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Assessment of the stability of new chemotherapeutic selenium-kojic acid derivative using HPLC and MS analysis

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ABSTRACT

The stability of the new chemotherapeutic selenium kojic acid derivative, 5-benzyloxy-2-selenocyanatomethyl-4-pyranone (P763), was evaluated in simulated gastric and intestinal fluids as well as in human plasma at 37^{o}C ± 1. A stability-indicating HPLC procedure with C8 RP column and a mobile phase of acetonitrile/water (1:1) at a flow rate 1 ml/min was used. The eluted P763 compound and its degradation products were detected at 254 nm. The accelerated stability profiles were established. Kinetic studies under controlled experimental conditions have proved that P763 underwent fast hydrolysis in simulated intestinal fluid (phosphate buffer solution, pH 7.4), however the compound showed an appropriate stability at gastric acid solution (0.1M HCl solution) and in human plasma. The kinetics have indicated that the compound's degradation followed pseudo first-order reaction with relatively higher k_{deg} and lower $t_{1/2}$ and t_{90} values in simulated intestinal fluid compared to those in simulated gastric acid solution and plasma. The degradation of P763 compound at basic solution was confirmed by MS and MS/MS analysis, where the characteristic signals of the molecular (m/z 322) and fragment (m/z 91, 198) mass ions of compound disappeared. The obtained results suggest that the stability of P763 compound should be considered for future biopharmaceutical and biological studies.

Keywords: kojic acid derivative, stability-indicating HPLC, stability profiles, MS/MS analysis, selenium-organic compound

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INTRODUCTION

Kojic acid, 5-hydroxy-2-hydroxymethyl-4-pyranone (Fig.1) provides a promising skeleton containing a polyfunctional heterocyclic oxygen-containing ring with several important centers enabling additional reactions [1]. The 4-pyranone skeleton, which is also a part of flavonoids is known to be associated with antineoplastic activity [2, 3]. BTMP was a 4-pyranone derivative with a proved cytotoxic activity [4]. The well-established chemical similarity in the chemical properties of inorganic and organic compounds of sulfur and selenium stimulated the chemical synthesis of two new selenium derivatives of kojic acid: namely; benzyl and methyl selenocyanate analogues [5]. Several organic and inorganic selenium compounds have also been reported as effective chemopreventive agents against multiple models of mammary tumorigenesis in mice and rats [6,7]. Recently, the first screening data of the antiproliferative/cytotoxic activities of novel selenium-derivatives of kojic acid have been published [8]. The cell growth inhibitory activities of the new selenium containing kojic acid derivatives were found to be preferentially aimed at the intracellular compartment rather than the plasma membrane, and thus they enlarged the group of novel antiproliferative active compounds [8]. The principle of preparing such selenium- kojic acid derivatives is based on the idea of molecule's decomposition into reactive moieties that interact with cell targets in cancer cells. Additionally, the selenium metal released, following molecule's decomposition, will pass into the cellular environment and produces its anticancer properties.

In addition to biological studies, assessment of the stability of the prepared kojic acid derivatives is important for biopharmaceutical and formulation studies. The stability of kojic acid derivative BTMP, has been investigated under acidic and basic conditions using HPLC and LC-MS analyses [9]. Therefore, before conduction of the biological and pharmaceutical studies of the newly-prepared selenium kojic acid derivatives, it is also necessary to evaluate the compound's stability under simulated biological conditions.

The purpose of the present study was to assess the stability of novel 5-benzyloxy-2-selenocyanatomethyl-4-pyranone, designated as P763 (Fig.1) in simulated gastric and intestinal fluids as well as in human plasma using a stability indicating HPLC procedure and also to prove the degradation process using MS analysis.

MATERIALS AND METHODS

MATERIALS

P763 (Fig.1) was prepared according to the reported method [5]. Characterization and elucidation of the chemical structure of P763 was confirmed by elemental and spectral analyses. Human plasma samples were kindly donated by Kuwait Central Blood Bank and were stored at -30^{o}C. All solvents were of HPLC grade and the reagents were of analytical grade. Water was purified by Milli-Q-System from Millipore Corporation Milford, MA, USA.

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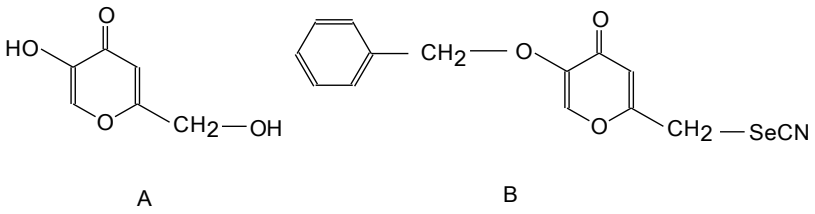

Fig. 1 Chemical structures of kojic acid (A) and selenium-kojic acid derivative, P763 (B)

HPLC AND CHROMATOGRAPHIC CONDITIONS

The stability studies were conducted using an isocratic HPLC (Waters Alliance 2695 LC, USA) connected to an auto-sampler (Waters, USA) and photodiode array detector (Waters 996, USA). The chromatographic analyses were performed at ambient temperature using RP C8 LC column (Waters, 150 mm length x 40 mm internal diameter, 5 µm particle size) and a mobile phase of acetonitrile/water (1:1) at a flow rate of 1 ml/min. A 20 µl aliquot of the sample was injected and the eluent was monitored at a λ=254 nm. The peak areas of the eluted compounds were automatically measured by the Millennium software and were used to calculate the remaining concentrations of P763 and the degradation products.

MASS SPECTROMETRIC (MS) ANALYSIS

MS analysis of P763 and the respective degradation products was performed using positive electrospray (+ESI) tandem MS (Quattro LC, Micromass, UK). The instrument tuning parameters were adjusted at 3.5 kV and 25 V for capillary voltage and cone voltage, respectively. The source and desolvation temperatures were 100^{o}C and 250^{o}C, respectively. The MS scans were in the range of m/z 50-400. Solutions of the P763 and its degradation products were prepared in the mobile phase acetonitrile/water/formic acid (80/20/0.05%) and the samples were directly injected into the + ES ionization probe at a flow rate 50 µl /min.

ACCELERATED STABILITY STUDY OF P763 IN SIMULATED GASTRIC AND INTESTINAL SOLUTIONS

A stock solution of P763 (1mg/ml) was prepared in acetonitrile. Six samples of 20 µl aliquots were diluted to 1 ml in 1.5-ml HPLC vials with either 0.1M HCl solution (simulated gastric fluid) or phosphate buffer solution, pH 7.4 (simulated intestinal fluid). The vials were well-mixed, incubated at 37^{o}C ±1 and aliquots of 20 µl were injected into HPLC at specified time intervals. The samples were drawn at 0.0 h and then at 1-hour intervals for 5 hours and then at 24 h in duplicate.

ACCELERATED STABILITY STUDY OF P763 IN HUMAN PLASMA

Six 20 µl aliquots of P763 (1 mg/ml) solution in methanol/water (1:1) were diluted with fresh and previously centrifuged human plasma in 1.5-ml HPLC vials. The vials were well-mixed

and incubated at 37°C ±1. A 50 µl aliquots were drawn at 0.0 h and then at 1-hour intervals for 5 h and at 24 h and transferred into 1.5-Eppendorff tubes and mixed with 250 µl of acetonitrile as protein precipitant. The samples were centrifuged at 980 g for 10 min and 20 µl aliquots of the clear supernatants were injected into HPLC.

STABILITY PROFILES

The stability profiles representing the degradation of P763 in simulated gastric and intestinal solutions and in human plasma, were established by plotting the log% of the remaining concentrations of P763 versus time in hours. The degradation kinetics i.e. the rate of degradation (K_{deg}), degradation half-life ($t_{1/2}$) and shelf-life (t_{90}) were determined from the stability profiles using the formulas: K_{deg} =2.303 x slope (h^{-1}), $t_{1/2}$= 0.693/K_{deg} (h), t_{90}= 0.105/K_{deg} (h), respectively.

PREPARATION OF BASE-INDUCED DEGRADATION PRODUCTS

In 10-ml tube, a 1-ml aliquot of P763 in methanol (1mg/ml) was mixed with 1 ml of phosphate buffer solution, pH 7.4. The mixture was kept in a water bath controlled at 37°C±1 for 24 h. Aliquots were taken and diluted with mobile phase (acetonitrile/water/formic acid (80/20/0.05%) and 20 µl aliquot was injected into mass spectrometer. The obtained MS and MS/MS profiles of the degraded solution of compound were compared with the MS and MS/MS spectra of P763 in mobile phase under the same measurement conditions.

RESULTS AND DISCUSSION

The HPLC chromatogram of P763 in acetonitrile/water (1:1) mobile phase exhibits a well-defined resolved peak at retention time 3.9 - 4.0 min. In simulated gastric fluid (0.1 M HCl solution), the HPLC chromatogram showed good stability as only a single peak of the analyte was observed without any detectable degradation products (Fig.2a). Based on peak area measurements, the acid solutions of P763 in 0.1 M HCl solution gave an approximately 24% reduction of the peak area following incubation at 37°C ±1 for 24 hours. In simulated intestinal fluid (phosphate buffer solution, pH 7.4), the HPLC chromatogram showed new peaks in addition to the compound's peak at retention times 5.1 min. and 5.7 min due to degradation (Fig.2b). The peak area of compound was significantly reduced up to 70% following incubation at 37°C ±1 for 24 hours. In human plasma, the rate of degradation of P763 compound was nearly similar to that in simulated gastric fluid, as the HPLC chromatogram exhibited no additional peaks of degradation products (Fig.2c). A decrease of approximately 22% of the peak area following incubation at 37°C ±1 for 24 hours was calculated. In order to assess the compound's stability, kinetic studies were performed under controlled experimental conditions of pH and temperature. The kinetic parameters (K_{deg}, $t_{1/2}$, t_{90}) were determined from the established accelerated stability profiles based on measuring the % reduction of the peak area of P763 compound at different time intervals compared to the initial peak area at 0.0 hours. Plotting of the log values of peak reduction, expressed as percentages, versus incubation time, expressed as hours, indicated pseudo first-order kinetics (Fig.3) as the reaction was induced by

the presence of base. Kinetic analysis indicated the values of degradation kinetic parameters (K_{deg}, $t_{1/2}$, t_{90}) of 0.058 h^{-1}, 11.9 h, 1.9 h (simulated gastric solution), 0.104 h^{-1}, 6.7 h, 1.0 h (simulated intestinal solution) and 0.045 h^{-1}, 15.4 h, 2.3 h (human plasma) (Table 1). The derived data suggested that the new P763 compound underwent distinct degradation by hydrolysis under basic buffer conditions at pH 7.4, however the compound showed better stability under acidic conditions and in human plasma at 37°C±1. The degradation of P763 was further proved by examining the MS and MS/MS profiles of P763 in basic and acidic solutions using positive electrospray tandem mass technology. As shown in Fig 4, the MS spectrum of P763 in acid showed a strong molecular mass ion of compound at m/z 322 [M+1]+ , a strong fragment mass ion at m/z 91 due to benzyl mass ion and a slightly elevated fragment mass ion at m/z 230 due to selenium - kojic acid mass ion. In MS/MS mode, P763 showed a specific and strong daughter mass ion signal at m/z 91 due to benzyl ion and a small daughter ion at m/z 198 due to a proposed condensation product of two selenium-kojic acid fragments (see the scheme at Fig. 6). The MS and MS/MS profiles of the base-induced degradation products showed no signals at m/z 322, 91 and 198 and only a small signal at m/z 230 was detected in MS profile (Fig 5). The MS and MS/MS data confirmed the HPLC results and supported the degradation process of P763 under basic conditions.

Table 1. Degradation kinetics of P763 in human plasma, simulated gastric and intestinal solutions at 37°C ±1 after incubation for 5 hours.

	K_{deg} (hr^{-1})	$t_{1/2}$ (hr)	t_{90} (hr)
Human Plasma	0.045	15.4	2.3
0.1M HCl solution (pH~1) (simulated gastric solution)	0.058	11.9	1.9
Phosphate buffer solution (pH 7.4) (simulated intestinal solution)	0.104	6.7	1.0

K_{deg} - rate constant of degradation
$t_{1/2}$ - half-life time of degradation
t_{90} - shelf life

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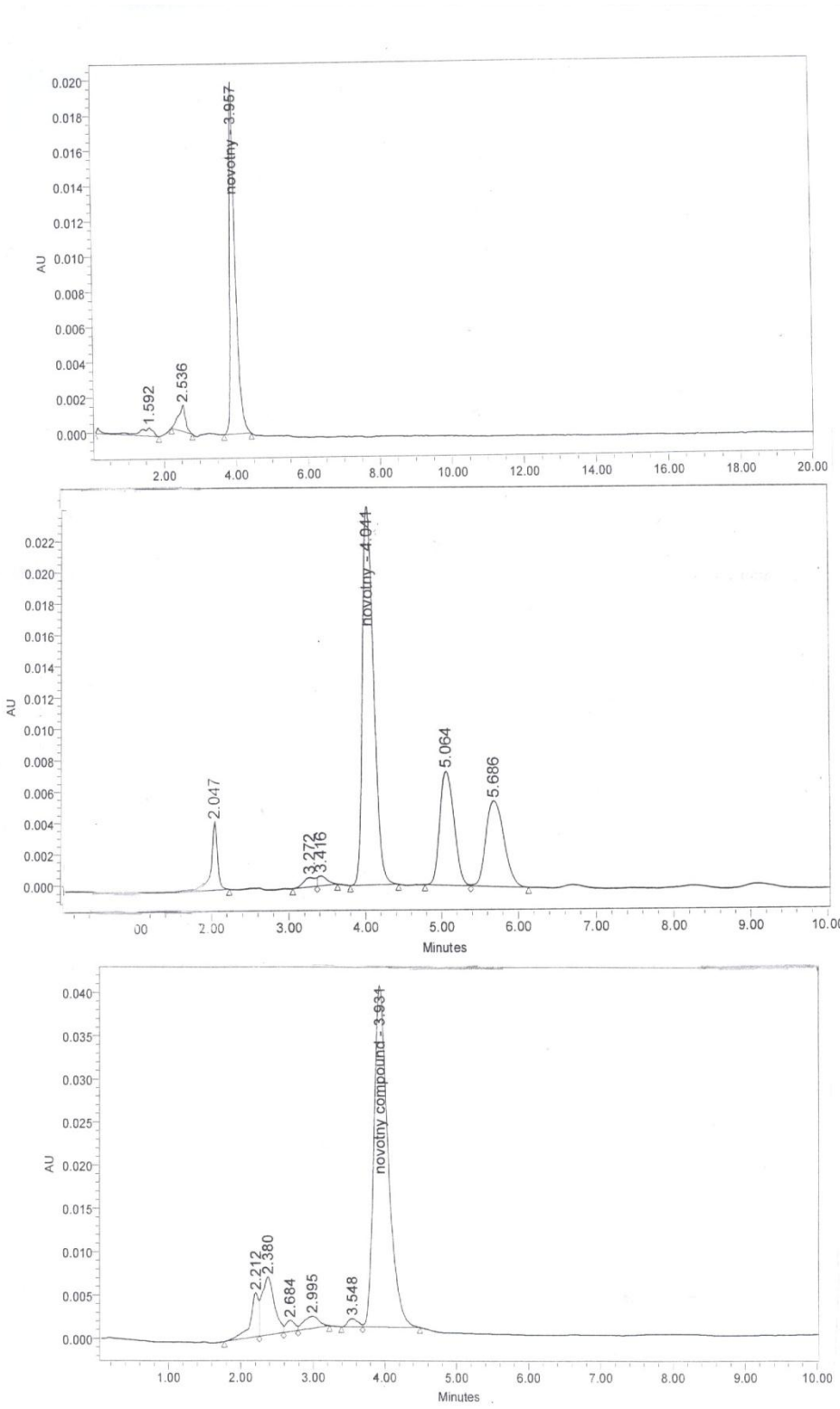

a. Simulated gastric solution

b. Simulated intestinal solution

c. Human plasma

Fig. 2. HPLC chromatograms of P763 in simulated gastric solution (a), simulated intestinal solution (b) and human plasma (c) at 37°C ±1 after incubation for 5 hours

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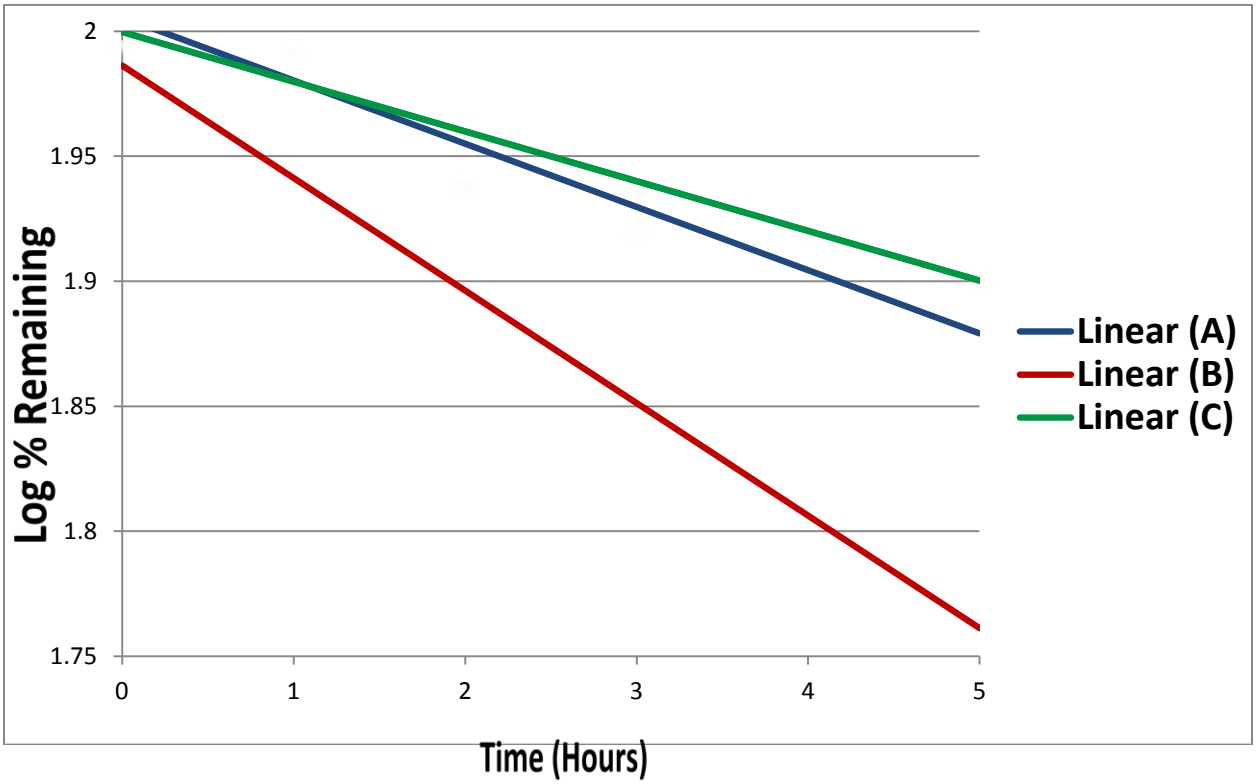
Fig. 3. Accelerated stability profiles of P763 in simulated gastric solution (A), simulated intestinal solution(B) and human plasma (C) at 37°C ±1 after incubation for 5 hours



(A): Simulated gastric solution
(B): Simulated intestinal solution
(C): Human Plasma

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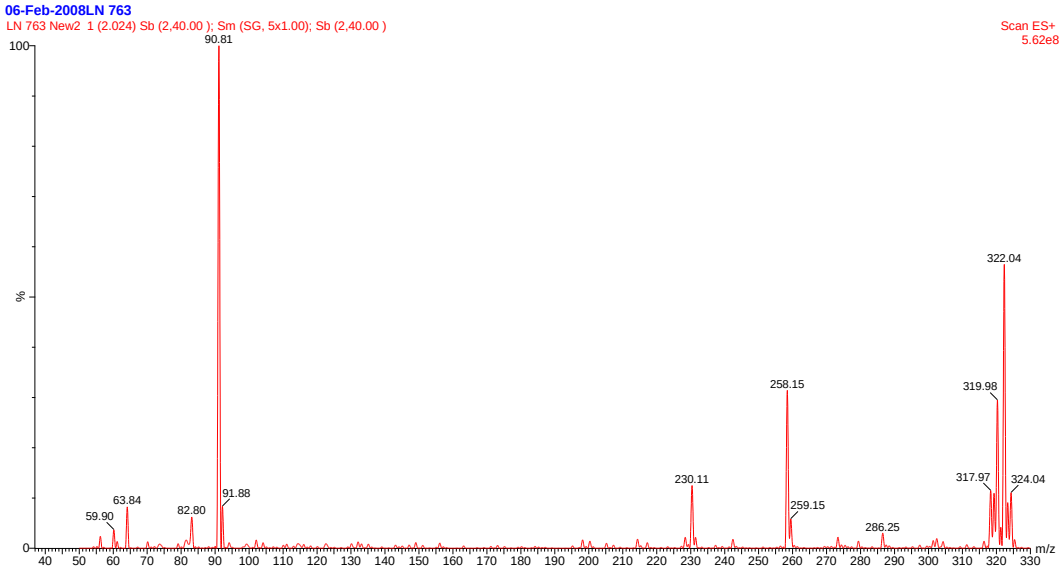
A



B

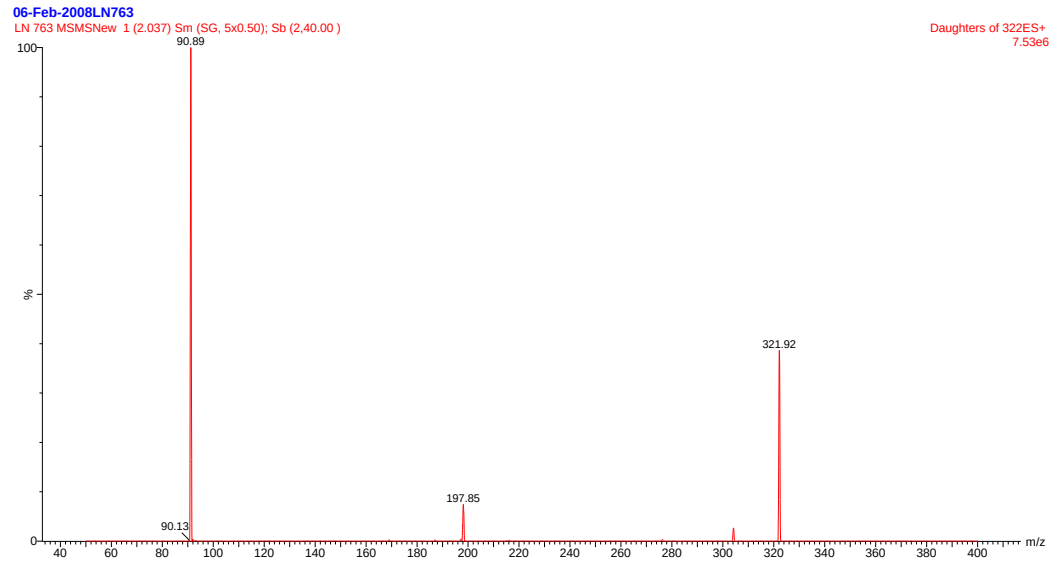


Fig. 4. MS (A) and MS/MS (B) scans of P763 using +ESI

Naturally occurring kojic acid was chemically used for the preparation of many derivatives possessing anticancer properties [4, 10]. Selenium-containing kojic acid derivatives were prepared in order to combine the anticancer properties of modified kojic acid molecule with the anticancer properties of the included-selenium metal [8] which was also known to affect cancer cells [11].

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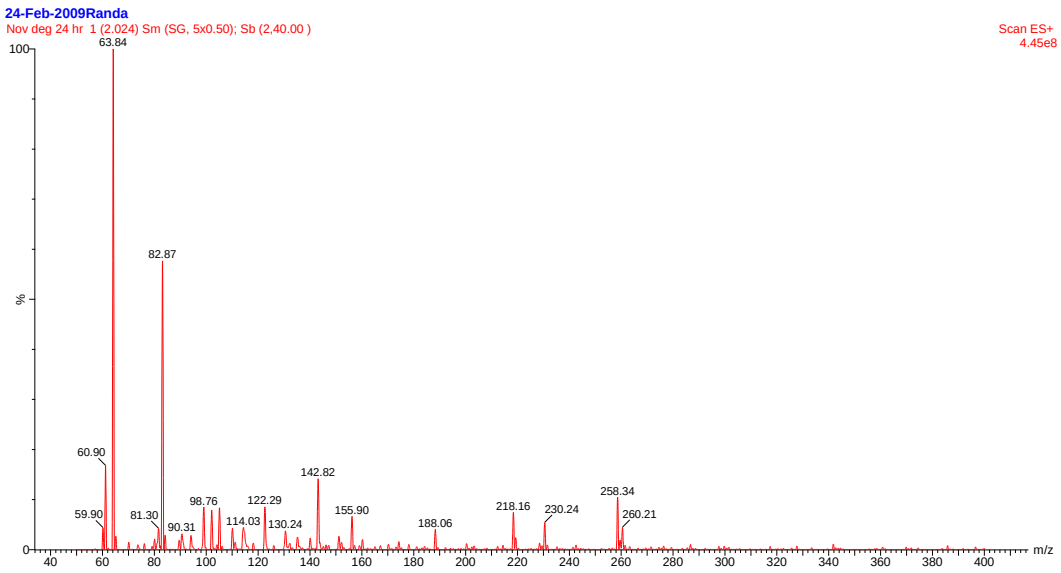

Fig. 5. MS scan of the degraded solution of P763 after incubation in phosphate buffer solution, pH 7.4 at 37°C±1 for 24 hours.

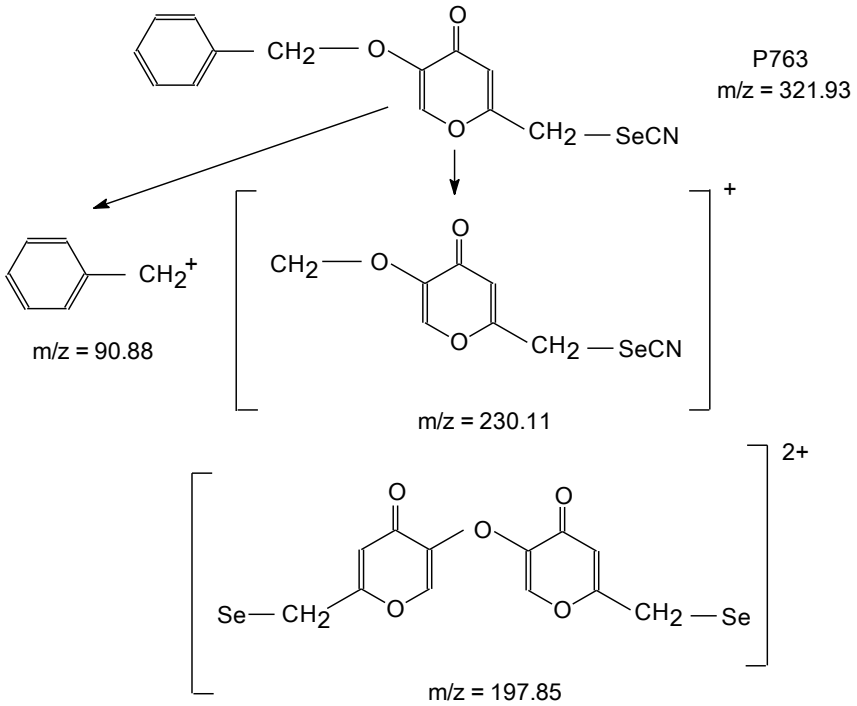

Fig. 6: Proposed fragmentation pattern of P763 as indicated from MS and MS/MS profiles

As the biological activities of these new kojic acid derivatives were confirmed [8], the importance of assessment the stability of these substances under simulated biological conditions of pH and temperature and in the presence of human plasma became obvious. For such investigations, a stability-indicating procedure should be applied. The developed HPLC procedure in our laboratory was proved to be stability-indicating as it permits measurement of

the examined compound in the presence of its acid- and base-induced degradation products or in the presence of biologically active constituents of human plasma. Using the developed method, it was possible to determine the degradation kinetics of compound at different pH conditions and in human plasma. The stability studies revealed that the compound P763 underwent base-induced hydrolysis with a significant chemical loss of product as indicated by the disappearance of the characteristic signals of compound in MS and MS/MS profiles. Moreover, the new small fragments at m/z 64 and 83 in the MS profile of base- induced degraded solution may suggest the formation of reactive free radical entities of P763 that accelerates compound's degradation (Fig.5).

In conclusion, the newly-prepared selenium-containing 4-pyranone derivative, P763, was appropriately stable in simulated gastric solution and in human plasma. The product's stability decreased in phosphate buffer solution, pH 7.4 of approximate 3 times less than that in acid solution or human plasma. The developed HPLC method proved to be a stability-indicating procedure for monitoring P763 in the presence of its degradation products and for kinetic studies. MS and MS/MS analyses were important to confirm the HPLC data and to prove the degradation process. The obtained results are important for future biological and biopharmaceutical studies of selenium-containing kojic acid derivatives.

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