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New Method to Measure the Carbamoylating Activity of Nitrosoureas by Electron Paramagnetic Resonance Spectroscopy

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A new method for measuring the carbamoylating activity of nitrosoureas and isocyanates using electron paramagnetic resonance (EPR) spectroscopy is described. The extent and time course of carbamoylation reaction of chloroethyl isocyanate and a series of 9 nitrosoureas toward amino group of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl were examined with both the EPR method and the HPLC method which has been proposed by Brubaker *et al.* [*Biochem. Pharmacol.* **35**:2359 (1986)]. Spin-labeled nitrosoureas we synthesized are included in this study since they have less toxicity or more efficiency than commercially available drug in some cases. The concentration of carbamoylated product was easily determined with the EPR spectra. There is a very high correlation ($r = 0.982$, $t = 2.58$, $N = 10$, $p < 0.001$) between the EPR and HPLC methods. Spin-labeled nitrosoureas showed lower carbamoylating activity than non-labeled analogues. The carbamoylating activity for these nitrosourea depended on the reactivity of isocyanate intermediate and almost independent of their half life. This rapid and simple EPR method is suitable for the detailed investigation of the rate and extent of carbamoylation reaction.

Keywords: Anticancer drug, nitroxide, EPR, nitrosourea, carbamoylation, carbamoylating activity

Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl-1-nitrosourea; A-TEMPO, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl; HPLC, high performance liquid chromatography; CENU, 1-ethyl-3-cyclohexyl-1-nitrosourea; CPNU, 1-isopropyl-3-cyclohexyl-1-nitrosourea; CBNU, 1-butyl-3-cyclohexyl-1-nitrosourea; SLCNU; 1-(2-chloroethyl)-3-[3-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea; SLMNU, 1-methyl-3-[3-(2,2,6,6-tetramethypiperidine-1-oxyl)]-1-nitrosourea; SLENU, 1-ethyl-3-[3-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea; SLPNU, 1-isopropyl-3-[3-(2,2,6,6- tetramethylpiperidine-1-oxyl)]-1-nitrosourea; SLBNU, 1-butyl-3-[3-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea; CEI, chloroethylisocyanate; 5′-AdThd, 5′-amino-5′-deoxythymidine

INTRODUCTION

2-Chloroethyl-1-nitrosoureas are highly active anticancer agents with a broad anti-tumor spectrum in experimental systems. Some of them, CCNU, BCNU, BCNU, and MeCCNU, have been applied for the treatment of human cancers, such

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as lymphomas, melanomas, a variety of hematological malignancies and some solid tumors, especially Hodgkin's disease and brain tumors.[1] New nitrosoureas, such as fotemustine,[2] tauromustine,[3] and perrimustine,[4] are under clinical trials. The clinical application of nitrosoureas, however, is still limited because of their delayed and cumulative toxic effects.[5] In the search for more active and less toxic analogues, various carrier molecules such as carbohydrates,[6] amino acids,[7] steroids-amino acid conjugates,[8] and nitroxide radicals[9] have been utilized for synthesizing new nitrosourea derivatives. However, the mechanisms of the anticancer action and toxicity of these drugs under physiological conditions are not fully understood.

It has been proposed that chloroethyl nitrosoureas spontaneously decompose to give alkylating (II) and carbamoylating (III) moieties (Fig 1A).[10] The compound II causes electrophilic alkylation and/or interstrand crosslink of DNA and the compound III carbamoylates amino and thiol groups in biological systems. These reactions are responsible for therapeutic efficacy and/or the toxic side effects.[10,11,12] Thus, a tremendous effort to determine alkylating and carbamoylating activities of these drugs has been made to elucidate the mechanisms of anticancer action and toxicity.[13] The standard method to assay carbamoylating activity has developed by Wheeler et al in 1974 using radiolabeled lysine.[13] However, since this method has a number of disadvantages, the HPLC method was later utilized by Brubaker *et al.*[14] The HPLC method has significant advantages in rapidity and simplicity over the radioisotope assay (Fig. 1B), but still carries some complexity in the assay process.

We describe here an EPR method which is more rapid, safe, and convenient. The method is based upon the carbamoylation of nitroxide (VI) to yield the corresponding spin-labeled urea (VII) (Fig. 1C). Instead of radioisotope, the stable nitroxide radical, 4-amino-2,2,5,5-tetramethylpiperidine-1-oxyl (A-TEMPO), was used and the concentration of the nitroxide radical was determined by the EPR technique which is much more sensitive than the spectrophotometric method. We measured the carbamoylating activity of CCNU and its analogues which have a different alkylating moiety and/or a spin-labeled carbamoylating moiety. Some of spin labeled nitrosoureas which we synthesized and tested have more antitumor activity and/or less toxic[15] than clinically used drugs such as CCNU. The detailed time course of the carbamoylation of these compounds was also determined with this method.

MATERIALS AND METHODS

CCNU was kindly gifted by Bristol-Myer Squibb Co. (Wallingford, CT, USA). Chloroethyl isocyanate (CEI) and A-TEMPO were purchased from Aldrich Chemical Co. (Tokyo, Japan). SLCNU, SLMNU, SLENU, SLPNU, SLBNU, CENU, CPNU, and CBNU were synthesized according to the procedures of Raikov *et al.*[9] and Gadzheva *et al.*[16,17] 5'-AdThd was purchased from the Sigma Chemical Co. (Tokyo, Japan).

Halflife Measurement of Nitrosoureas

The halflife ($\tau_{1/2}$) of nitrosoureas was determined through the Wheeler's spectrophotometric method.[13] Briefly, the solution of nitrosoureas (0.26–0.27 mM) in 0.9 ml acetone, 0.9 ml of 0.1 M phosphate buffer pH 7.2, and 35 ml of distilled water were incubated at 37°C. The decrease in absorbance was monitored at wavelength of about 230 nm corresponding to the absorbance maximum of each compound. Measurements were carried out on an LKB spectrophotometer (Sweden).

Chromatographic Procedures

Preparative layer chromatography (PLC) analysis was performed on silica gel 60 F-254 PLC plates (2 mm in thickness). HPLC analysis was performed on a Gilson chromatographic system using Whatman Partisil ODS-2 column (4.6 ×

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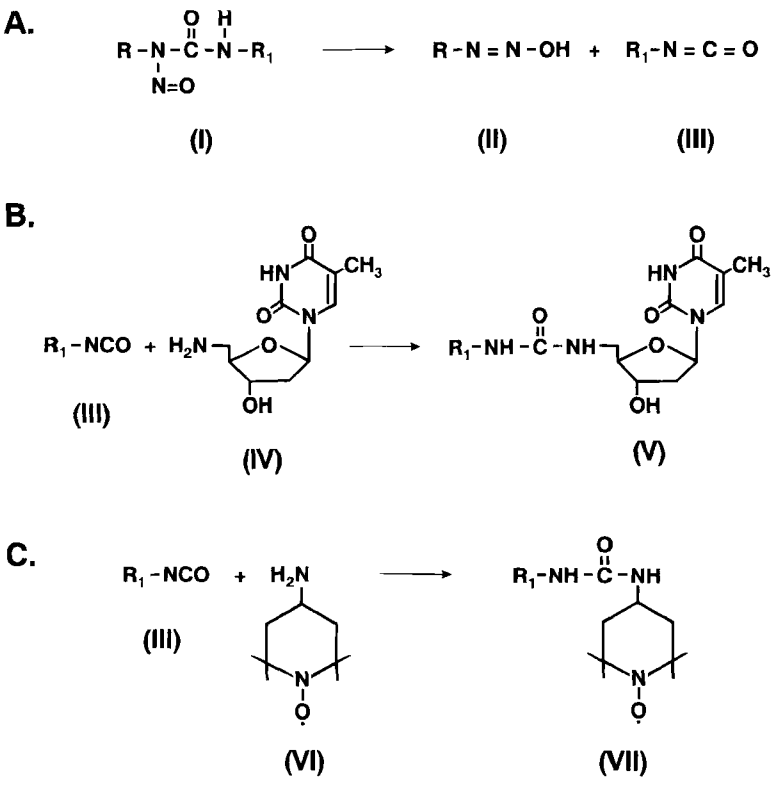

FIGURE 1 (**A**) Decomposition of alkylnitrosoureas (I) to yield an alkyldiazohydroxide (II) and isocyanate (III); (**B**) Principle of Brubaker's method to determine carbamoylating activity of nitrosoureas and isocyanate using the carbamoylation on the amino group of 5'-AdThd (IV); (**C**) Determination of carbamoylating activity of nitrosoureas and isocyanate using the carbamoylation on the amino group of A-TEMPO (VI). Resultant spin-labeled compound (VII) is assayed with EPR. Nitrosoureas (I) used in this study are listed in Table 1 with the substituents, R and R_1.

150 mm, 5 μm). Mobile phase A was 95% 10mM KH_2PO_4 (pH 3.0) / 5% methanol and mobile phase B was 55% 10 mM KH_2PO_4 (pH 3.0)/45% methanol. A linear gradient of O–100% B was used for 10 min at a flow rate of 1.00 ml/min at ambient temperature.

EPR Measurements

EPR measurements were made at room temperature (23 ± 1)°C on a JEOL JES-FE2XG spectrometer (Tokyo, Japan) under the following conditions: microwave frequency, 9.42 GHz; magnetic field, 334.5 ± 5 mT; time constant, 0.1 sec; microwave power, 8 mW; field modulation width, 0.125 mT; amplitude, 5 × 100; sweep time, 2 min / 10 mT. EPR parameters such as a nitrogen hyperfine coupling constant (a_N), a g-value (g), and an exchange energy (J) were determined with the signal of external Mn^{2+} standard. In this microwave frequency, the apparent g-values of third and fourth Mn^{2+} signals are 2.033 and 1.981, respectively. These signals are presented in Fig. 2. EPR spectra were acquired through A/D converter board and processed with a personal computer, on which signal intensity was calculated and signal area was obtained by duplicated integration of the corresponding signal.

Measurement of Carbamoylating Activity

A-TEMPO (50 μmol) was dissolved in 500 μl of ethanol and 4 ml of 0.1 M PBS (pH 7.4) for a stock solution. To 450 μl of this stock solution was

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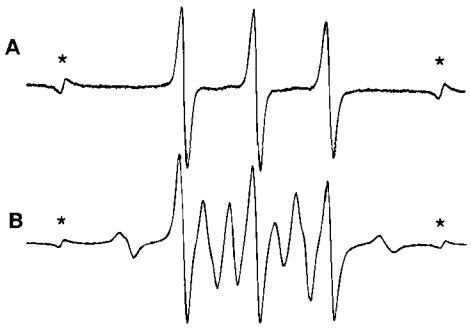

FIGURE 2 EPR spectra of the carbamoylated products. (**A**) Mononitroxide radical generated after incubation of CENU with A-TEMPO at 37°C for 6 hr. (**B**) Binitroxide radical generated after incubation of SLCNU with A-TEMPO at 37°C for 6 hr. The signals pointed with * correspond to the third and fourth signals of an external instrumental Mn^{2+} standard.

added 5 μmol of the corresponding nitrosourea dissolved in 50 μl of ethanol, giving a final mixture of A-TEMPO (10 mM) and nitrosourea (10 mM). For chloroethyl isocyanate (CEI), dioxane was used for dissolving medium instead of ethanol. The reaction mixtures were incubated at 37°C in a water bath and aliquots were withdrawn for analysis each hour for 6 hours. The chromatographic procedure was performed as follows: Ten μl of each mixture was spotted on PLC plates by microdispencer and the chromatograms were developed with chloroform/methanol (9:1, v/v). After drying, the chromatograms were visualized on UV light at 254 nm. The smears corresponding to a nitroxide radical were scraped off and extracted with 1 ml of methanol/1N NaOH (5:1, v/v). Nitroxides present at different position from A-TEMPO or corresponding nitrosourea were attributed to the products of carbamoylation. Then each extract was sucked into a flat quartz cell (180 μl in volume, JEOL, Tokyo, Japan) for EPR measurement. The standard curve for the assay of A-TEMPO was made using the signal height or area of A-TEMPO with a known concentration, relative to the signal of Mn^{2+} in MgO used as an internal EPR standard.

Carbamoylating activity was determined by the following equation and expressed as a percent carbamoylation of A-TEMPO:

$$\frac{[A-TEMPO]_0}{[Y]_0} - \frac{[A-TEMPO]_T}{[Y]_0} \times 100 \quad (1)$$

where $[A\text{-}TEMPO]_0$, $[A\text{-}TEMPO]_T$, and $[Y]_0$ are the concentrations of A-TEMPO at time 0, at time T, and the concentration of compound Y at time 0, respectively. To verify this method, carbamoylating activity was also measured with the method of Brubaker et al described elsewhere.[14] Briefly, a stock solution of 5′-AdThd was prepared by dissolving 5 μmol of 5′-AdThd in 450 μl of PBS. To 450 μl of the stock solution was added 5 μmol of the compound tested in 50 μl of vehicle. The vehicle used for each compound was the same as that used in EPR method. The reaction mixture was incubated at 37°C in water bath for 6 hour and 10 μl of each mixture was injected for HPLC analysis. The standard curve of 5′-AdThd assay was constructed by triplicate injections at three concentrations in the range of interest. Carbamoylating activity was calculated with the following equation and expressed as a percent carbamoylation of 5′-AdThd:

$$\frac{[5'-AdThd]_0}{[Y]_0} - \frac{[5'-AdThd]_6}{[Y]_0} \times 100 \quad (2)$$

where $[5'\text{-}AdThd]_0$, $[5'\text{-}AdThd]_6$, and $[Y]_0$ are the concentrations of 5′-AdThd at time 0, at 6 hours incubation, and the concentration of compound Y at time 0, respectively.

Statistical Analysis

Statistical difference between carbamoylating activities were evaluated using a single factor ANOVA with the Newman-Keuls multiple comparison test.[18]

RESULTS AND DISCUSSION

It is shown that nitroxide radical itself is not mutagenic nor carcinogenic and relatively nontoxic.[19] Many nitroxide radicals readily pass cell membranes and can even cross the blood brain barrier

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at the site of diseased tissues.[20] Furthermore, a nitroxide radical moiety in the spin labeled compounds imparts a beneficial influence on the antineoplastic activity of a drug.[15] On the basis of these data, we have been synthesizing spin labeled analogues of CCNU and examining the activity and toxicity of these analogues.[9,16,17,21] Then, it was found that some are less toxic than CCNU which has been used in clinical cases.[17] It was suggested that the anti-oxidant action of nitroxide may be responsible for the beneficial effects of spin labeled compounds.[21] Furthermore, spin labeled amino acid nitrosourea derivatives were remarkably active against the experimental tumor model.[22] In spite of these promising features of spin labeled nitrosoureas, their carbamoylating activity has not been fully evaluated. Thus, we compared the carbamoylating activity of spin-labeled nitrosoureas with that of non-labeled analogues for clarifying the action of spin labeled nitrosoureas. The list of 9 nitrosoureas and CEI which we examined is shown in Table I. SLCNU is a spin-labeled chloroethyl analogue of CCNU containing the stable nitroxide TEMPO instead of the cyclohexyl analogue of CCNU containing the stable nitroxide TEMPO instead of the cyclohexyl group. SLMNU, SLENU, SLPNU, SLBNU, CENU, CPNU and CBNU are either spin labeled or non labeled analogues of CCNU that contain alkyl residues instead of the chlorethyl residue.

EPR and HPLC Determination of the Carbamoylating Activity

In the PLC analysis of the incubation medium, we obtained three spots on each chromatogram, which corresponded to unreacted A-TEMPO, unreacted nitrosourea, and carbamoylated product. We performed EPR measurement for all spots visualized on PLC plates and the following EPR spectra were obtained (Fig. 2). The EPR spectra of A-TEMPO and all spin labeled nitrosoureas had symmetric three lines attributable to mononitroxide radicals. The products of carbamoylation of CEI, CCNU, CENU, CPNU and CBNU gave symmetric three lines in their EPR spectra which are compatible with mononitroxide radicals (Fig. 2A). The a_N and g of these species were 1.67 mT and 2.006, respectively, which are reasonable values for A-TEMPO moiety in methanol-1N NaOH mixture (5:1, v/v). The products of carbamoylation of SLCNU, SLMNU, SLENU, SLPNU and SLBNU yielded their EPR spectra of nine lines (Fig. 2B), since their carbamoylating moiety with nitroxide radical reacted with A-TEMPO and a binitroxide radical, N,N'-bis[4-(1-oxyl-2,2,6,6-tetramethylpiperidyl)]urea, was generated. Biradical has two

TABLE I Carbamoylating activity of nitrosoureas and chloroethyl isocyanate

Compound	R[a]	R_1[a]	$\tau_{1/2}$[b] (min)	Carbamoylating Activity (%)[c] EPR method[d]	HPLC method[e]
CEI[f]	———	———	—	81.66 ± 0.37	85.98 ± 0.35
CCNU	$ClCH_2CH_2$-	cyclohexyl	54	63.17 ± 1.99	70.61 ± 0.66
CENU	CH_3CH_2-	cyclohexyl	192	70.61 ± 2.54	79.18 ± 0.49
CPNU	$(CH_3)_2CH$-	cyclohexyl	185	66.16 ± 0.14	75.84 ± 0.18
CBNU	$CH_3(CH_2)_3$-	cyclohexyl	270	72.62 ± 1.31	81.52 ± 0.69
SLCNU	$ClCH_2CH_2$-	TEMPO[g]	31	45.71 ± 2.76	45.92 ± 0.36
SLMNU	CH_3-	TEMPO	79	55.57 ± 0.43	57.14 ± 4.59
SLENU	CH_3CH_2-	TEMPO	75	54.46 ± 0.17	56.00 ± 0.02
SLPNU	$(CH_3)_2CH$-	TEMPO	18	55.12 ± 2.37	56.08 ± 0.09
SLBNU	$CH_3(CH_2)_3$-	TEMPO	83	50.84 ± 0.02	52.88 ± 0.23

a) Substituents of R and R_1 in corresponding nitrosourea shown in Fig. 1A (I). b) Half-life of nitrosourea in 0.1 M PBS, pH 7.4
c) Expressed as the mean ± S.E. (n = 3). d) Expressed as a percentage of reacted A-TEMPO for 6 hours. e) Expressed as a percentage of reacted 5—AdThd for 6 hours. f) chloroethyl isocyanate g) 2,2,6,6-tetramethylpiperidine-1-oxyl

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spins in a molecule and intramolecular spin-spin exchange interaction gives additional EPR signals. The a_N, g and spin-spin exchange energy (J) of this biradical were estimated as 1.67 mT, 2.006, and 1.79 mT, respectively. The a_N and J values of this biradical are largely dependent on solvent used[23] and the estimated values are in the reasonable range for our methanol-water mixture. The alkylation of A-TEMPO did not occur because there was no additional spots which should yield a triplet EPR signal responsible for the parent compounds or carbamoylating products. Thus, it is indicated that alkylating activity of the nitrosoureas does not interfere with the assay of carbamoylating activity. In the case of non-labeled nitrosoureas, we measured the concentrations of carbamoylated product [P], initial A-TEMPO $[A\text{-}TEMPO]_0$ and unreacted A-TEMPO $[A\text{-}TEMPO]_T$ using the signal area of the triplet EPR spectrum. In the case of spin labeled nitrosoureas which yield binitroxide as carbamoylated products, [P] cannot be directly evaluated using a mononitroxide (A-TEMPO) concentration standard. Since it was proved in non-labeled nitrosoureas that [P] was equal to the concentration of reacted A-TEMPO which can be calculated by $[A\text{-}TEMPO]_0$ $[A\text{-}TEMPO]_T$, [P] for all nitrosoureas was obtained as this concentration difference and then a percent carbamoylation was calculated as Eq. (1).

The HPLC chromatogram of the medium incubated for or 6 hour is shown in Fig. 3. The concentration of 5'-AdThD could be easily determined with a well-defined peak as described elsewhere.[14] The carbamoylating activity was estimated with the decrease in this peak as described before. The data obtained with this HPLC method using 5'-AdThd should be cautiously evaluated since 5'-AdThd can be alkylated by these nitrosoureas, which may interfere with the measurement of carbamoylated products.

The Carbamoylating Activities of Nitrosoureas

The carbamoylating activity of nitrosoureas measured with both EPR method and HPLC method under identical conditions are listed in Table I. Chloroethyl isocyanate was included as an indicator of the maximum value for carbamoylation obtainable in each method. Clearly, the order of these compounds in carbamoylating activity was identical in both methods. However, values obtained by the EPR method were slightly lower than the values of the HPLC method, indicating that the reactivity of A-TEMPO toward isocyanate residues may be lower than that of 5'-AdThd. The values obtained with these two methods have an exact linear correlation, the regression equation of which is $Y = 10.00 + 0.78X$, where X and Y are percent carbamoylating activities by the HPLC and EPR methods, respectively ($r = 0.982$, $N = 10$, $P < 0.001$, $t = 2.58$). The results of the statistical analysis on these carbamoylating activities measured with the EPR method show that the order of carbamoylating activity is SLCNU < SLBNU, SLENU, SLPNU, SLMNU < CCNU, CPNU, CENU, CBNU < CEI. The probabilities associated with inequality are $p < 0.05$, $p < 0.005$, and $p < 0.001$, respectively. Although there was no significant difference between the carbamoylating activity of CCNU and CPNU, CCNU had significantly lower activity than CENU and CBNU ($p < 0.025$ and $p < 0.005$, respectively). CPNU also had significantly lower activity than CBNU ($p < 0.05$). The data measured with the HPLC method gave the same statistical results. Carbamoylating activity of the nitrosoureas strongly depends on the chemical structures of the isocyanates produced in their decomposition process and was independent of CEI. The carbamoylating activity of all spin-labeled nitrosoureas SLCNU, SLENU, SLPNU and SLBNU is lower than their non-labeled corresponding analogues CCNU, CENU, CPNU and CBNU. The carbamoylating activity of CENU, CPNU and CBNU was similar to that of their chloroethyl analogue CCNU since the same carbamoylating moiety, that is, cyclohexyl isocyanate is produced in all of their decomposition process. Just as the non-labeled analogue, there was no significant difference in the carbamoylating activities of four spin labeled nitrosoureas (SLMNU, SLENU, SLPNU, and SLBNU) because

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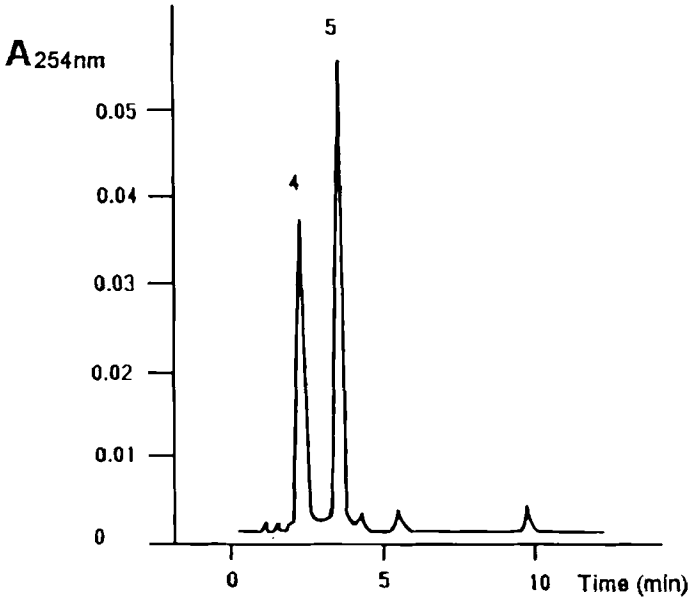

FIGURE 3 HPLC chromatogram for the assay of carbamoylating activity using Brubaker's method.[13] After incubation of SLCNU with the assay reagent 5'-AdThd at 37°C for 6 hr, an aliquot was injected. The extent of carbamoylation was measured by the decrease in the 5'-AdThd peak (4) from its initial concentration. Peak (5) is due to the major carbamoylation product.

the same spin labeled isocyanate is responsible for the reaction of carbamoylation. The significant lower activity of SLCNU than other spin labeled nitrosourea and of CCNU than CENU or CBNU indicate that large alteration of substituent R causes a secondary effect on the carbamoylating activity of these compounds although the main factor to determine the carbamoylating activity is the nature of isocyanate produced.

Although both of carbamoylating activity and alkylating activity seem to be involved in the anti-cancer activity of these compounds, it has been reported that the anti-cancer activity is mainly responsible for their alkylating activity and the contribution of carbamoylating activity is small.[13] The carbamoylating activity is rather related to the toxicity of these compounds. Thus, the compounds with high alkylating activity and low carbamoylating activity is suggested to have a high therapeutic index.

The present results supported above discussion. The carbamoylating activity of SLCNU is lower than that of CCNU, whereas the anticancer activity of SLCNU is three times higher than that of CCNU.[20] On the other hand, SLCNU has 7 times lower toxicity than CCNU.[10] These data indicate that the carbamoylating activity is correlated to the toxicity and is not the main cause of anticancer activity. The higher anticancer activity of SLCNU has been attributed to its lower or appropriate lipophilicity.[20]

Time Course of Carbamoylation and Half Life

The half life of the nitrosoureas was also determined with the spectrophotometric method[13] and the results are listed in Table I. We followed the time course of carbamoylation for each compound studied (Fig. 4). These data clearly show the detailed relationship between the carbamoylating activity and the time course of decomposition for each nitrosourea. For CEI, the reaction was completed within a few minutes (Fig. 4A) since CEI and the amino group of A-TEMPO can react directly with each other and its extent of carbamoylation is independent of decomposition process involved in nitrosoureas. CEI provides the upper limit of carbamoylation for each

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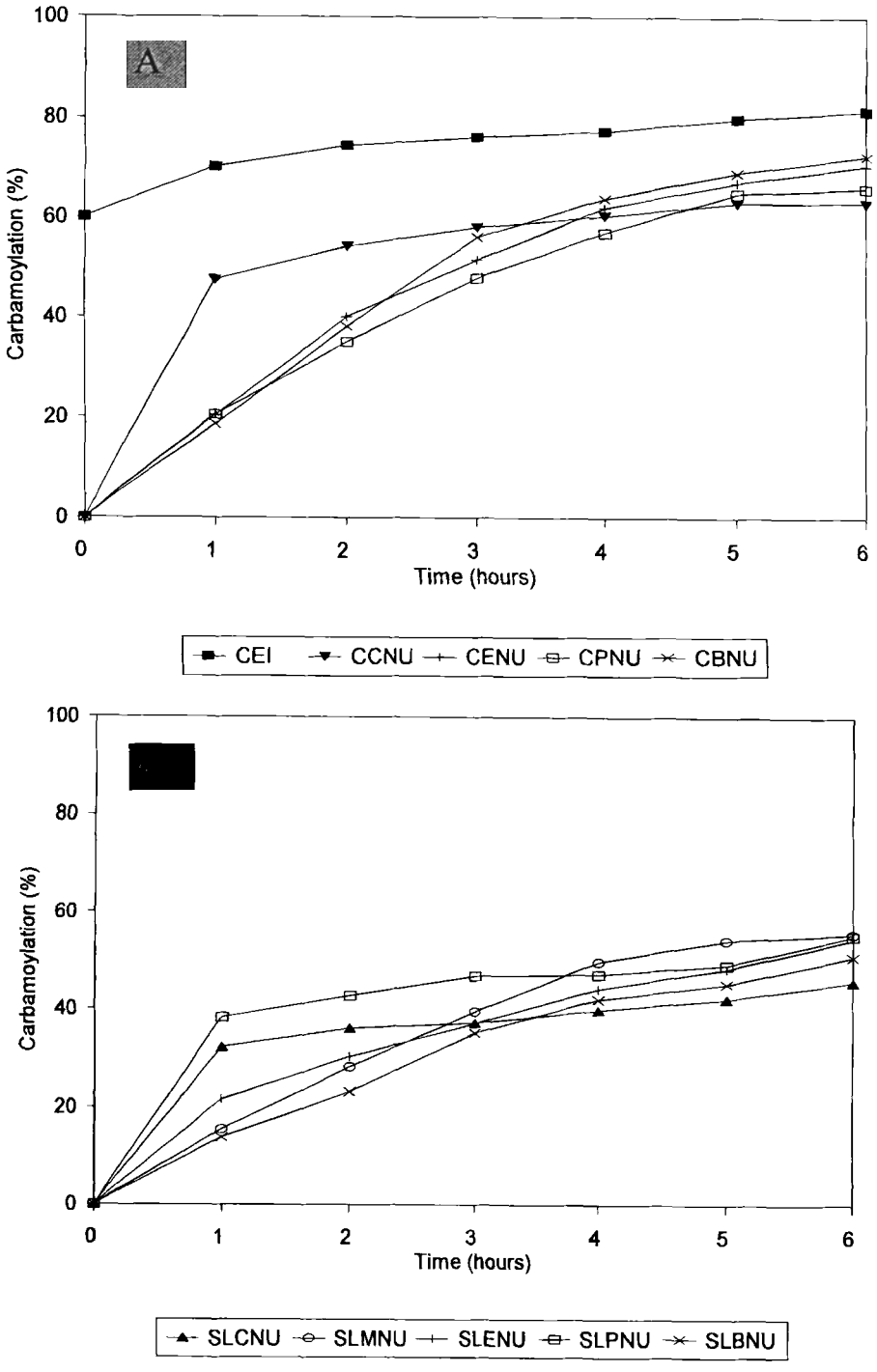

FIGURE 4 Time course for the carbamoylation of A-TEMPO by CEI, CCNU, CENU, CPNU and CBNU, (**A**). Time course for the carbamoylation of A-TEMPO by SLCNU, SLMNU, SLENU, SLPNU and SLBNU, (**B**). The compounds were incubated at 37°C with A-TEMPO in PBS (pH 7.4). Aliquots were taken every hour, analyzed by EPR method and the extent of carbamoylation determined by the decrease in the concentration of A-TEMPO. Values represent the mean of three experiments. The S.E. s associated with the data of this figure are within 6% of the presented values.

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nitrosourea in whole time course. The fact that CEI cannot completely carbamoylate A-TEMPO suggests that the isocyanate itself may decompose through other reaction such as hydration competitively to the carbamoylation. The non-labeled nitrosoureas shown in Fig. 4A demonstrated that the time course of carbamoylation is closely dependent on their half life. The carbamoylation reaction of CCNU, the half life of which was determined as 54 min, was completed within 2 hour, while that of CENU, CPNU and CBNU, which has much longer half life, continued up to 4 hours. The difference in their half life is attributable to the difference in their alkylating moiety R: CENU, CPNU, and CBNU are not chlorinated as CCNU. Since the intermediate generated from chlorinated R has been assumed to be vinyl carbonium ion,[13] which is much more stable than alkyl carbonium ion generated from non-chlorinated R, this intermediate difference may affect the half life.

However, the carbamoylating activity of these nitrosoureas approached that of CCNU after 4 hr incubation (Fig. 4A) independent of their halflife, indicating that cyclohexyl isocyanate generated in the all decomposition process carbamoylates A-TEMPO in the same extent. The plots of the time course of carbamoylation reaction by spin-labeled nitrosoureas demonstrated the similar relationship between their halflife and the initial rate of carbamoylation reaction (Fig. 4B). The extent of carbamoylation by SLMNU, SLENU, SLPNU and SLBNU strongly depended on their halflife, a rate of decomposition of parent drug. After 4 hour incubation, the extent of carbamoylation of these compounds converged on the same value. Consequently, the final carbamoylating activity is basically independent of their halflife variation caused by their alkylating moiety and dependent on the nature of generated isocyanate.

The halflives in Table I also show their strong dependence on the substituent R_1. It is suggested that the decomposition of nitrosourea is initiated by base-catalyzed proton abstraction on the nitrogen adjacent to R_1,[14] which is rationalized by the fact that increased basicity of the solvent accelerates the decomposition.[24] The proton abstraction leaves a negative charge on the nitrogen of the intermediate. The electron-donating nature of substituent R_1 destabilizes the negative charge and so decreases the rate of decomposition. Since TEMPO is less electron-donating than cyclohexyl group, the halflives of spin labeled nitrosourea may be shorter than that of non-labeled analogues.

Wheeler *et al.*[13] also established the linear correlation between the carbamoylating activity and the halflife of nitrosourea for the nitrosoureas with different R_1 and fixed R, indicating that the change in halflife and the carbamoylating activity originates from the same characteristics of R_1. The carbamoylating activity is directly correlated to the reactivity of isocyanate produced (III in Fig. 1). Isocyanate can react with methanol or water which we used as a solvent more slowly than with an amino group. Thus, the carbamoylating activity reflects the relative efficacy of the reaction with solvent and A-TEMPO. The electron-donating nature of R_1 may decrease positive charge on the carbon of isocyanate and make it more inert against nucleophilic attack on the carbon atom, which makes it stable in our solvent. Stabilization of isocyanate gives more chance for isocyanate to react with A-TEMPO, which increases carbamoylating activity.

The new assay method for carbamoylating activity presented in this paper has been shown to have advantages in promptness and simplicity with keeping the identical ability in sensitivity with that of the wide spread methods of Wheeler *et al.* and Brubaker *et al.* Determination can be made within several minutes in the laboratory where an EPR machine is equipped. No radio-isotope is required. It also proved that this EPR method is useful for detailed investigations of the rate as well as the extent of carbamoylation.

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