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Short communication

Development and validation of a sensitive liquid chromatographic method for the analysis of a novel radioprotectant: ON 01210.Na

Parina P. Fernandes [a], Manoj Maniar [b], Alekha K. Dash [a,*]

[a] *Department of Pharmacy Sciences, Creighton University Medical Centre, Omaha, NE 68178, USA*
[b] *Palm Pharmaceuticals, Fremont, CA 94539, USA*

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Abstract

ON 01210.Na is a chlorobenzylsulfone derivative with potential property to mitigate the effects of accidental or intentional exposure to life threatening levels of radiation. A simple and sensitive HPLC method was developed and validated for the assay of ON 01210.Na. The isocratic system used a mobile phase consisting of acetonitrile:0.1% trifluroacetic acid in water (60:40, v/v) at a flow rate of 1 ml/min. The method used a C-18 Gemini column (250 mm × 4.6 mm) with column effluents monitored at 254 nm. Forced degradation of the drug was achieved by autoclaving ON 01210.Na with 0.05N HCl, 0.05N NaOH or 1.5% (v/v) hydrogen peroxide. The assay validation parameters evaluated include specificity, linearity, precision, accuracy and sensitivity. The retention time of the drug and the other effluents were well within 7 min. Standard curves were linear over the concentration range of 10–500 μg/ml. The R.S.D. values for the within-day and day-to-day precision ranged from 0.4 to 2.5 and 2.2 to 4.4%, respectively. The R.S.D. for accuracy measurement ranged from 0.85 to 1.7%. The critical level, the detection level and the determination level for this assay were 2.86 ± 0.67, 5.69 ± 0.67 and 15.6 ± 1.8 μg/ml, respectively. A simple, sensitive and stability indicating HPLC assay was developed and validated for the analysis of a novel radioprotectant. This method was used to evaluate the aqueous as well as solid-state stability of this drug during autoclaving.

Keywords: Radioprotectant; ON 01210.Na; HPLC analysis; Forced degradation; Validation

1. Introduction

Uncontrolled exposure to radiation from a possible nuclear battlefield, outer space travel or even accidents presents the greatest threat and challenge to the civilized world today. The detrimental effect of ionizing radiation to normal human cells was known to the public as early as 1896, just after discovery of X-rays. Ionizing radiation may include electromagnetic radiation like X-rays, gamma rays or particulate radiation such as neutrons and alpha particles that have enough energy to ionize atoms and molecules. The consequences of ionizing radiation on cells and tissues are a complex phenomena, and death from radiation is the result of sequences of events which occurs within a fraction of second to several weeks. The first step in this process is the transfer of radiation energy from the source to atoms or molecules in its path, which results in a chemical modification in macromolecules inside the cell that is critical for biological function.

One of the most challenging tasks of radiobiology today is the development of pharmacological agents that can repair or eliminate the early damage produced in cells and tissues by ionizing radiation. With the recent advancement of science, the task of developing specific and more effective radioprotectants is progressing rapidly to address the challenging tasks we have ahead [1]. The search for these agents started as early as 1949 [2]. The primary objectives of these agents is to substantially increase survival and enhance post-attack effectiveness on military personnel on a nuclear battle field. These agents are intended to be self-administered shortly before or after the radiation exposure to reduce early molecular, cellular and tissue damage. Protection depends on the ability of chemical agents to reduce the intracellular concentration of free radicals and reactive oxygen species that are produced within the first millisecond after irradiation. The first generation chemical agents used as radioprotectors are compounds with antioxidant and scavenging potentials. At present amifostine (Ethyol®) is the only FDA approved drug for

* Corresponding author. Tel.: +1 402 280 3188; fax: +1 402 280 1883.
E-mail address: adash@creighton.edu (A.K. Dash).

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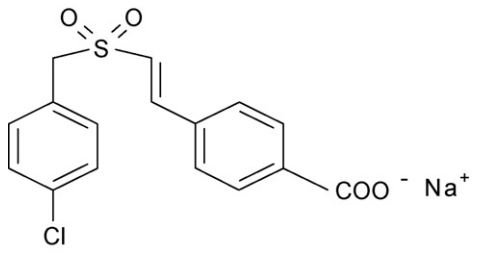


Fig. 1. Structure of (*E*)-4-carboxystyryl-4-chlorobenzylsulfone, sodium salt (ON 01210.Na).

reducing xerostomia after radiation therapy of cancer, and one of the most potent radioprotectants available at the present time [3–5]. However, its use as a radioprotectant has been restricted because of its serious adverse effects [6].

Various novel compounds are currently under clinical investigation as radioprotectants that can accelerate recovery of tissue stem cells and their precursors after radiation exposure. ON 01210.Na (Onconova Therapeutics, Princeton, NJ) is a novel derivative of chlorobenzylsulfone that is under clinical investigation and is known to repair DNA. The structure of this compound is shown in Fig. 1 and has a molecular weight of 358.8 with an aqueous solubility of 8–10 mg/ml at room temperature [7]. It has been shown to protect during life threatening levels of radiation exposure. The pre-clinical studies have demonstrated that ON 01210.Na provides radioprotection of mice in a dose-dependent manner in contrast to amifostine which did not provide adequate protection at the equivalent dose level [7]. This makes the study on the novel drug ON 01210.Na urgent and necessary. However, no LC methods are currently available for the analysis of this group of compounds.

The objectives of this study are: (i) to develop and validate a HPLC assay that can support pre-formulation, and formulation development for this drug, (ii) to elucidate the stability indicating nature of this assay procedure by performing LC analysis of forced degraded samples of ON 01210.Na exposed to drastic acidic, alkaline and oxidizing conditions and (iii) to apply this method for evaluating the solid-state, and solution stability of this radioprotectant.

2. Experimental

2.1. Chemicals and reagents

ON 01210.Na was received from Onconova Therapeutics Inc. (Princeton, NJ, USA); acetonitrile, water (HPLC Grade), hydrogen peroxide 30% (v/v) in water were obtained from Fischer Scientific (Saint Louis, MO, USA). Reagent grade sodium hydroxide and hydrochloric acid were purchased from Fischer Scientific (Fairlawn, NJ, USA); trifluoro acetic acid (TFA), 98%, spectrochemical grade was obtained from Sigma Chemicals (Saint Louis, MO, USA).

2.2. Chromatographic equipments

Two chromatographic systems were utilized in this investigation. Gradient mode was used during the initial developmental stage and isocratic system for method optimization and validation.

2.2.1. Gradient system

The chromatographic system used in gradient mode consisted of a Shimadzu (Columbia, MD, USA) LC-10 AT VP pump programmed by a system controller (SCL-10 AVP), an auto-injector SIL-10 ADVP, a column oven CTO-10 ASVP, diode array detector SPD-M 10 AVP, operated by Class VP & 2.1 SP1 software system. Separation was achieved using a Gemini 5 μ-C-18 110A column 250 mm × 4.6 mm (Phenomenex, Torrance, CA, USA). The initial HPLC method development process utilized a gradient system with the following mobile phase composition: mobile phase A, water containing 0.1% TFA and mobile phase B, 100% HPLC grade acetonitrile. The gradient used was a linear gradient from 0% B to 100% B over 30 min. A photodiode array detector was used and the effluents were monitored at three wavelengths 230, 254 and 320 nm, respectively. The flow rate was maintained at 1.0 ml/min.

2.2.2. Isocratic system

For chromatographic separation in the isocratic mode, the HPLC system consisted of a Shimadzu LC system (Columbia, MD, USA) equipped with a UV–vis spectrophotometric detector SPD-6 AV, an auto-injector SIL-10 ADVP. The mobile phase consisted of 60:40 (v/v) of acetonitrile:water containing 0.1% (v/v) TFA. The apparent pH of the mobile phase was 2.5. The flow rate of the mobile phase was maintained at 1.0 ml/min. The effluents were monitored at 254 nm. Peak area response was used for the quantitation of the ON 01210.Na.

2.3. Preparation of standard and sample solutions for HPLC analysis

The stock standard solution (1000 μg/ml) was prepared by dissolving 0.10 g of ON 01210.Na in 100 ml 60:40 (v/v) of acetonitrile:water. No TFA was added to the aqueous phase. Various standard solutions were then prepared by diluting the above stock solution with mobile phase without TFA to yield nominal concentrations over a range of 10–500 μg/ml. The apparent pH of this solution was 6.9.

2.4. Forced degradation studies

Forced degradation of ON 01210.Na in solution was carried out under three drastic conditions. For forced degradation with a strong acid or a base, samples were sealed in glass ampoules and autoclaved in a Tattnauer autoclave (Ronkonkoma, NY, USA). The autoclaving condition used was 121 °C, 31 psi pressure, for 30 min. Solutions that appeared cloudy during autoclaving were filtered through 0.1 μm syringe filter (Whatman Inc., Florham Park, NJ, USA) before injecting into the HPLC system. However, forced degradation with an oxidizing agent (1.5% hydrogen peroxide) was carried out in an oven for safety reasons. The sample was incubated at 50 °C in an oven (Stabeltherh, Blueisland, IL, USA) over a period of 2 h.

2.4.1. Sample preparation for the forced degradation studies

One milliliter of the 500 μg/ml aqueous solution of ON 1210.Na was mixed with 1 ml of 0.1N HCl. This solution was sealed into a 5 ml glass vial and autoclaved. After autoclaving, the sample was filtered through a 0.1 μm filter and 20 μl of the filtered sample was analyzed by HPLC. The same sample preparation procedure was adopted for degradation studies with 0.05N NaOH solution except that no filtration prior to HPLC analysis was needed for this condition. For forced degradation with hydrogen peroxide, 30% (v/v) of the hydrogen peroxide solution was diluted to 3% (v/v) with distilled water. One milliliter of the 500 μg/ml standard solution was mixed with 1 ml of diluted (3%, v/v) hydrogen peroxide and incubated at 50 °C in the oven over a period of 2 h. The samples were filtered through a 0.1 μm filter and 20 μl of the filtered sample was analyzed by HPLC.

2.5. Application

This method was used to evaluate the stability of ON 01210.Na during autoclaving both in the solid-state and in solution.

2.5.1. Solid-state stability

Solid-drug sample was placed in a crimped vial and autoclaved. The autoclaving condition was exactly the same as described earlier. After autoclaving, a known weight of the sample was used to make a solution of the drug at a concentration of 400 μg/ml. Twenty microliter of the above solution was injected onto HPLC.

2.5.2. Stability in solution

A solution of ON 01210.Na was prepared in HPLC grade water at a concentration of 200 μg/ml. This solution was sealed into a 5 ml glass ampule and autoclaved. The autoclaving condition was exactly the same as described previously. After autoclaving, the sample was filtered through a 0.1 μm syringe filter and 20 μl of the filtered sample was analyzed by HPLC.

3. Results and discussion

3.1. Chromatography

The initial HPLC method development process utilized a gradient system with a photodiode array detector. Column effluents were monitored at three wavelengths 230, 254 and 320 nm, respectively at a mobile phase flow rate of 1.0 ml/min. The retention time of ON 01210.Na was found to be around 23 min. This condition was also suitable for the detection of the parent compound along with the other impurities present in the sample. A known concentration of the ON 01210.Na solution (100 μg/ml) was analyzed at three different wavelengths (230, 254 and 320 nm) and the absolute peak area at these wavelengths were determined. The results of this study indicated that analysis of the column effluents at 254 nm is more sensitive as compared to the other two wavelengths. Therefore, column effluents were monitored at 254 nm for the entire study. Even though the retention time of the drug was more than 20 min for this gradient system, the degradation products with a forced degradation study with 0.1N NaOH were well resolved and detected under these conditions. The next objective of the method development process was to reduce the chromatographic time without sacrificing the resolution between ON 01210.Na and its impurities/degradation products. Hence, efforts were focused on developing an isocratic system utilizing the various combinations of acidified water and acetonitrile as the mobile phase. Three mobile phase compositions were selected for this study. They were: 20:80, 40:60 and 60:40 (v/v) of acetonitrile:water containing 0.1% TFA, respectively. The drug was dissolved in solutions of acetonitrile:water without 0.1% TFA with similar proportions as mentioned above. The retention time of ON 01210.Na for the first two mobile phase compositions was more than 8 min whereas with 60:40 composition it was around 5 min at a flow rate of 1 ml/min. The resolution between ON 01210.Na peak and peaks for three possible impurities were good. The retention times of these impurity peaks were 4.1, 6.2 and 6.8 min, respectively. Therefore, the third mobile phase composition was selected for further evaluation. Representative chromatograms of the mobile phase alone (data not shown) and the drug in the diluting solution are shown in Fig. 2 No interference from the mobile phase was detected which is an indicative of the specificity of this assay procedure.

3.2. Establishing stability indicating aspect of the developed method

3.2.1. Forced degradation with 0.05N HCl

Fig. 3(a) depicts a typical chromatogram of a forced degraded sample with 0.05N HCl after autoclaving. Decrease in the absolute peak area of the parent peak and appearance of any extra peaks due to possible degradation products were monitored. There was less than 10% decreased in the absolute peak area of the parent peak upon autoclaving ON 01210.Na in presence of strong acid. This method was able to identify many more additional peaks, possibly due to the degradation products along with the parent peak.

3.2.2. Forced degradation with 0.05N NaOH

Significant degradation was noticed under forced degradation with a strong base. A representative chromatogram from such a study is shown in Fig. 3(b) A major degradation product was noticed in the chromatogram at the retention time of 3.4 min.

3.2.3. Forced degradation with 1.5% (v/v) hydrogen peroxide solution

Fig. 3(c) represents the chromatogram of a sample degraded with an oxidizing agent at 50 °C for 2 h. Interestingly, no parent peak was noticed at the retention time of 5.0 min. Instead two extra peaks, possibly due to the degradation product were noticed at the retention time of 2.5 and 4.2 min, respectively.

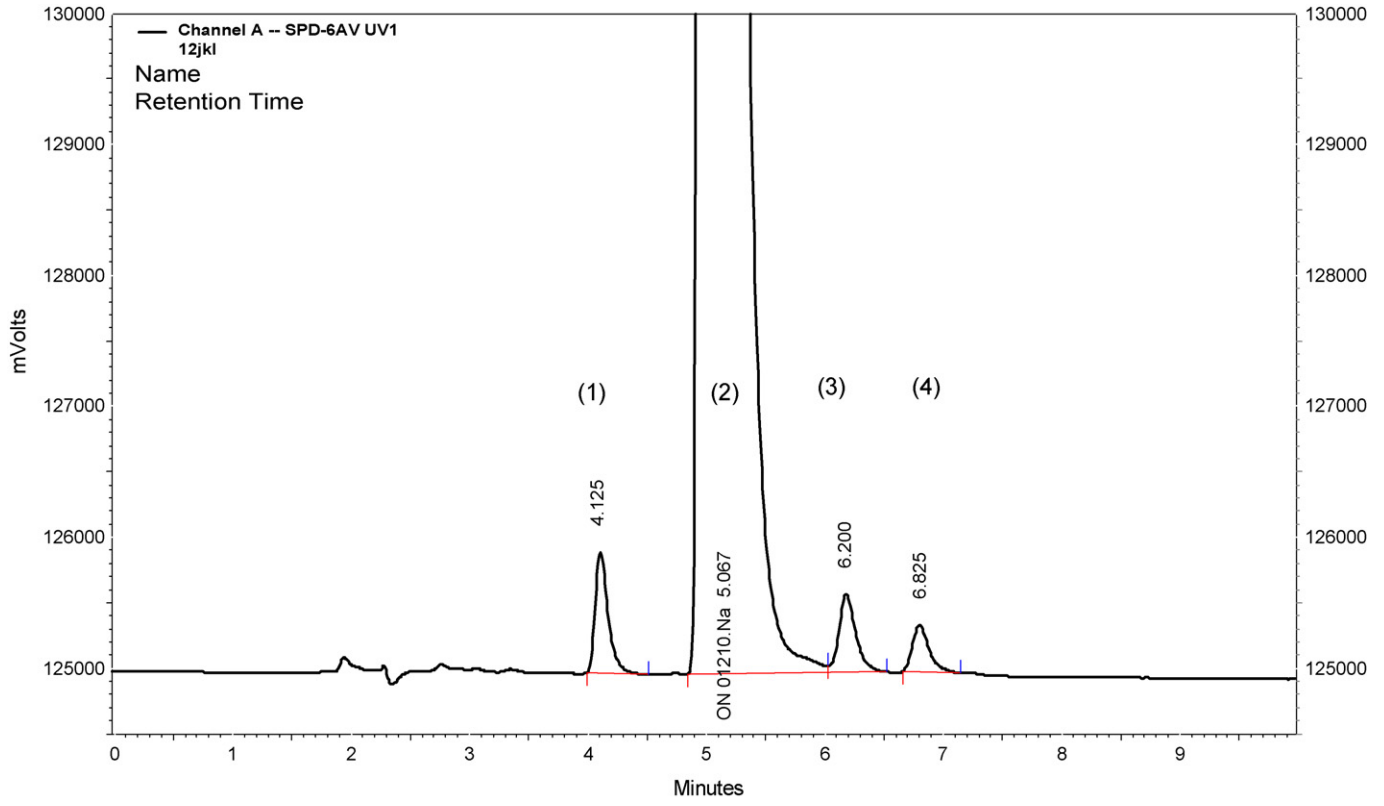


Fig. 2. A representative chromatogram of ON 01210.Na using an isocratic system of acetonitrile:water containing 0.1% TFA 60:40 showing the drug peak eluting at 5 min and its three major impurities eluting at 4, 6 and 6.5 min.

All of the above forced degradation studies have clearly indicated that the HPLC method developed for ON 01210.Na is indeed stability indicating. The peak of ON 01210.Na is from a single component and none of the degradation products co-elute with parent drug peak.

3.3. Assay validation

3.3.1. Linearity

Standard curves were constructed by plotting peak area versus concentration of the drug. Standard curves for ON 01210.Na was linear over the concentration range of 10–500 μg/ml. The equation of the standard curve correlating the peak area (PA) to the drug concentration (C in μg/ml) in this range was $PA = 7372.3C + 0.14556$, $R^2 > 0.999$.

3.3.2. Precision

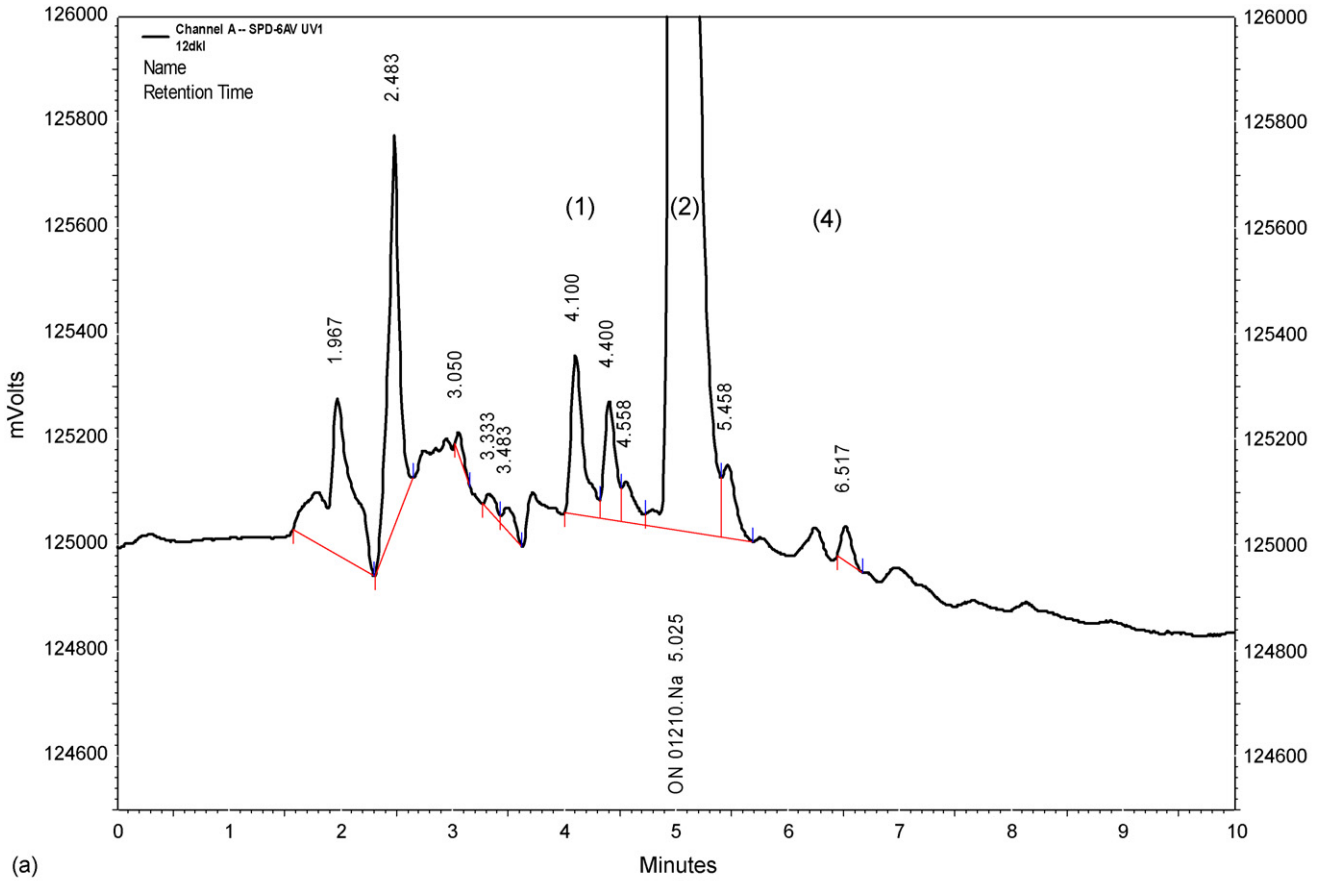
Within-day precision of the assay was determined by analysis of replicate ($n = 4$) samples of five different standard solutions on the same day. To determine day-to-day precision, the same solutions were analyzed on 5 different days over a period of 12 days. The variability in the peak area at each concentration is presented in Table 1 Within-day relative standard deviation (R.S.D.) values for the ON 01210.Na assay ranged from 0.4 to 2.5%. The day-to-day precision R.S.D. values for ON 01210.Na were 2.1–4.4%. During this period, the stock solution and standard solutions were stored under room temperature (23 °C).

3.3.3. Accuracy

Three quality control samples (QCs) for ON 01210.Na were placed at room temperature (23 °C) over a period of 15 days. These samples were analyzed five times during this time and the accuracy of the assay was determined by comparing the measured concentration to its nominal value. The results of this study are depicted in Table 2. The accuracy of this method was found to be between 97.7 and 101.6% The R.S.D.'s for accuracy measurement ranged from 0.85 to 1.7%.

3.3.4. Sensitivity

The lowest limit of reliable assay measurement criteria described by Oppenheimer et al. was used to determine the sensitivity parameters [8]. Six different standard curves were used in this calculation. The critical level was defined as the assay response above which an observed response is reliably recognized as detectable. This value is also considered as the threshold value, defining detection. If the measured value exceeds this value, then the presence of analyte is detected, otherwise it is not. This was 2.86 ± 0.33 μg/ml (mean ± S.D.). The detection level is the actual net response, which may be expected to lead to detection. This is the least value of the true concentration that is "nearly sure" to produce a measured value that results in detection. This was 5.69 ± 0.67 μg/ml (mean ± S.D.). The determination level is the concentration at which the measurement precision will be satisfactory for quantitative determination and was found to be 15.6 ± 1.79 μg/ml (mean ± S.D.) for a level of precision of 10% R.S.D.



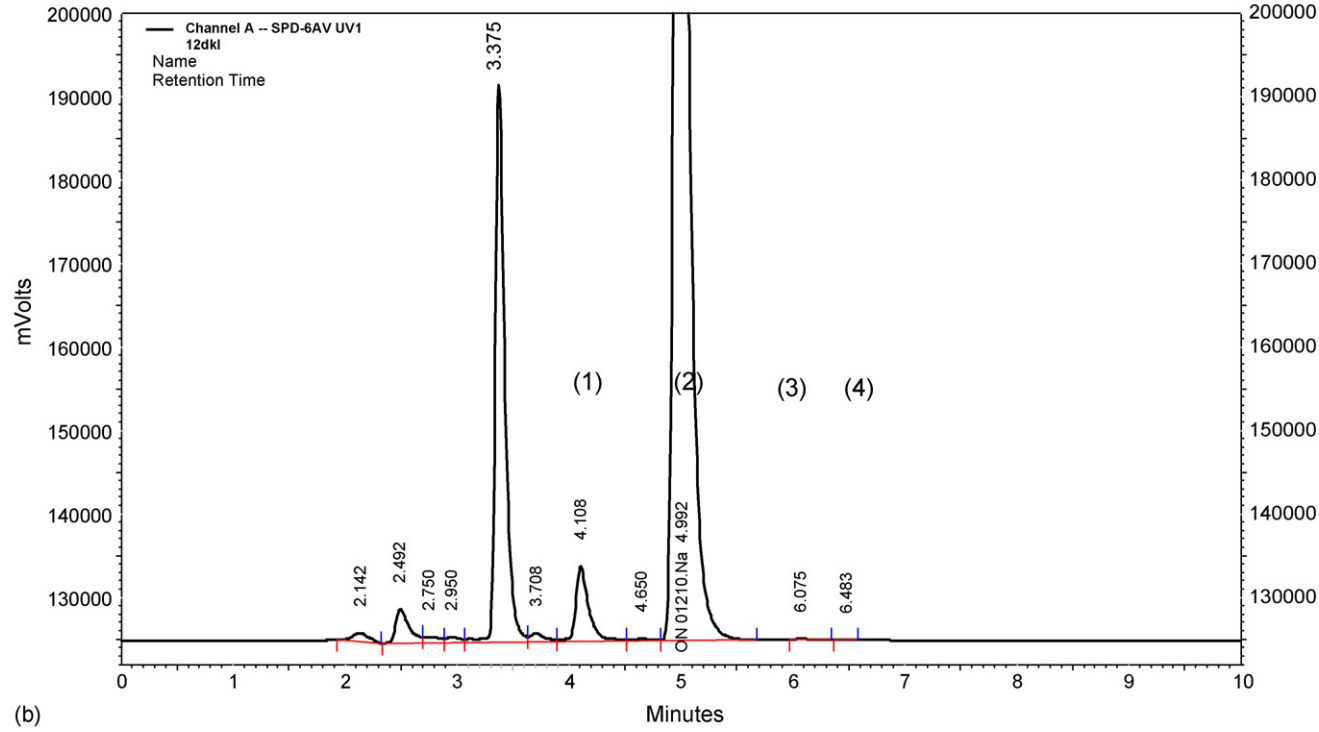


Fig. 3. Forced degradation study with (a) 0.05N HCl after autoclaving showing the appearance of many more additional peaks due to possible degradation products, (b) 0.05N NaOH after autoclaving and (c) 1.5% hydrogen peroxide. The spiked sample with ON 01210.Na was injected onto HPLC after incubation with hydrogen peroxide for 2 h.

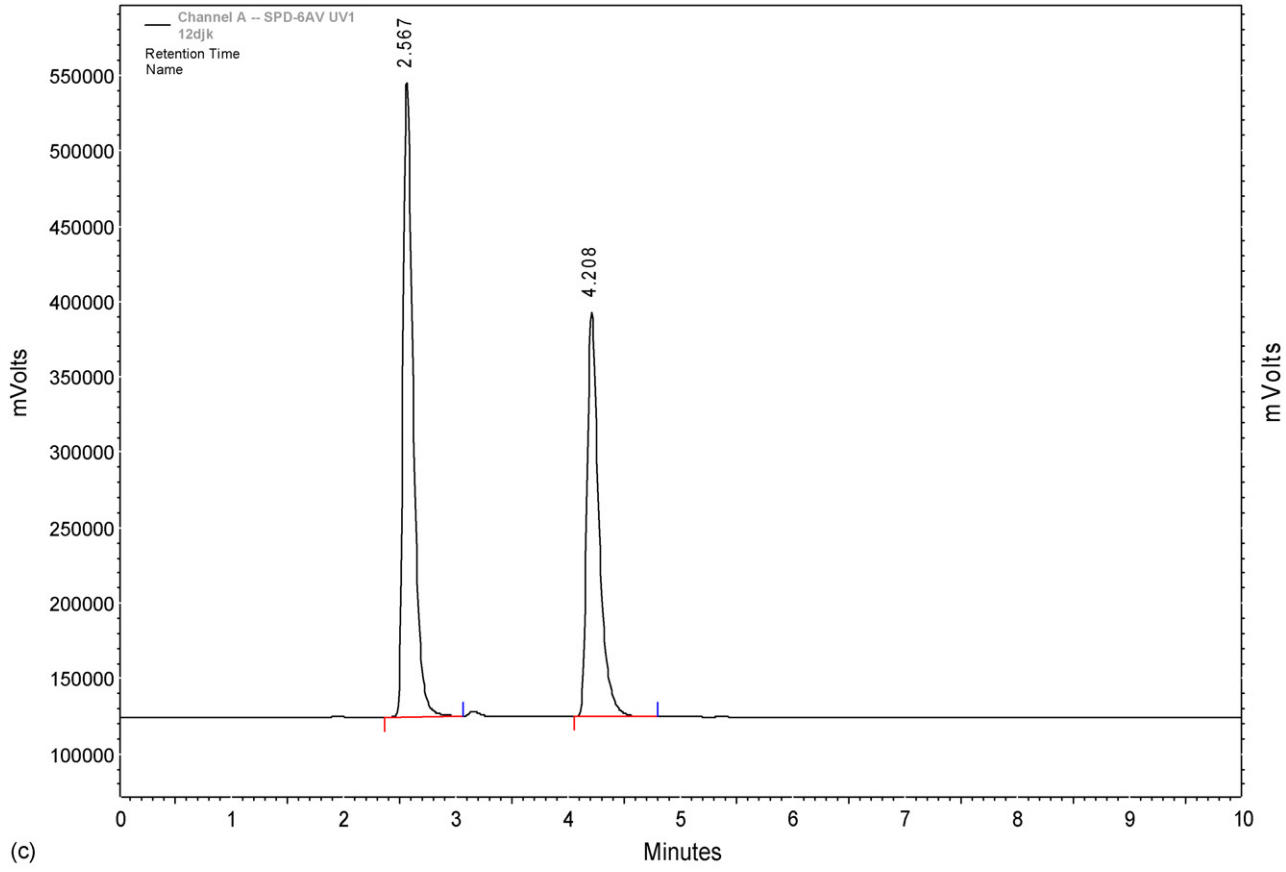


Fig. 3. (*Continued*)

Table 1
Within-day and day-to-day analytical precision for ON 01210.Na

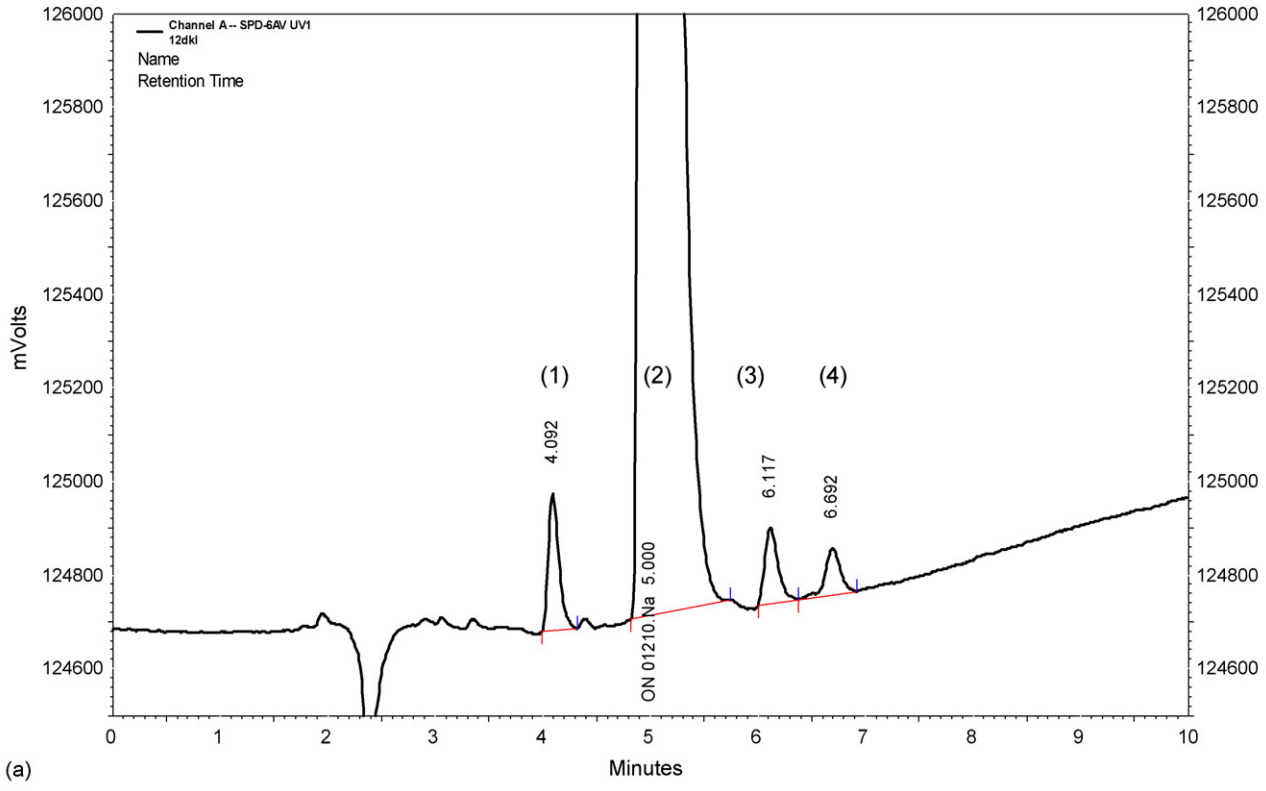
Concentration (μg/ml)	Within-day		Day-to-day	
	Mean peak area[a]	R.S.D. (%)	Mean peak area[b]	R.S.D. (%)
0	0	0	0	0
10	79531.0	0.8	82255.8	4.4
50	385264.5	2.5	393338.8	2.5
100	759900.8	0.6	772519.5	2.6
200	1498462.0	0.4	1522404	2.1
500	3666046.0	0.5	3723656	2.1
Slope	7321.1 ± 37.1	0.5	7434.1 ± 156.1	2.1

[a] $n = 4$.
[b] $n = 6$, over a period of 12 days.

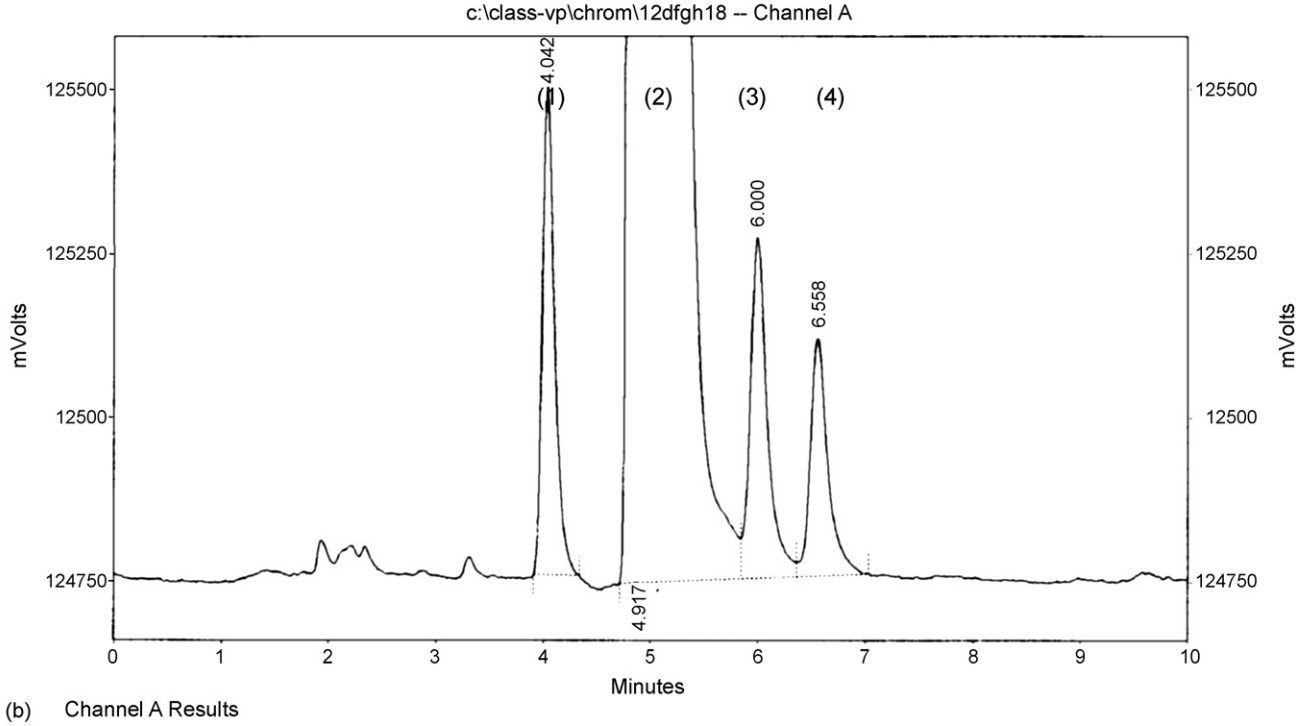
Table 2
Accuracy in the analysis of ON 01210.Na in quality control samples

Actual concentration (μg/ml)	Measured concentration (μg/ml)[a]	Accuracy[b]
20	19.5 ± 0.3	97.7 ± 1.7
250	254.1 ± 2.2	101.6 ± 0.9
400	398.3 ± 3.4	99.6 ± 0.8

[a] Mean ± S.D.; $n = 5$.
[b] Accuracy = (measured concentration/actual concentration) × 100.



(a)



(b) Channel A Results

Fig. 4. A representative chromatogram of sample (a) ON 01210.Na (solid) sample after autoclaving and reconstituted in the mobile phase without TFA and injected onto HPLC and (b) ON 01210.Na in water after autoclaving showing no extra degradation peaks.

4. Applications

Since the desired route of administration for this radioprotectant is the parenteral route, evaluation of the stability of this drug during autoclaving was essential. The HPLC method developed and validated was used to determine the stability of this drug during autoclaving both in solid as well as in solution.

4.1. Stability of ON 01210.Na in the solid-state

Solid drug sample after autoclaving in a crimped vial was further evaluated for any degradation using the developed HPLC method. The sample after autoclaving was used to make a standard solution of 400 μg/ml and injected onto HPLC. There was no reduction in the absolute peak area of the parent peak, and also no additional peaks were detected in the chromatogram as shown in Fig. 4(a). Based on this observation, it seems that the solid drug ON 01210.Na is stable under the autoclaving conditions.

4.2. Stability of ON 01210.Na in aqueous solution

The developed LC method was further utilized to determine the stability of ON 01210.Na in aqueous solution during autoclaving. Drug solution was prepared in HPLC grade water at a concentration of 200 μg/ml. All the peaks detected in the chromatogram before and after autoclaving were noted with their corresponding retention time and absolute peak areas. The absolute peak area of the peak after autoclaving and appearance of any additional peaks due to possible degradation were monitored. Representative chromatogram of injected solutions after autoclaving is shown in Fig. 4(b). No reduction in the absolute peak area or appearance of additional peaks was observed after autoclaving this drug in aqueous solution, indicating its stability in solution after autoclaving.

5. Conclusions

A sensitive, HPLC assay was developed and validated for the analysis of ON 01210.Na in aqueous solution using a C-18 Gemini column. This method was found to be stability indicating and used to evaluate the aqueous as well as solid-state stability of this drug during autoclaving. The drug was found to be stable during autoclaving in both conditions.

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