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Extra-Weak Chemiluminescence of Drugs. IX.[1] Extra-Weak Chemiluminescence of Neocarzinostatin Injection

KIYOTO EDO, KUNIHITO SAITO,* MAYUKO KATO, YURIKO AKIYAMA-MURAI, HIDEAKI SATO, and MICHINAO MIZUGAKI

Department of Pharmaceutical Sciences, Tohoku University Hospital, 1-1 Seiryo-machi, Sendai 980, Japan

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The extra-weak chemiluminescence (CL) of the antitumor antibiotic neocarzinostatin (NCS) in buffer was measured in order to evaluate the stability of NCS injection. Under conditions where NCS is labile, such as heat treatment, high pH or the presence of a thiol compound, NCS generated higher extra-weak CL than under conditions where it is stable (0.1 M acetate buffer pH 4.0). On the other hand, the extra-weak CL generated from NCS in buffer decreased in the presence of disaccharides such as sucrose and maltose, which are known to stabilize NCS. This CL measurement may be used as a monitor for evaluating the stability of injections as well as solid drugs such as tablets, capsules, *etc.*, as reported previously (Mizugaki *et al.*, 1985).

Keywords——extra-weak chemiluminescence; neocarzinostatin; drug-stability; injection; antitumor antibiotic

Evaluation of drug stability is in general difficult and requires a considerable time. Recently, we have introduced an analytical method involving the measurement of the extra-weak chemiluminescence (CL) for studies on drugs.[2] Namely, we have detected strong emission of extra-weak light from comparatively unstable drugs such as imipramine hydrochloride,[3,4] kampo-extracted herbal drugs[1,5,6] and neocarzinostatin (NCS).[7]

In our previous paper, the stability of NCS powder was investigated by using this analytical method. A high emission intensity was detected from NCS in the dark at room temperature. This antibiotic[8] is composed of a protein moiety (apo-neocarzinostatin (apo-NCS), molecular weight (MW) 11000)[9] and a non-protein chromophore (neocarzinostatin chromophore (NCS-chr), MW 659)[10] in the molar ratio of $1:1$.[11-13] This high emission intensity of NCS was due to NCS-chr but not apo-NCS.[7] In addition, it is suggested that one of the emission species of NCS-chr may be a singlet oxygen.

Since our previous papers have dealt with the extra-weak CL of drugs in the solid state, we describe, in this paper, the extra-weak CL generated in solution by NCS, with easy detection of the biologically inactive product, apo-NCS, by high-performance liquid chromatography.

Materials and Methods

Chemicals——NCS and apo-NCS were gifts from Kayaku Antibiotics Research Co., Ltd. All the other chemicals were purchased from commercial sources, and were of analytical grade.

Apparatus——Quantitative detection of the extra-weak CL was performed by single photon counting at extra-weak sensitivity using an OX-70 chemiluminescence analyzer (Tohoku Electronic Industrial Co., Ltd., Sendai, Japan), which was equipped with a low noise photomultiplier (HTV R878) and a 50 mm diameter photocathode (Hamamatsu Photonic Co., Hamamatsu, Japan). The photocathode was cooled by a thermoelectric cooler to minimize noise. Samples, which were weighed at least 3 h before and protected from air in the dark at room temperature, were placed

in a stainless steel dish-type cell 50 mm in diameter and dissolved in 3 ml of various buffers or in the presence of various chemicals, and then single photoelectron pulses were counted under atmospheric conditions at various temperatures. The results are arbitrarily expressed as the mean values of observed photoelectrons for 10 s after subtracting the dark counts (about 670 counts/10 s).

Determination of Emission Spectrum——The spectrum of chemiluminescence was measured by the previously described method.[7]

Quantitative Detection of NCS by Liquid Chromatography——The Pharmacia FPLC system was employed for all the studies reported here.[14]

Residual NCS (%) was calculated from the integral of absorbance at 280 nm: % of NCS = [NCS] × 100/([NCS] + a[apo-NCS]), where [NCS] and [apo-NCS] are the integral values of NCS and apo-NCS, respectively. The relative integral value, a, of apo-NCS with respect to NCS is 2.

Results

Extra-Weak CL of NCS in Buffer

The relationship between the concentration of NCS in 0.1 M acetate buffer pH 4.0, at which the biological activity of NCS was stable,[15] and the spontaneous emission intensity generated from NCS at 50 °C was examined (Fig. 1). The CL intensity generated from NCS increased linearly in parallel with NCS concentration in the range from 0 to 32 mg/ml and was 700 counts/10 s/mg.

Temperature Dependency of the Extra-Weak CL Generated from NCS in Buffer

At high temperature, NCS was converted to apo-NCS after the release of NCS-chr, with the loss of its biological activities, and this release and degradation of NCS-chr were shown to be temperature-dependent.[15,16] The temperature-dependent degradation of NCS in 0.1 M acetate buffer (pH 4.0) from 20 °C to 80 °C was examined by monitoring of the extra-weak CL intensity (Fig. 2). The CL intensity of NCS increased linearly in a temperature-dependent manner. After the extra-weak CL of NCS in buffer had been measured, the residual NCS was determined by FPLC. As shown in Fig. 2, the degradation of NCS to apo-NCS is temperature-dependent. The above data and the previous report[7] strongly suggest that the extra-weak CL generated from NCS in buffer is responsible for the degradation of NCS, especially NCS-chr after the release of NCS-chr from the NCS complex.

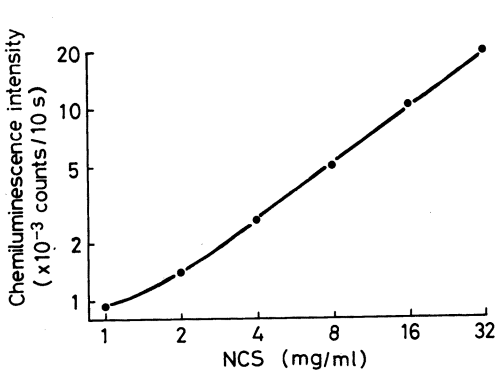

Fig. 1. Dose–Response Relation of the Extra-Weak CL of NCS in Buffer at 50 °C

The extra-weak CL of NCS in 3 ml of 0.1 M acetate buffer (pH 4.0) just after dissolution was counted in the dark at 50 °C.

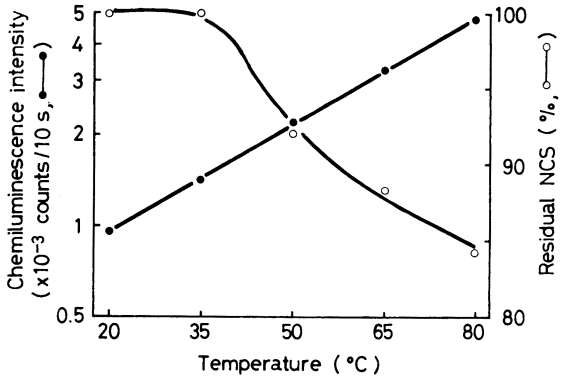

Fig. 2. Temperature Dependency of the Extra-Weak CL Generation of NCS in Buffer

The extra-weak CL of 3 ml of NCS (12 mg) in 0.1 M acetate buffer (pH 4.0) was measured at various temperatures between 20 °C and 80 °C. Residual NCS was estimated by FPLC as described in our previous report.[14]

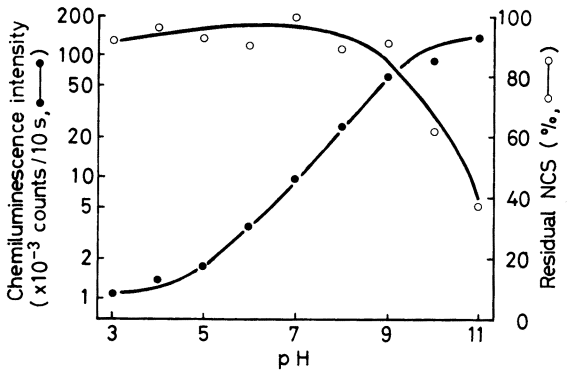

Fig. 3. Extra-Weak CL Generated from NCS in Buffer at Various pH Values at 50 °C

The extra-weak CL of 3 ml of NCS (12 mg) in the Britton–Robinson buffer at various pH values between 3 and 11 was measured at 50 °C.

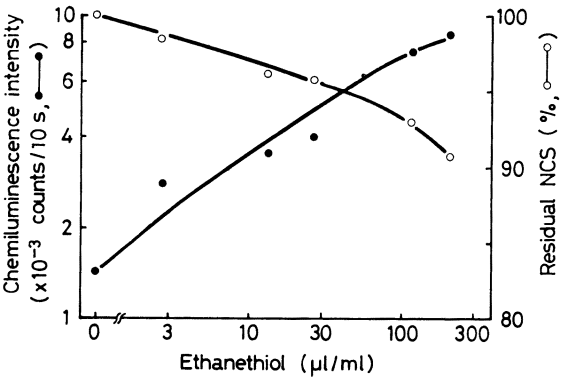

Fig. 4. Extra-Weak CL Generated from NCS in Buffer at pH 4.0 in the Presence of Ethanethiol

The extra-weak CL of NCS (12 mg/ml, 3 ml) in 0.1 M acetate buffer (pH 4.0) was measured at 50 °C in the presence of various amounts of ethanethiol.

Effect of pH on the Extra-Weak CL Generated from NCS Solution

The effect of pH on the CL generation from NCS in buffer was examined. The extra-weak CL of NCS immediately after dissolving NCS (12 mg) in the buffer at various pH values (3 ml) was measured at 50 °C. As shown in Fig. 3, the extra-weak CL of NCS in buffer was about 1000 counts/10 s at pH 3. This extra-weak CL increased in proportion to increase of the pH, and at pH 11 reached 100000 counts/10 s. After the measurements of extra-weak CL of NCS in various pH buffers, the residual NCS was estimated to evaluate the correlation between the extra-weak CL intensity and the residual NCS. NCS released its chromophore above pH 8, and the residual NCS amounted to 37% at pH 11.

Effect of Ethanethiol on the CL Generation from NCS in Buffer

It is known that NCS easily loses its biological activity in the presence of thiol compounds.[17] Therefore, the effect of ethanethiol on the extra-weak CL generation from NCS in buffer was tested (Fig. 4). The extra-weak CL generated from NCS (4 mg/ml, 3 ml) in 0.1 M acetate buffer (pH 4.0) increased in parallel with the amount of added ethanethiol. On the other hand, the residual NCS decreased with increasing ethanethiol added to the NCS solution just after measuring the CL intensity of NCS in the buffer.

Effect of Disaccharide on Chemiluminescence Generated from NCS in Buffer

It was reported that NCS was stabilized by disaccharides such as sucrose, maltose, *etc.*[18] The effects of these disaccharides on the extra-weak CL generation from NCS in buffer are shown in Table I. The extra-weak CL of NCS in 0.1 M acetate buffer (pH 4.0) at 50 °C decreased from 1153 to 356 and 785 counts/10 s, after the addition of sucrose and maltose, respectively.

TABLE I. Effect of Disaccharide on Extra-Weak Chemiluminescence Generated from NCS in Buffer

Disaccharide	Chemiluminescence intensity[a]
None	1152
Sucrose (500 mg)	356
Maltose (500 mg)	785

a) The extra-weak CL intensities generated from NCS in 0.1 M acetate buffer (pH 4.0, 12.5 mg/2 ml) were measured in the presence of disaccharide at 50 °C.

Spectral Analysis of Extra-Weak Chemiluminescence Generated from NCS in Buffer

To elucidate the active species which causes the light emission from NCS in buffer, spectral analysis of the extra-weak CL generated from NCS (32 mg) in 0.1 M acetate buffer (pH 4.0, 3 ml) was performed with a filter spectral analyzer as described previously.[7] The emission intensity at 460, 570, and 590 nm was high as compared to other ranges (data not shown). This emission spectrum of CL from NCS in buffer resembles that of NCS powder.[7]

Discussion

There is no convenient means to evaluate drug stability. We applied the CL technique to the evaluation of the stability of drugs in tablets and capsules as a first screening system.[2] Mizuno *et al.* reported that comparatively unstable drugs such as adenosine triphosphate (ATP), ascorbic acid, *etc.* generated extra-weak CL in buffer solution.[19] Now, we wish to evaluate the scope and limitations of this analytical method for evaluating drug stability in solution.

NCS, which is used as an anticancer injection in Japan, generated comparatively high extra-weak CL.[7] The CL generated from NCS has been measured to clarify the relationship between the stability of the injection and the extra-weak CL intensity, since the relationship between the biological activities and stability of NCS is well understood.[15,16]

The extra-weak CL generated from NCS in 0.1 M acetate buffer (pH 4.0) at 50 °C increased in a dose–response fashion (Fig. 1) and showed temperature dependency between 20 and 80 °C (Fig. 2). These data are consistent with the fact that in general drugs are labile to heat. It has been reported that the NCS injection was unstable at high temperature because in buffer the NCS-chr was easily released from NCS.[20,21] In fact, Fig. 2 shows that the residual NCS monitored by FPLC decreased after heat treatment. In addition, under conditions where the drug is unstable (pH more than 8.0), high CL intensity was observed (Fig. 3). These observations are in agreement with the view that high extra-weak CL is observed from aqueous solutions of unstable drugs. This hypothesis is also supported by the result that the extra-weak CL generated from NCS in buffer decreased in the presence of disaccharides, sucrose and maltose, which are stabilizers of NCS (Table I).

On the other hand, it was reported that sulfhydryl compounds cause the release of NCS-chr.[17] A low concentration of ethanethiol enhanced the extra-weak CL generated from NCS but a high concentration of ethanethiol suppressed the enhancement of generation of the extra-weak CL from NCS in buffer. The two disulfide bonds of apo-NCS were presumably reduced by ethanethiol and the free NCS-chr concentration increased to generate the high extra-weak CL from NCS at a low concentration of ethanethiol. On the other hand ethanethiol at a high concentration may trap the oxygen dissolved in the buffer or react with NCS-chr to form CL-insensitive derivatives.

The spectral distribution of CL generated from NCS in buffer resembled that of NCS

powder. Autoxidation by active oxygen seems to be responsible for the generation of extra-weak CL from NCS in buffer as well as CL from NCS powder.[7,22]

The above experimental results indicated that NCS generated more extra-weak CL under conditions where it was labile than under conditions where it was stable. Therefore, the best conditions for preparation of stable injections may be evaluated by measuring the extra-weak CL of injections.

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