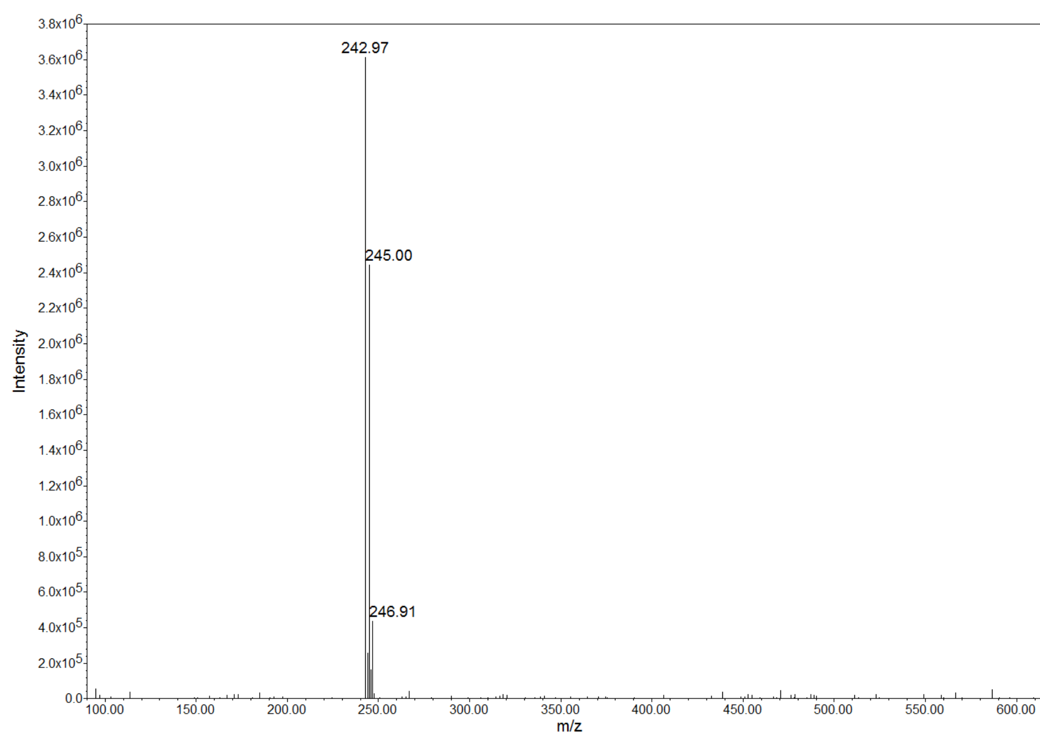

Figure 14. Negative ion mode mass spectrum of ethacrynic acid derivative (t_R = 6.80 min) formed in incubation of the ethacrynic acid investigational sample with hydrogen peroxide

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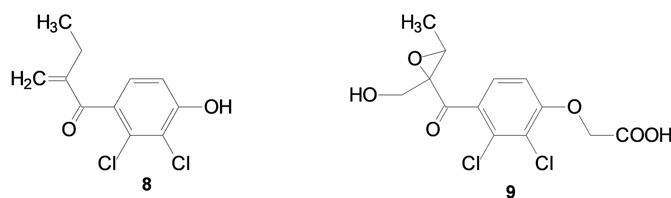

Figure 15. Proposed structures of compounds (**8** and **9**) formed in incubation of the ethacrynic acid investigational sample with hydrogen peroxide

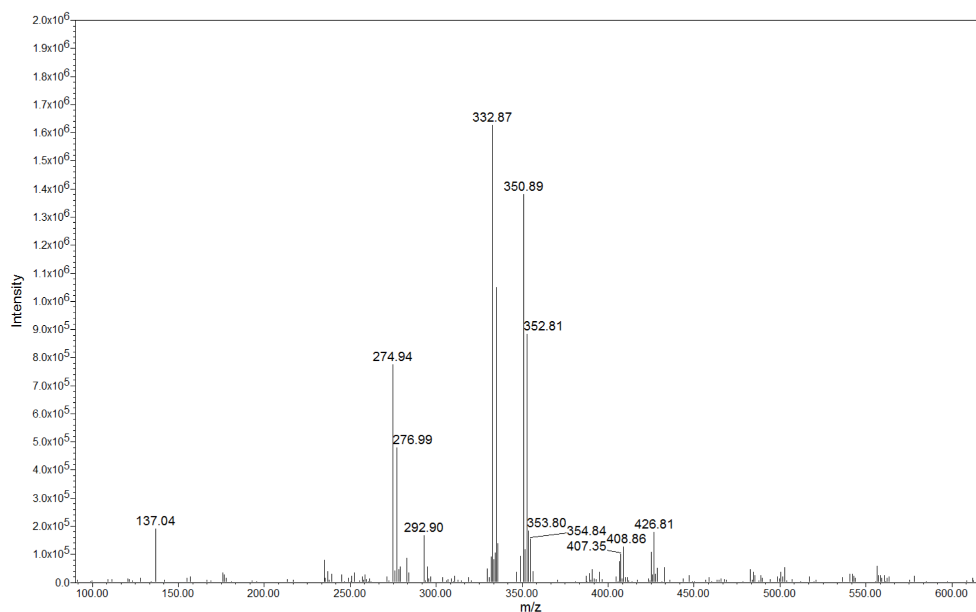

Figure 16. Negative ion mode mass spectrum of ethacrynic acid derivative (t_R = 2.30 min) formed in incubation of the ethacrynic acid investigational sample with hydrogen peroxide

identification of the other oxidation products, need further synthetic and instrumental analyses.

According to the above, the newly developed HPLC-UV-Vis conditions turned to be a stability-indicating method for investigation hydrolytic and oxidative forced degradation studies of ethacrynic acid. For the future application of the method in our drug development program, validation of if has been performed with the following results.

Linearity was studied by preparing standard solution of ethacrynic acid at seven different concentrations from 0.5 to 500 μg/mL (0.5; 1; 10; 50; 100; 250; 500 μg/mL). The method was linear in the examined range. The equation of the regression line is y = 8.9523x + 3.1103, where y is peak area, and x is concentration, r^2 = 1.0000. Precision was calculated from the data of repeatability and intermediate precision. The evaluation was performed by calculating the relative standard deviation (RSD%). Data for repeatability and intermediate precision are summarized in Table 1 and Table 2, respectively. Limit of detection (LOD) was taken as the concentration producing a detector signal that could be clearly distinguished from the baseline noise (3 times the baseline noise). The limit of quantification (LOQ) was taken as the concentration that produced a detector signal ten times greater than the baseline noise. The LOD and LOQ values of ethacrynic acid were found to be 0.2 μg/mL and 0.5 μg/mL, respectively.

4 Conclusions

An isocratic stability-indicating HPLC assay method was developed to quantitate ethacrynic acid in solution formulations. Utilizing the method, the main hydrolytic (**3**, **4**, and **5**) and one of the oxidative (**7**) degradation products could be identified and separated. The structure of the separated degradation products was confirmed by negative ion-mode HPLC-MS. The hydrolytic results confirmed the previously reported sequence of the formation of **5**. At the lower temperature, hydrogen peroxide can react with ethacrynic acid to form the epoxide derivative **7**. At the higher temperature, several other oxidation products were also formed. The validated analytical method can be used to quantitate ethacrynic acid in a solution formulation in the 0.5 - 500 μg/mL concentration range.

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