In Vivo Kinetic Analysis of Covalent Binding between N-Acetyl-L-Cysteine and Plasma Protein through the Formation of Mixed Disulfide in Rats

Daisuke Harada,[1,3] Shinsaku Naito,[1] Isao Hiraoka,[1] and Masaki Otagiri[2]

Received October 4, 2001; accepted January 29, 2002

Purpose. This investigation was undertaken to study the relationship between plasma drug clearance and covalent protein-binding kinetics of N-acetyl-L-cysteine (NAC).

Methods. NAC was intravenously administered to rats via a bolus injection or continuous infusion. Plasma concentrations of protein-unbound and total NAC were analyzed using a compartment model, taking into consideration of the protein binding process, and the apparent first-order binding and dissociation rate constants (k_{on} and k_{off}) were obtained.

Results. Plasma total NAC after a bolus injection showed biphasic elimination with an inflection point at 1 hr. After 1 hr, NAC was largely present in the covalent protein-bound form. During the steady state of the infusion, approximately 30%–40% of plasma NAC bound with protein covalently. The k_{on}, k_{off}, and the elimination rate constant of protein-unbound drug (k_e) were 0.23, 0.57, and 4.3 hr^{-1}. The dissociation half-life of NAC from protein estimated from k_{off} was in agreement with the elimination half-life of plasma total NAC. This suggests that the dissociation of NAC from protein rate-limited the drug elimination in plasma ($k_{off} < k_e$).

Conclusion. We demonstrated that plasma total drug clearance is kinetically limited by covalent protein binding. The compartmental model described here is useful for analyzing its kinetics *in vivo*.

KEY WORDS: N-acetyl-L-cysteine; plasma protein; covalent binding; mixed-disulfide; kinetic analysis; compartmental method.

INTRODUCTION

Covalent binding between a drug or its metabolites and plasma (tissue) protein is an undesirable phenomenon. As the result of the formation of such stable bonds may be recognized as a structural alteration of protein by the immune system (i.e., "hapten" formation) (1), the hypersensitive (allergic) reactions are often caused. Therefore, it is thought that the accurate evaluation and/or prediction of protein binding of compounds that bind covalently to protein are very important for overcoming allergic adverse effects.

In conventional pharmacokinetic evaluation methods, protein-drug binding is typically reported only as the ratio of protein-bound or free (protein-unbound) drug to the total drug concentration in plasma or tissue, and the temporal properties of binding and dissociation are neglected because they are extremely rapid reactions (2) compared with the disposition processes such as metabolism and excretion. However, in the case of drugs that contain a thiol (SH) group(s), which include antihypertensive agents (3,4), antirheumatoid agents (5), and cytoprotective agents (6,7), time-dependent disulfide bond formation of these drugs with plasma protein has been observed (8–10). In addition, because this covalent binding is labile, a time-dependency has also been observed in the dissociation of drugs from protein. Therefore, the involvement of these kinetic factors should be examined for the protein binding of SH-containing drugs and more suitable pharmacokinetic evaluation methods should be developed.

N-Acetyl-L-cysteine (NAC) is an SH-containing drug (11–13), and the covalent binding of NAC to plasma protein, in rats, has been examined, using a dithiothreitol-reductive HPLC method (10). In the present study, we evaluated the *in vivo* pharmacokinetics of NAC, as a typical SH drug, based upon an analysis of a compartment model, taking into consideration of the covalent protein binding process.

MATERIALS AND METHODS

Chemicals

NAC was purchased from Ajinomoto Co., Inc. (Tokyo, Japan). L-Thioproline (used as the internal standard) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dithiothreitol (DTT) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The saline solution and lactated Ringer's solution used were obtained from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals or solvents were analytical or HPLC grade.

Animal Experiments

Animals were treated in a manner consistent with the *Principles of Laboratory Animal Care* (NIH publication #85-23, revised 1985).

Bolus injection study: Fasted male Sprague-Dawley rats (7 weeks old, Charles River Japan, Kanagawa, Japan) received NAC (in saline solution, 8.25, 41.25, and 206.25 μmol/ml/kg body weight) via a tail vein over a 5-s period. Then animals were anesthetized and approximately 7 ml of blood was collected from the postcaval vein of each rat using a heparinized syringe. The time points collecting blood were 2.5, 5, 15, and 30 min and 1, 2, 4, and 6 h after injection, and 3 or 5 animals were used at each time point. The blood was immediately centrifuged to obtain a plasma sample.

Continuous infusion study: Fasted rats (9–10 weeks old) received NAC (in lactated Ringer's solution, 7, 21, and 63 μmol/10 ml/kg body weight/h) via a tail vein continuously over a 24-h period using a syringe pump (SP-100s, JMS Co., Ltd., Hiroshima, Japan). Blood samples (0.5 ml) were collected repeatedly from the jugular vein via a catheter. Blood collection was performed 3 or 4 times per animal with three different sampling schedules: (1) 1 h after the start of the infusion and 10 min and 2 h after the end of the infusion, (2) 10 min and 4 h after the start of the infusion and 30 min and 4 h after the end of the infusion, and (3) 30 min and 24 h after the start of the infusion and 1 and 24 h after the end of the infusion. The blood was immediately centrifuged to obtain plasma sample.

[1] Naruto Research Institute, Otsuka Pharmaceutical Factory, Inc., 115 Tateiwa, Muya-cho, Naruto, Tokushima 772-8601, Japan.

[2] Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan.

[3] To whom correspondence should be addressed. (haradada@otsukakj.co.jp)

ABBREVIATIONS: NAC, N-acetyl-L-cysteine; DTT, Dithiothreitol.

0724-8741/02/0500-0615/0 © 2002 Plenum Publishing Corporation

Determination of Plasma NAC Levels

As described in our previous report (10), plasma concentrations of reduced, protein-unbound, and total NAC were determined.

Briefly, reduced NAC was determined by the non-reduction method. Plasma proteins were precipitated by the addition of perchloric acid and removed by centrifugation. Perchloric acid, which may cause damage to HPLC instruments, was removed from the supernatant as a crystal of potassium perchlorate by the addition of $KHCO_3$. Then the supernatant was acidified with phosphoric acid to stabilize the SH group of NAC, and finally subjected to HPLC. Total NAC was determined by the total reduction method. Following alkalization of the plasma with NaOH, all disulfides were reduced by adding DTT. Reduction was terminated by adding perchloric acid. Subsequent treatment of the supernatant was the same as that used in the non-reduction method. Protein-unbound NAC was determined by the low-molecular-weight disulfide reduction method. A portion of the supernatant acquired by the nonreduction method was neutralized with $KHCO_3$ and rendered alkaline with NaOH. DTT was then added to reduce the low-molecular-weight disulfides. The reduction was terminated by adding phosphoric acid, and the final sample was subjected to HPLC analysis.

That all of the NAC added to the plasma was recovered in the supernatant after protein precipitation was confirmed by the total reduction method. This indicates that the NAC, which is non-covalently bound to protein, was liberated from the protein by the addition of perchloric acid and thorough mixing. Therefore, the non-covalently bound NAC is referred to as "protein-unbound" NAC in this study.

HPLC reverse phase ion-pair chromatography, combined with a post-column derivative method using iodoplatinate was employed to detect SH groups. The mobile phase (0.1 mol/l sodium phosphate buffer at pH 2.2 containing 2.5 mmol/l sodium 1-octanesulfonate: methanol at 20:1 [v/v]) was delivered to the guard and analytical columns (STR-ODS II, 4.6 mm i.d., 10 and 150 mm length, respectively, Shinwa Chemical Industries, Ltd., Kyoto, Japan) at 0.6 ml/min. The column eluent was mixed with the reaction medium (0.1 mol/l sodium phosphate buffer at pH 2.2 containing 100 μmol/l hexachloroplatinate [IV] [$H_2PtCl_6 \bullet 6H_2O$, Wako Pure Chemical Industries] and 10 mmol/l potassium iodide). The extent of decolorization of the reaction medium was monitored at 500 nm.

Data Expression and Calculations

Plasma concentrations of NAC reported in this study are given in terms of SH group equivalents. The low-molecular-weight disulfide and protein-bound concentrations were calculated from actual measurements according to the following equations.

[Low-molecular-weight disulfide] = [Protein-unbound] – [Reduced]

[Protein-bound] = [Total] – [Protein-unbound]

Because the concentrations are given as SH equivalents, the actual concentrations of the compounds differ from the indicated protein-unbound, total, and low-molecular-weight disulfide concentrations, which may include disulfide dimers.

Kinetic Analysis

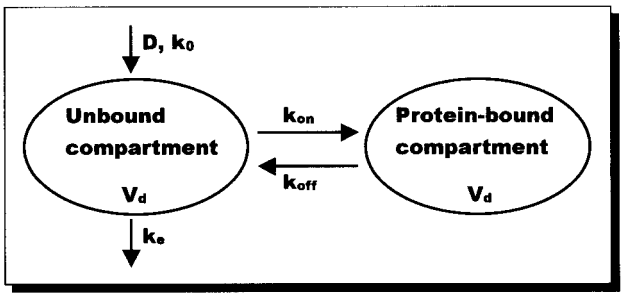
Pharmacokinetic parameters were determined by both a compartmental method and a non-compartmental method.

Figure 1 shows the compartment model including protein-binding process used in this study. In this model, the protein- unbound compartment and the protein-bound compartment were established. These compartments were connected by the apparent first-order binding rate constant (k_{on}) and the dissociation rate constant (k_{off}). In addition, drug elimination was expressed by multiplying the protein-unbound compartment by the first-order elimination rate constant (k_e). Based on this model, simultaneous differential equations expressing the drug transition between these compartments were expressed as below:

$$dX_u / dt = -(k_{on} + k_e) \cdot X_u + k_{off} \cdot X_b$$

$$dX_b / dt = k_{on} \cdot X_u - k_{off} \cdot X_b$$

where X_u is drug amount in the unbound compartment and X_b is drug amount in the protein-bound compartment. Then, these differential equations were solved by the conventional method using Laplace transform, and the equations express-



[Bolus injection]

$$\text{(i)}\quad C_u = \frac{D(\alpha - k_{off})}{V_d(\alpha-\beta)} \exp^{(-\alpha t)} + \frac{D(k_{off} - \beta)}{V_d(\alpha-\beta)} \exp^{(-\beta t)}$$

$$C_b = \frac{Dk_{on}}{V_d(\beta-\alpha)} \exp^{(-\alpha t)} + \frac{Dk_{on}}{V_d(\alpha-\beta)} \exp^{(-\beta t)}$$

$$\text{(ii)}\quad C_t = \frac{D(\alpha - k_{off} - k_{on})}{V_d(\alpha-\beta)} \exp^{(-\alpha t)} + \frac{D(k_{off} + k_{on} - \beta)}{V_d(\alpha-\beta)} \exp^{(-\beta t)}$$

[After infusion]

$$\text{(iii)}\quad C_u = \frac{k0(\alpha - k_{off})}{V_d(\alpha-\beta)\alpha} \exp^{(-\alpha t')} + \frac{k0(k_{off} - \beta)}{V_d(\alpha-\beta)\beta} \exp^{(-\beta t')}$$

$$C_b = \frac{k0k_{on}}{V_d(\beta-\alpha)\alpha} \exp^{(-\alpha t')} + \frac{k0k_{on}}{V_d(\alpha-\beta)\beta} \exp^{(-\beta t')}$$

$$\text{(iv)}\quad C_t = \frac{k0(\alpha - k_{off} - k_{on})}{V_d(\alpha-\beta)\alpha} \exp^{(-\alpha t')} + \frac{k0(k_{off} + k_{on} - \beta)}{V_d(\alpha-\beta)\beta} \exp^{(-\beta t')}$$

[During infusion]

$$C_u = \frac{k0}{V_d k_e}\left[1 + \frac{\beta - k_e}{(\alpha-\beta)} \exp^{(-\alpha t'')} + \frac{k_e - \alpha}{(\alpha-\beta)} \exp^{(-\beta t'')}\right]$$

$$C_b = \frac{k0k_{on}}{V_d\alpha\beta}\left[1 + \frac{\beta}{(\alpha-\beta)} \exp^{(-\alpha t'')} + \frac{\alpha}{(\beta-\alpha)} \exp^{(-\beta t'')}\right]$$

$$\text{(v)}\quad C_t = \frac{k0(k_{off} + k_{on})}{V_d\alpha\beta}\left[1 + \frac{\beta(k_{off} + k_{on} - \alpha)}{(\alpha-\beta)(k_{off} + k_{on})} \exp^{(-\alpha t'')} + \frac{\alpha(\beta - k_{off} - k_{on})}{(\alpha-\beta)(k_{off} + k_{on})} \exp^{(-\beta t'')}\right]$$

Fig. 1. A compartment model including protein binding process and theoretical equations that describe plasma NAC concentrations. (C_u, plasma protein-unbound NAC concentration; C_b, plasma protein-bound NAC concentration; C_t, plasma total NAC concentration; t, time after bolus injection; t', time after end of infusion; t", time after start of infusion; V_d, distribution volume; D, dose of NAC in injection; k_0, dosing rate of NAC in infusion; α and β are hybrid parameters, where $\alpha + \beta = k_{on} + k_{off} + k_e$ and $\alpha \times \beta = k_{off} \times k_e$.)

ing time-drug concentrations during various experimental periods were finally obtained (Fig. 1). However, distribution volumes for two compartments could be different but were assumed to have the common values because the analysis was performed based entirely upon the plasma concentrations.

Equations i to v were entered into the computer program "Multi", which is a non-linear least-squares method analytical program (14), using the five parameters, α, β, k_{on}, k_{off}, and V_d, as program variables. The actual values for the plasma protein-unbound and total NAC concentrations after the bolus injection, after the infusion, and during the infusion were entered simultaneously. An approximate calculation using the *Simplex* algorithm was then initiated after entering the appropriate initial values for the five parameters. Finally, the values of the five parameters were determined by an approximate calculation using the *Gauss-Newton* algorithm.

In the case of noncompartmental analysis, the elimination half-life is usually calculated from the slope of the time-concentration (log value) plot. The area under the concentration-time curve (AUC) is calculated using the trapezoidal method.

RESULTS

Plasma NAC Levels in the Bolus Injection Study

Figure 2 shows the plasma concentrations of reduced, protein-unbound, and total NAC as well as the calculated concentrations of the low-molecular-weight disulfide form and the mixed-disulfide with protein in rats, after a bolus injection of 206.25 μmol/kg. The AUC for the plasma total- and protein-unbound NAC (AUC_t and AUC_u) were approximately in proportion to the dose of NAC, and the total body clearance of the plasma total- and protein-unbound NAC ($Cl_{t,\ total}$ (=Dose/AUC_t) and $Cl_{u,\ total}$ (=Dose/AUC_u)) upon the bolus injection of 8.25, 41.25, 206.25 μmol/kg were 1279, 920, 1261 ml/kg/hr and 1856, 1392, 1553 ml/kg/hr, respectively. The total NAC showed a biphasic elimination curve. Specifically, the total NAC decreased rapidly up to 1 h with a half-life of 13–16 min, and subsequently decreased relatively slowly with a half-life of approximately 2 h Protein-unbound NAC showed almost the same values as total NAC for the first 15 min, but differences were observed at later time points. Based on the difference between the total and protein-unbound concentrations, we conclude that covalently protein-bound NAC through the formation of a mixed-disulfide was the main constituent of plasma NAC (approximately >70% of the total NAC) after 1 h (Fig. 3). Although plasma NAC consisted almost entirely of reduced molecules (the parent drug) immediately after administration, low-molecular-weight disulfide molecules rapidly became the main constituent within15 min to 1 h (Fig. 3).

In brief, plasma NAC after bolus injection is present in the form of two different disulfides: a low-molecular-weight form and as a mixed-disulfide with protein. In addition, the main constituent of plasma NAC was converted from the low-molecular-weight disulfide form to the protein-involved disulfide form within 1 h after administration, when total NAC elimination switched from the rapid phase to the slow phase. This suggests that the covalent binding of NAC to protein is a factor in the rate of total drug elimination from plasma.

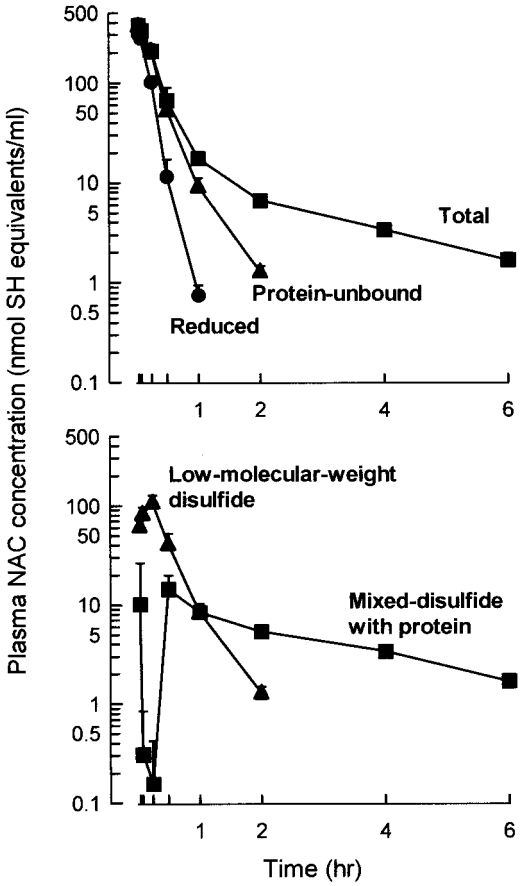


Fig. 2. Plasma concentrations of reduced, protein-unbound, and total NAC (upper panel) and calculated concentrations (see Materials and Methods) of the low-molecular-weight disulfide form and mixed-disulfide with the protein form (lower panel) after a bolus injection at 206.25 μmol/kg. Plasma reduced and protein-unbound NAC values below the quantitative measurement range of HPLC analysis were treated as zero in the calculations. Each point represents the mean and S.D. of three rats.

Plasma NAC Levels in the Continuous Infusion Study

Figure 4 shows the plasma concentrations of protein-unbound and total NAC and the calculated concentration of covalently protein-bound in rats during and after an infusion

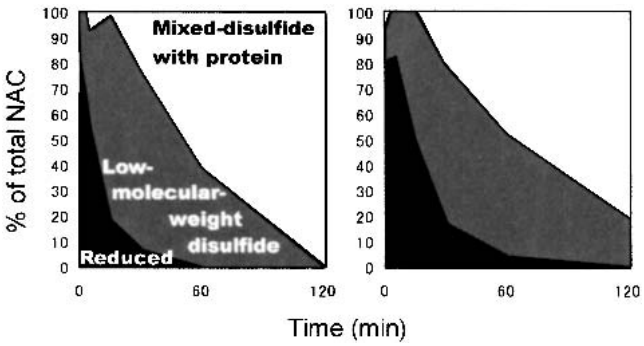


Fig. 3. Composition of plasma total NAC after bolus injection. NAC doses are 41.25 μmol/kg (left panel) and 206.25 μmol/kg (right panel). Plasma reduced and protein-unbound NAC values below the quantitative measurement range of HPLC analysis were treated as zero in the calculations.

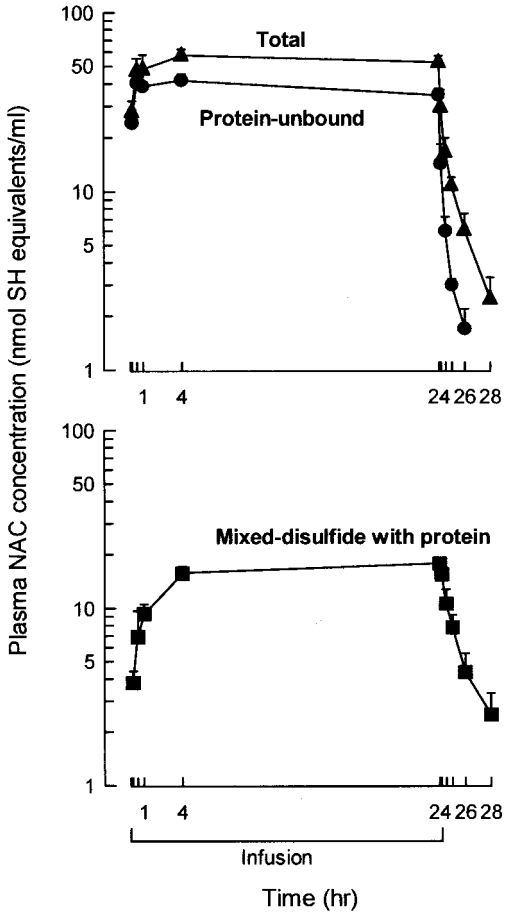


Fig. 4. Plasma concentrations of protein-unbound and total NAC (upper panel) and calculated concentration of mixed-disulfide with the protein form (lower panel) in the infusion study at 63 μmol/kg/hr. Plasma protein-unbound NAC values below the quantitative measurement range of HPLC analysis were treated as zero in the calculations. Each point represents the mean and S.D. for three rats.

of 63 μmol/kg/hr. The plasma total- and protein-unbound NAC already reached steady-state concentrations ($C_{t,\ ss}$ and $C_{u,\ ss}$) at 4 h after the start of infusion and these were in proportion to the dosing rate of NAC, respectively. The $Cl_{t,\ total}$ (= Dosing rate/$C_{t,\ ss}$) and $Cl_{u,\ total}$ (= Dosing rate/$C_{u,\ ss}$) upon the infusion of 7, 21, 63 μmol/kg were 1111, 1047, 1095 ml/kg/hr and 1471, 1634, 1507 ml/kg/hr. After the end of the infusion, plasma NAC was eliminated with a half-life of approximately 1 h Although the contribution of the protein-bound form to total NAC (i.e., the covalent protein-binding ratio) was relatively low in the steady state during infusion (approximately 30–40%), this value increased rapidly after completion of the infusion (approximately 60–70%) (Table I).

Compartmental Analysis

The compartmental analysis was performed using data obtained from the bolus injection study and the continuous infusion study simultaneously. Because three doses were employed in both studies, analyses for 9 (3 × 3) pairs of doses were performed. The mean ± SD values of the kinetic parameters are shown in Table II. The fitting curves were in good agreement with the experimentally determined values for plasma NAC (Fig. 5). The obtained k_{on}, k_{off}, and k_e values

Table I. Contribution of Covalently Protein-Bound NAC to Total NAC (Covalent Protein-Binding Ratio) in Plasma in the Infusion Study

	Covalent protein binding ratio (%)		
Time	7 μmol/kg/h[a]	21 μmol/kg/h	63 μmol/kg/h
During infusion			
10 min	0.0 ± 0.0	10.5 ± 5.5	14.0 ± 3.8
30 min	11.4 ± 3.5	18.1 ± 3.2	14.4 ± 4.2
1 hr	7.4 ± 1.7	21.7 ± 2.4	20.1 ± 6.2
4 hr	23.6 ± 10.7	36.1 ± 3.0	27.3 ± 0.2
24 hr	34.9 ± 3.0	40.3 ± 3.8	33.9 ± 0.8
After infusion			
10 min	46.9 ± 12.3	56.9 ± 0.8	52.3 ± 2.3
30 min	NC[b]	65.2 ± 3.8	63.9 ± 0.6
1 hr	NC	NC	71.7 ± 5.0
2 hr	NC	NC	71.3 ± 5.9

Note: Each point represents mean ± S.D. of three rats.
[a] Dosage rates of NAC (*see* MATERIALS AND METHODS section).
[b] Not Calculated.

were 0.23, 0.57, and 4.3 (hr^{-1}), respectively. The dissociation half-life of the protein-NAC conjugate was estimated to be approximately 1.2 h ($0.693/k_{off}$), which is consistent with to the elimination half-life of plasma total NAC in the terminal phase of bolus injection and after infusion. The total body clearance (1663 ml/kg/h, $k_e \times V_d$) is consistent with the values for the clearance of protein-unbound NAC ($Cl_{u,\ total}$) calculated by non-compartmental analysis.

DISCUSSION

In the present study, we have established a compartment model to analyze the covalent protein-binding kinetics of NAC in rat *in vivo* as simply as possible under following assumptions. Theoretically, the volume of distribution (V_d) is expressed by the following equation:

$$V_d = V_P + V_T \cdot f_{u,\ P} / f_{u,\ T}$$

where V_P is plasma volume, V_T is tissue volume, $f_{u,\ P}$ is the unbound fraction in plasma, and $f_{u,\ T}$ is the unbound fraction in tissue. Therefore, V_d of NAC in rat could be altered accompanying with time-dependent change of plasma protein binding ratio of NAC through a covalent bond observed in the present study. However, it is considered that proteins having covalent binding ability of SH-compounds must exist not only in plasma but also in inter-cellular fluid and in cellular fluid, e.g., albumin in inter-cellular fluid, fatty-acid bind-

Table II. Pharmacokinetic Parameters Determined by Compartmental Analysis

Parameters[a]	
k_{on} (h^{-1})	0.23 ± 0.13
k_{off} (h^{-1})	0.57 ± 0.18
k_e (h^{-1})	4.3 ± 0.5
V_d (ml/kg)	391 ± 67
Cl_{total} (ml/kg/h)[b]	1663 ± 159

[a] Means ± S.D. of 9 analyses (*see* MATERIALS AND METHODS section).
[b] $k_e \times V_d$

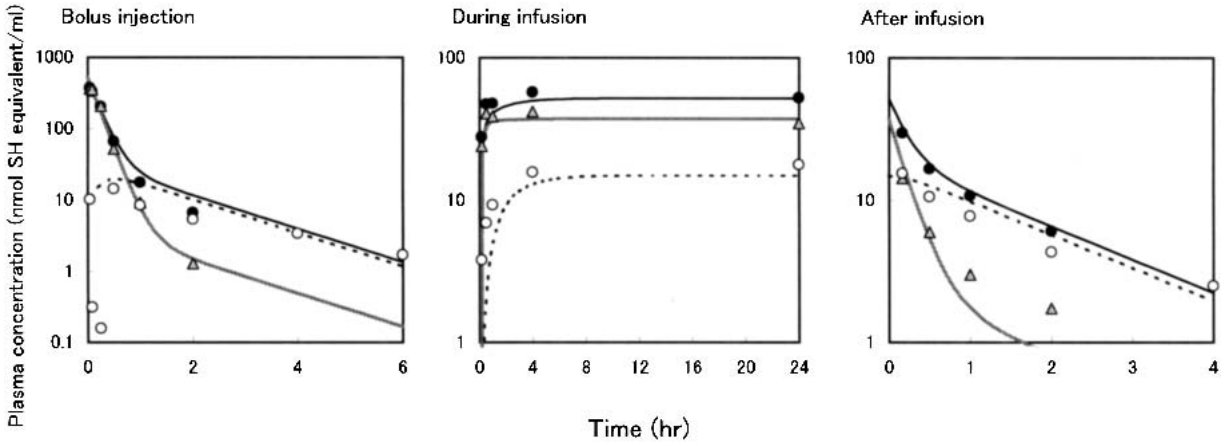


Fig. 5. Fitting curves calculated from the theoretical equations shown in Fig. 1. The parameters shown in Table II were obtained from this fitting analysis. Solid lines, gray lines, and broken lines indicate the theoretical values for total NAC, protein-unbound NAC, and covalently protein-bound NAC, respectively. Symbols indicate the plasma NAC levels determined in the bolus injection study (206.25 μmol/kg) and the infusion study (63 μmol/kg/hr). Closed circles, open gray triangles, and open circles indicate plasma total NAC, protein-unbound NAC, and covalently protein-bound NAC, respectively.

ing protein in liver (15), and myoglobin in skeletal muscle (16). These ex-plasma protein binding should, if possible, be taken into consideration to kinetic analysis, but it is difficult actually to determine protein binding in various tissues. Therefore, we assumed that ex-plasma covalent protein binding of NAC is reversible similar to plasma and that equilibrium is established consistently between protein binding processes in plasma and in ex-plasma. We analyzed based upon this hypothesis that ($f_{u,P}$ / $f_{u,T}$) and V_d are time-independent steady values. Moreover, we have preliminary observed using radiolabeled compounds that NAC was very rapidly converted to L-cysteine and that most of NAC distributed as this metabolite in body at 1 h after administration to rat (unpublished data). It seems uncertain based only on the plasma data whether a peripheral compartment, to which unbound NAC distributes, should be established into the present model. However, we assumed that distribution of NAC to peripheral compartment is negligible from the above physiological background.

One of the covalent protein-binding sites through a disulfide bond in plasma has been identified as the 34th cysteine residue of albumin (17,18). Considering the high concentration of albumin in plasma, the main covalent protein-binding site of SH drugs, including NAC, would be expected to be this cysteine residue on the albumin molecule. The present results suggest that covalent binding is not the result of a direct reaction between reduced NAC and protein but, rather, to a disulfide interchange reaction of the low-molecular-weight disulfide form, because the formation of covalent bonds progressed after the reduced form of NAC rapidly altered to its disulfide form. Definitely, it can be considered that covalent protein binding and the dissociation mechanisms for NAC should not be attributed to a first-order reaction but, rather, to a high-order reaction that is affected not only by the NAC-disulfide concentration but also by the concentrations of proteins and intrinsic SH compounds. However, in this study, it was possible to analyze these reactions as an apparent first-order reaction. This might be due to the fact that the levels of proteins and intrinsic SH compounds in plasma were substantially higher than the levels of NAC and therefore had a negligible effect on the analysis.

The pharmacokinetic properties of NAC, including protein-binding kinetics, in rats are shown diagrammatically in Figure 6.

Immediately after the bolus injection, plasma NAC is present almost entirely in the protein-unbound form (reduced molecules and low-molecular-weight disulfides), which eliminates very rapidly. On the other hand, because the formation of protein-bound NAC is much slower than the rate of elimination of the unbound drug ($k_{on} << k_e$), plasma levels of protein-bound NAC are very low and contribute only slightly to total NAC elimination. However, at later time points, covalent protein binding reaches a steady state and the binding ratio is determined by the equilibrium between k_{off} and k_e. Specifically, the dissociation of the protein-NAC conjugate is much slower than the elimination of unbound NAC ($k_{off} << k_e$), and, as a result, the covalent protein-binding ratio is relatively high. In other words, plasma drug elimination is rate-limited by the dissociation of NAC from protein. In the steady state during infusion, elimination of the unbound drug is counterbalanced by the continuous supply of NAC (via infusion) and the covalent protein-binding ratio results in equilibrium between protein binding and the dissociation rate (approximately 29% [$k_{on}/(k_{on}+k_{off})$]). After the infusion is complete, the pharmacokinetic behavior of NAC is similar to that seen for a bolus injection.

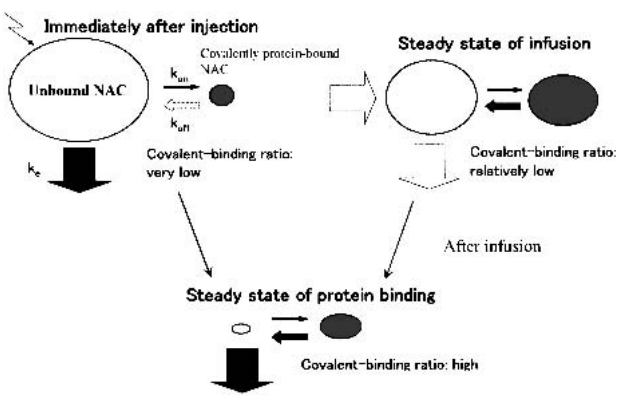


Fig. 6. Diagram showing the pharmacokinetics of NAC, including its covalent plasma protein-binding kinetics.

The Cl_{total} obtained from a compartmental analysis ($= k_e \times V_d$) is theoretically considered to express the plasma protein-unbound NAC clearance. In fact, this was in good agreement with the protein-unbound clearance ($Cl_{u,\ total}$) calculated from a non-compartmental analysis. On the other hand, the plasma total NAC clearance ($Cl_{t,\ total}$) was smaller than these unbound clearances, because the dissociation of NAC from protein restricted the total drug elimination in plasma as mentioned above. However, the difference between the total- and protein-unbound clearances is small, suggesting that the contribution of the time-dependent protein binding to the pharmacokinetics of NAC is relatively low.

NAC is converted to its metabolites such as L-cysteine or glutathione, and these metabolites probably have pharmacological effects similar to NAC in the body. Consequently, the pharmacological active form of NAC is still unclear. Therefore, it is not so easy to discuss the pharmacological effect of NAC directly related to the time-dependent protein binding of NAC. To answer to the above critical points, the pharmacokinetic-pharmacodynamic (PK-PD) analysis including protein-binding kinetic analysis of drug, which only parent-compound has a pharmacological effect, will be necessary. In this point of view, a selenium-containing antioxidative agent, ebselen, is a very interesting ligand, because it binds to albumin through an Se-S bond, resembling a disulfide bond, and the albumin-drug conjugate itself is responsible for the pharmacological effects (19). Upon the development of such drug, a kinetic analysis of the time-dependent protein binding will be important to establish an appropriate pharmacokinetic model. The *in vivo* kinetic analytical method shown in the present study is estimated to be theoretically applicable to the analysis of the time-dependent protein binding of other drugs, such as ebselen or several SH-containing drugs.

The present results kinetically prove a lability of the covalent protein binding of NAC through a mixed-disulfide. This corresponds to the fact that any allergic effect of NAC is not observed in animal toxicity test using rat (in-house data). Probably, it is considered that the duration and/or the level of the covalent bonds formed in the rat was not enough to form "hapten", which induces several allergic side-effects (1) (Although the immunogenicity of the albumin-NAC complex itself is unclear). On the other hand, cases of allergic side effects such as urticaria etc., have been reported upon NAC therapy in human, although they are rare (20). In addition, the pharmacokinetic data of NAC studied by Olsson *et al.* (21) indicates that the elimination half-life of plasma NAC in human is considerably longer than that in rat. Therefore, the species differences in pharmacokinetics of NAC including its time-dependent protein binding should be elucidated. The protein-binding kinetic study of NAC using rat and human sera and albumin is currently under way.

REFERENCES

1. B. K. Park and N. R. Kitteringham. Drug-protein conjugation and its immunologic consequences. *Drug. Metab. Rev.* **22**:87–144 (1990).
2. G. R. Wilkinson. Plasma and tissue binding considerations in drug disposition. *Drug. Metab. Rev.* **14**:427–465 (1983).
3. C. I. Johnston, J. A. Millar, B. P. McGrath, and P. G. Matthews. Long-term effects of captopril (SQ14 225) on blood pressure and hormone levels in essential hypertension. *Lancet* **2**:493–496 (1979).
4. C. A. Fink, Y. Qiao, C. J. Berry, Y. Sakane, R. D. Ghai, and A. J. Trapani. New alpha-thiol dipeptide dual inhibitors of angiotensin-I converting enzyme and neutral endopeptidase EC 3.4.24.11. *J. Med. Chem.* **38**:5023–5030 (1995).
5. E. C. Huskisson. Recent drugs and the rheumatic diseases. *Rep. Rheum. Dis.* **54**:1–4 (1974).
6. J. W. Purdie. A comparative study of the radioprotective effects of cysteamine, WR-2721, and WR-1065 in cultured human cells. *Radiat. Res.* **77**:303–311 (1979).
7. N. Susa, S. Ueno, and Y. Furukawa. Protective effects of thiol compounds on chromate-induced cytotoxicity in HeLa cells. *J. Vet. Med. Sci.* **54**:281–288 (1992).
8. R. Narazaki, K. Harada, A. Sugii, and M. Otagiri. Kinetic analysis of the covalent binding of captopril to human serum albumin. *J. Pharm. Sci.* **86**:215–219 (1997).
9. R. Narazaki, M. Harada, K. Hanada, and M. Otagiri. Covalent binding between bucillamine derivatives and human serum albumin. *Pharm. Res.* **13**:1317–1321 (1996).
10. D. Harada, S. Naito, Y. Kawauchi, K. Ishikawa, O. Koshitani, I. Hiraoka, and M. Otagiri. Determination of reduced, protein-unbound, and total concentrations of N-acetyl-L-cysteine and L-cysteine in rat plasma by post-column ligand substitution high-performance liquid chromatography. *Anal. Biochem.* **290**:251–259 (2001).
11. G. Boman, U. Bäcker, S. Larsson, B. Melander, and L. Wåhlinder. Oral acetylcysteine reduces exacerbation rate in chronic bronchitis: Report of a trial organized by the Swedish Society for Pulmonary Diseases. *Eur. J. Respir.* **64**:405–415 (1983).
12. L. F. Prescott, R. N. Illingworth, J. A. J. H. Critchley, M. J. Stewart, R. D. Adam, and A. T. Proudfoot. Intravenous N-acetylcysteine: The treatment of choice for paracetamol poisoning. *Br. Med. J.* **2**:1097–1100 (1979).
13. B. Särnstrand, A. Tunek, K. Sjödin, and A. Hallberg. Effects of N-acetylcysteine stereoisomers on oxygen-induced lung injury in rats. *Chemico-Biological Interactions* **94**:157–164 (1995).
14. K. Yamaoka, Y. Tanigawara, T. Nakagawa, and T. Uno. A pharmacokinetic analysis program (Multi) for microcomputer. *J. Pharmacobiodyn.* **4**:879–885 (1981).
15. T. Sato, K. Baba, Y. Takahashi, T. Uchiumi, and S. Odani. Rat liver fatty acid-binding protein: Identification of a molecular species having a mixed disulfide with cysteine at Cysteine-69 and enhanced protease susceptibility. *J. Biochem.* **120**:908–914 (1996).
16. Y. Enoki, Y. Ohga, H. Ishidate, S. Sakata, H. Kohzuki, and M. Hattori. Mixed disulfide formation in myoglobin of mouse. *Comp. Biochem. Physiol.* **113B**:149–155 (1996).
17. D. C. Carter and J. X. Ho. Structure of serum albumin. In C. B. Anfinsen, J. T. Edsall, F.M. Richards, and D.S. Eisenberg (eds.), *Advances in Protein Chemistry,* vol. 45, Academic Press, San Diego, California, 1994 pp. 153–203.
18. R. Narazaki, T. Maruyama, and M. Otagiri. Probing the cysteine 34 residue in human serum albumin using fluorescence techniques. *Biochim. Biophys. Acta* **1338**:275–281 (1997).
19. V. Ullrich, P. Weber, F. Meisch, and F. von Appen. Ebselen-binding equilibria between plasma and target proteins. *Biochem. Pharmacol.* **52**:15–19 (1996).
20. USP-DI Vol. 1. *Drug information for the health care professional, 20th ed.*, Micromedex, Inc., Colorado, 2000.
21. B. Olsson, M. Johansson, J. Gabrielsson, and P. Bolme. Pharmacokinetics and bioavailability of reduced and oxidized N-acetylcysteine. *Eur. J. Clin. Pharmacol.* **34**:77–82 (1988).