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Characterization of a novel degradation product of 2,2′-dithiobis[*N*-isoleucylbenzamide], an inhibitor of HIV nucleocapsid protein zinc fingers

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Abstract

Zinc finger motifs have been found to be important in a variety of protein structures including transcription factors and viral nucleocapsid proteins. Recently, it was demonstrated that various aromatic disulfides effectively remove the metal ion from the zinc finger, resulting in an alteration of tertiary structure in this region of the protein, thereby inhibiting transcription. Among these compounds, 2,2′-dithiobis[*N*-isoleucylbenzamide] exhibits activity against human immunodeficiency virus (HIV)-type 1 in vitro and has been selected for preclinical development as an anti-HIV agent. Analysis of this agent by reversed-phase high-performance liquid chromatography (HPLC) indicated a significant quantity of two additional compounds. Identifying the parent disulfide was accomplished by scanning eluting peaks with positive ion thermospray ionization (TSP) mass spectrometry (MS). Solution-induced disproportionation of the disulfide into its sulfydryl monomer was demonstrated by treating the drug with dithiothreitol (DTT) prior to HPLC analysis. TSP-MS analysis of the remaining chromatographic peak suggested a molecular weight of 265, which, with ^{1}H-nuclear magnetic resonance (NMR) data of the isolated material, allowed us to elucidate the chemical structure as *N*-isoleucyl-benzisothiazolone. Contact with stainless steel, such as that employed in an HPLC system, was found to accelerate degradation of the parent disulfide to the benzisothiazolone. © Published by Elsevier Science B.V.

Keywords: 2,2′-dithiobis[*N*-isoleucylbenzamide]; Benzisothiazolone; Antiviral agents; Liquid chromatography; Mass spectrometry; Nuclear magnetic resonance spectroscopy

1. Introduction

Shortly after the discovery of the ‘zinc-finger motif’ in 1985 [1], many proteins were found to

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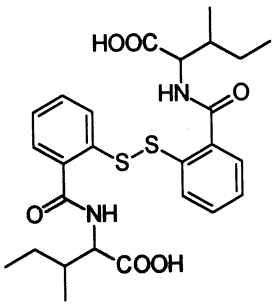
contain amino acid sequences that facilitate folding about a zinc ion [2], with identification of no less than ten types of zinc-binding domains [3]. Although surprisingly widespread [4], zinc-finger motifs are nonetheless important in a variety of protein structures including transcript factors and viral nucleocapsid proteins [5]. It has been recently demonstrated that various aromatic disulfides effectively remove the metal ion from the zinc finger, resulting in an alteration of tertiary structure in this region of the protein, thereby inhibiting transcription [6]. Among these compounds, 2,2′-dithiobis[*N*-isoleucylbenzamide] (**I**) (see Fig. 1) exhibits activity against HIV-1 in vitro and has been subsequently selected for preclinical development as an anti-HIV agent [7,8]. As a prelude to developing a specific assay for **I** in biological fluids, its chemical structure and purity were confirmed using desorption chemical ionization (DCI) mass spectrometry (MS) and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. However, reversed-phase high-performance liquid chromatography (HPLC) analysis of freshly prepared pH 7 aqueous solution of **I** showed three significant chromatographic peaks. The structural identification and origin of each of these compounds are described in the present report.



I

Fig. 1. Chemical structure of 2,2′-dithiobis[*N*-isoleucylbenzamide] (**I**).

2. Experimental

2.1. Reagents and chemicals

2,2′-Dithiobis[*N*-isoleucylbenzamide] (NSC 672 594) was obtained from the Pharmaceutical Resources Branch, DTP, DCTDC, National Cancer Institute (Bethesda, MD, USA). All other reagents (such as dithiothreitol (DTT), ammonium formate, formic acid, and potassium phosphate), solvents, and deuterated solvents were purchased in the highest purity available from Aldrich (Milwaukee, WI, USA) and used as received. Distilled water was deionized and stripped of dissolved organics by passage through mixed-bed resins and activated carbon (Hydro Water Systems, Rockville, MD, USA).

2.2. Chromatography

High-performance liquid chromatography (HPLC) was performed at ambient temperature using an HP Series 1050 pump and autosampler fitted with a 500-μl sample loop (Hewlett-Packard, Palo Alto, CA, USA). Samples were subjected to analysis on a 4-μm Nova-Pak C_8 column (150 × 3.9 mm I.D.) (Millipore, Milford, MA, USA) by isocratic elution with acetonitrile–ammonium formate (pH 3.7; 0.15 M) (35:65, v/v). At a flow rate of 1.0 ml/min, UV spectra of the column effluent were acquired every 1000 ms using an HP Model 104014 photodiode array detector. At a flow rate of 0.7 ml/min, the column effluent was first monitored for UV absorbance at 252 nm, and was then monitored by thermospray ionization (TSP) MS with scanning detection, as described below.

2.3. Mass spectrometry

The column effluent was passed directly into an HP 5989A mass spectrometer equipped with a thermospray LC/MS interface (Hewlett-Packard). The thermospray ion source was operated in the filament-assisted mode with either positive or negative ion detection. Operating temperatures for the interface were as follows, ion source, 280°C; probe stem 114°C; and probe tip, 246°C.

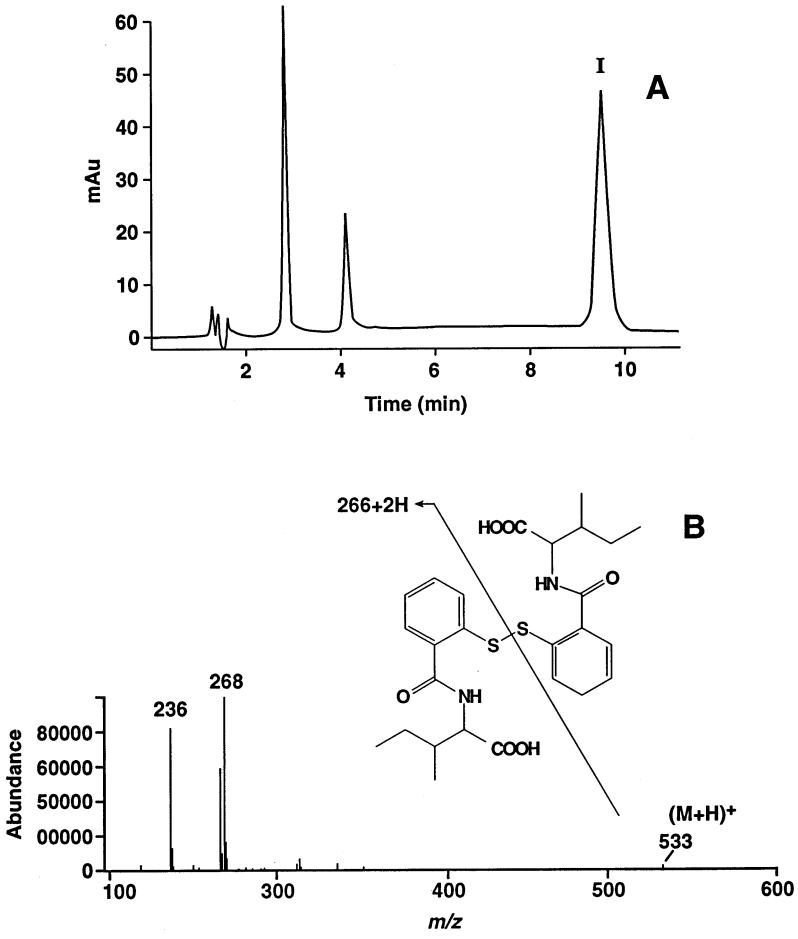


Fig. 2. Liquid chromatogram of **I** determined with UV detection at 252 nm (A). The positive ion TSP-MS of the third chromatographic peak (B) shows ions at m/z 533 (the $[M+H]^+$ ions expected for **I**) and prominent ions at m/z 268, as would be predicted for fragmentation of the labile –S–S– bond.

Nominal resolution mass spectra (from m/z 200 to 600) were acquired at the rate of 0.8 scans per s with the electron multiplier set at 2500 V. Using methane as the reagent gas, nominal resolution positive-ion desorption chemical ionization (DCI) mass spectra (from m/z 100 to 800) were obtained using a Finnigan TSQ 70 mass spectrometer (San Jose, CA). Samples were introduced into the ion source using a high temperature direct exposure probe.

2.4. ^1H-nuclear magnetic resonance spectroscopy

Normal Fourier transform (F.t.) ^{1}H-NMR spectra (both ^{1}H spectra and ^{1}H–^{1}H homonuclear correlation (COSY) spectra) were acquired using a Varian VXR-500 S spectrometer, equipped with a Sun Microsystem data system, at 25°C. Samples were dissolved in deuterated solvents. Residual ^{1}H signal of the solvent was used as an internal reference in determining the relative chemical shift (δ) values.

3. Results and discussion

Reversed-phase HPLC analysis (Fig. 2A) indicated a significant quantity of two additional compounds in the presence of **I**. Identifying the most

strongly retained peak in the chromatogram (retention time of 9.6 min) as the parent disulfide was easily accomplished by detecting the eluting chromatographic peaks with positive ion TSP-MS. Ions corresponding to the expected $[M+H]^+$ are observed at m/z 533 (Fig. 2B), whereas the prominent ions observed at m/z 268 predictably result from fragmentation of the labile –S–S– bond.

It was further suspected that a simple solution-induced reduction of disulfide **I** would lead to the production of its sulfydryl monomer. Treating a disulfide (RS-SR) with dithiothreitol (DTT) results in nearly quantitative conversion to the corresponding sulfydryl (RS-H). Thus, HPLC analysis of a solution of **I** after treatment with DTT (Fig. 3) allowed rapid identification of the second chromatographic peak (which eluted at 4.1 min) as *N*-isoleucylbenzamide-2-thiol, **II**. Unexpectedly, the earliest eluting peak was also converted quantitatively to **II** when treated with DTT. Although a structural relatedness to **I** was thus indicated, the identity and origin of the chromatographic peak exhibiting a retention time of 2.7 min were not so easily deduced, and therefore further analysis was necessary.

Identifying the earliest eluting chromatographic peak began with on-line analyses using UV and

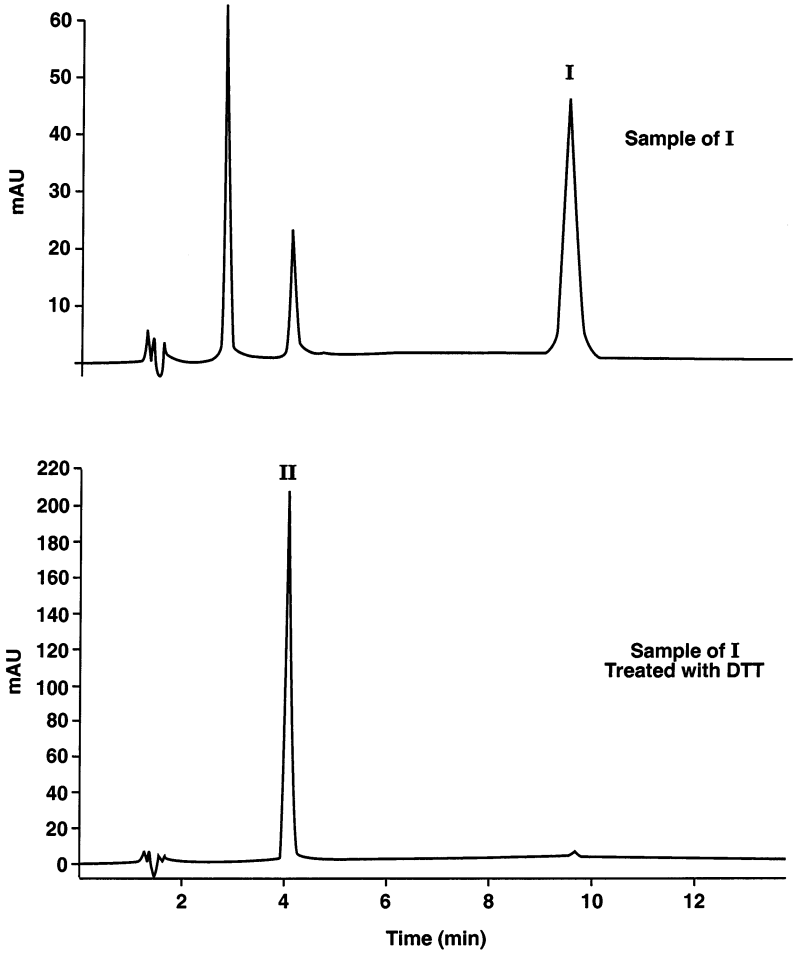


Fig. 3. HPLC analysis of **I** before and after treatment with DTT allows identification of the second chromatographic peak as *N*-isoleucylbenzamide-2-thiol, **II**.

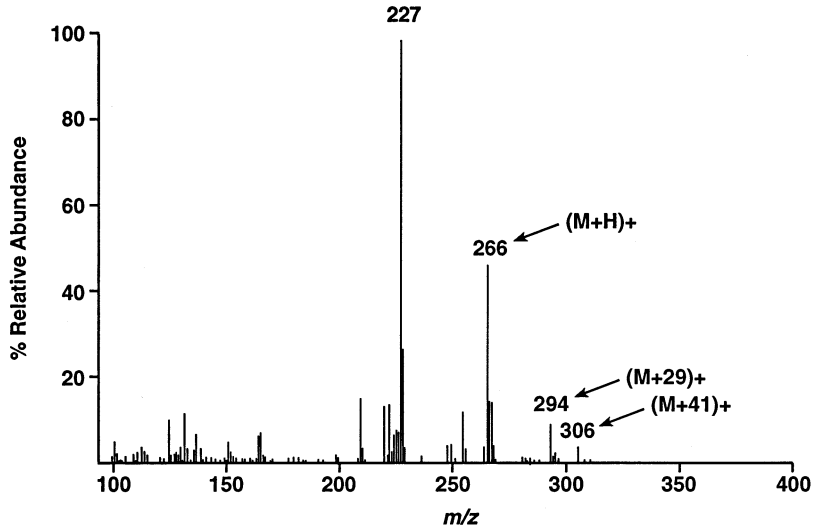


Fig. 4. Desorption chemical ionization (DCI)-mass spectrum of the earliest eluting peak, using methane as the reagent gas. Assigning $(M+H)^+$, $(M+29)^+$, and $(M+41)^+$ to the ions appearing at m/z 266, 294, and 306, respectively, confirms the molecular weight of 265 d previously proposed from thermospray ionization mass spectrometric data.

TSP-MS. The UV absorption maximum in spectra acquired during elution of the peak at 2.7 min occurred at a significantly longer wavelength (322 nm) than that found for **I** (290 nm), indicating the presence of a more extensive system of conjugated chemical bonds. The TSP-mass spectra possessed a base peak at m/z 266 with positive ion detection and at m/z 264 with negative ion detection, suggesting a molecular weight of 265 d.

When incubated in moderately acidic aqueous solution (pH 5–6), **I** was observed to slowly but quantitatively convert to the earliest eluting chromatographic peak, which was then carefully isolated and purified by solid phase extraction. Using methane as the reagent gas, the positive-ion DCI-mass spectrum (Fig. 4) of the isolated bulk material was acquired. In chemical ionization (CI) MS, ionization of a sample is accomplished by reaction of the sample with ionic species, which are generated in situ by bombarding a 'reagent gas' such as methane with high energy electrons [9]. When methane, for example, is bombarded at a low pressure (about 10^{-5} Torr) with high-energy electrons, methane is ionized and ions of m/z 16 are formed. If the pressure of methane is increased to about 1 Torr, ion–molecule reactions occur between these ions and other methane molecules. These ion–molecule reactions lead to formation of a 'reagent gas plasma' which is composed of ions of m/z 17 $(CH_4+H)^+$, m/z 29 $(C_2H_5)^+$, and m/z 41 $(C_3H_5)^+$, with relative abundances in a ratio of about 8:7:1, respectively. Sample molecules are then ionized by reaction with these plasma ions. In the case of thermally stable sample molecules which are characteristically aromatic and possess hetero atoms such as nitrogen, ionization with these plasma ions can lead to formation of relatively abundant $(M+H)^+$, $(M+C_2H_5)^+$, and $(M+C_3H_5)^+$ ions, representing $(M+1)^+$, $(M+29)^+$, and $(M+41)^+$, respectively [10]. Thus, assigning $(M+H)^+$, $(M+29)^+$, and $(M+41)^+$ to the ions appearing at m/z 266, 294, and 306, respectively, supported the molecular weight assignment of 265 d proposed previously by positive and negative ion TSP-MS.

The ^{1}H-NMR spectrum was then obtained and compared to the ^{1}H-NMR spectrum of **I** (Fig. 5). The ^{1}H-NMR spectrum of **I** (Fig. 5A) shows a set of signals (●) for a four-spin, AA′BB′ system of aromatic protons and another set of signals (■) for protons belonging to the *N*-isoleucyl group. A solitary signal (▲) at 8.72 ppm was assigned to the proton attached to the nitrogen atom. The ^{1}H-NMR spectrum of the unknown (Fig. 5B)

shows much similarity to the spectrum of **I**, however, there are subtle differences. One set of signals (■) indicates an *N*-isoleucyl group, which is also present in **I**. The other set of signals (●) suggest an *ortho*-substituted aromatic ring. Connectivity among ●s and connectivity among ■s was established from COSY experiments. However, differences in chemical shift values indicate a change in one or both substituents relative to those possessed by **I**. Finally, and quite significantly, the N–H (▲) signal found in the spectrum of **I** is conspicuously absent in the spectrum of the unknown.

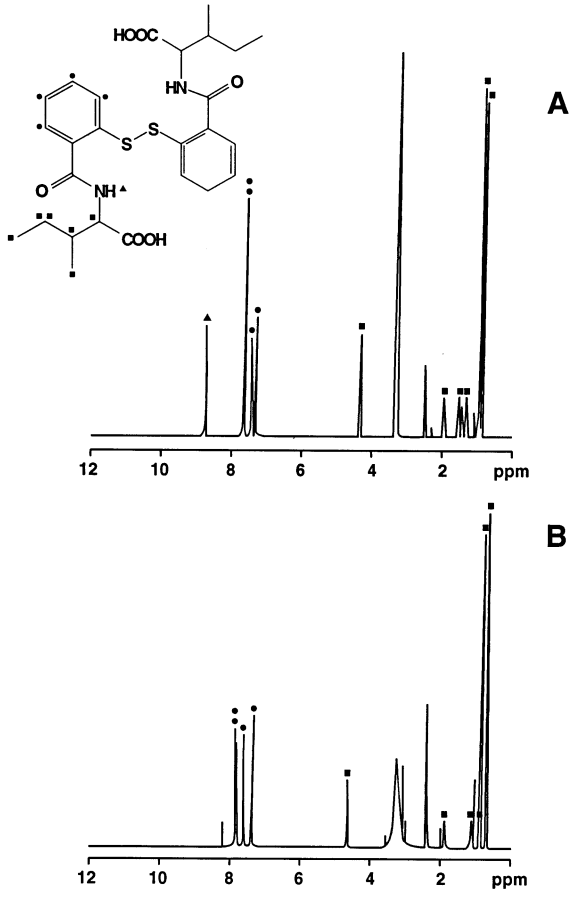


Fig. 5. The 500 MHz ^{1}H-NMR spectra of **I** (A) and of the earliest eluting peak (B) using DMSO-d_6 as solvent. Similarities between the spectra include the presence of an *ortho*-substituted aromatic ring and an isoleucyl group. Conspicuously absent from the spectrum of the unknown is the signal for N–H, which is observed in the spectrum of **I** at 8.72 ppm.

From these data, the earliest eluting chromatographic peak is proposed to be — *isoleucyl-benzisothiazolone*. Forming such a structure requires an unusual nucleophilic intramolecular displacement of the parent disulfide to occur. The scheme shown in Fig. 6 can thus be used to mechanistically describe the formation of *N*-isoleucyl-benzisothiazolone **III** from the parent disulfide **I** in aqueous solution.

A similar intramolecular transformation yields benzisothiazolone when di-thiosalicylamide is reacted with bromine [11]. In the present case, however, it was determined that accelerated formation of **III** occurs when an aqueous solution of **I** contacts a stainless steel surface, such as that employed in an HPLC system. When a solution of **I** was examined by ^{1}H NMR spectroscopy, **III** was obviously absent. However, when the same solution of **I** was purposefully drawn through a stainless steel needle, the rapid formation of **III** was clearly evident upon re-analysis. Passing a fresh solution of **I** through a titanium needle, however, does not produce a detectable level of **III**.

4. Conclusions

The propensity of 2,2′-dithiobis[*N*-isoleucylbenzamide] **I** to degrade to the sulfydryl **II** and the benzisothiazolone **III** has several direct implications upon pharmacokinetic studies. It is entirely plausible that all three species could be present in systemic circulation following intravenous administration of **I** to an animal or human subject. Thus, an analytical method permitting the specific determination of all three compounds in plasma would be most desirable for characterizing the pharmacokinetic behavior of **I**. The development of a specific assay for the quantitation of any of these compounds necessarily depends upon establishing conditions to sufficiently stabilize **I** as well as prevent its formation from the reaction between **II** and **III**. Unfortunately, a preliminary evaluation of the influence of pH on these reactions suggested that conditions affording optimal stability might be mutually exclusive. Briefly, the rate of disulfide disproportionation diminishes

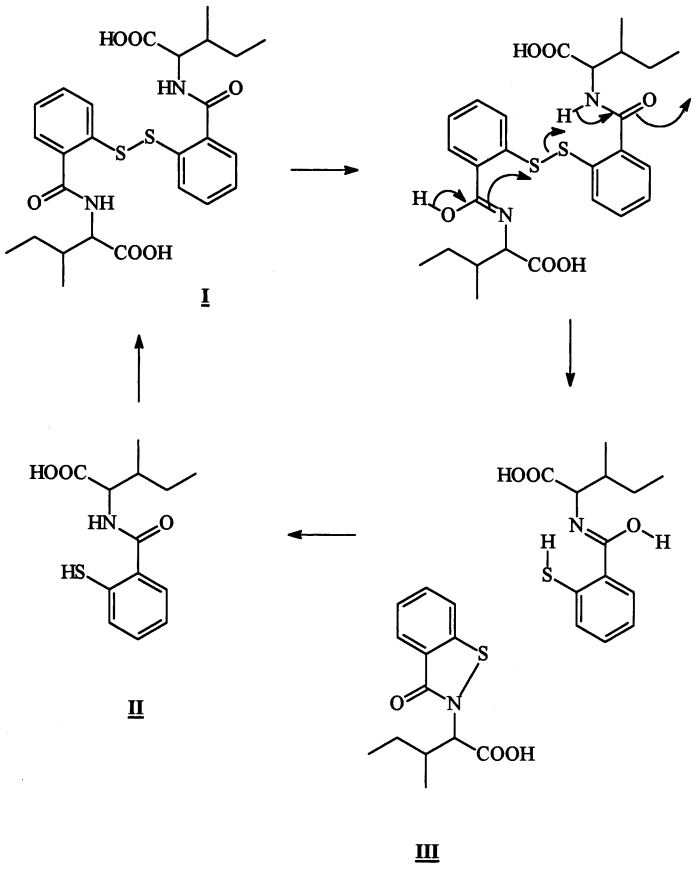


Fig. 6. Proposed route of degradation of **I** in aqueous solution, leading to formation of sulfydryl **II** and benzisothiazolone **III**.

considerably with decreasing pH, to the point where **I** is relatively stable in solutions with pH < 2.5. However, **II** and **III** react rapidly in moderately acidic solution to afford **I**. This behavior effectively precludes the ability to ascertain the actual concentrations of the individual compounds in samples that potentially contain both the sulfydryl **II** and benzisothiazolone **III** using a chromatographic procedure which requires preliminary isolation of the analytes from the sample matrix. For this reason, the approach that we have taken to assay **I** in biological fluids involves converting all drug-related species that are not covalently bound to plasma protein to a single compound, the sulfydryl **II**, prior to HPLC analysis [7]. Secondly, both the 2,2′-dithiobenzamides [12,13] and the benzisothiazolones [13] have been shown to possess the ability to induce the ejection of zinc from zinc fingers of nucleocapsid protein, NCp7, subsequently inhibiting HIV replication [14]. Other disulfide-containing compounds have also demonstrated this activity [15,16], while the corresponding sulfydryl species do not [15]. Thus, the actual composition of the dosage form requires characterization to establish whether a significant fraction of the disulfide **I** has degraded to **II** and **III**, not only prior to administration, but also if the dosing solution comes into contact with a stainless steel surface as it is being delivered.

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