Pharmacokinetics of the antineoplastic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in the aqueous and vitreous of rabbit

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1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), an anticancer drug, was assayed in the aqueous and vitreous of rabbits after intravenous injection, subconjunctival injection, and topical application. Pharmacokinetic parameters were calculated from the time-course curves. The penetration into aqueous and vitreous across the blood-aqueous barrier and the blood-retinal barrier after intravenous injection was related to the anatomic dimensions of the eye (the volume of tissue involved and the surface area through which BCNU diffuses). To evaluate the three routes of administration, the area under the time-course curve was calculated from the simulated time-course curve. The results suggest that topical application of BCNU is the best and subconjunctival injection the second best route for treating an iris tumor, and that intravenous injection is the best route for choroidal and retinal tumors. (INVEST OPHTHALMOL VIS SCI 23:199-208, 1982.)

Key words: pharmacokinetics, area under the curve, anticancer drug, BCNU, carmustine, intravenous injection, subconjunctival injection, topical application, intraocular penetration

Carmustine [NSC-409962; 1,3-bis(2-chloroethyl)-1-nitrosourea; BCNU] is a lipid-soluble drug that decomposes rapidly in vivo to yield alkylating and carbamoylating intermediates at physiologic pH. It is one of the most effective chemotherapeutic agents for a variety of human cancers, especially brain tumors. As part of our investigations on chemotherapy for ocular malignancies, we showed that BCNU is effective against Brown-Pearce epithelioma implanted in the anterior chamber of rabbit eyes when the drug is administered directly or by a sustained-delivery system consisting of an episclerally implanted silicone balloon. BCNU was also found to be effective against Greene melanoma transplanted into the anterior chamber of rabbit eyes when periocular and intravenous administration routes were combined.

To our knowledge, the intraocular penetration of BCNU has not been evaluated according to route of administration—intravenous injection, subconjunctival injection, or topical application. This article reports the time course of BCNU in the aqueous and vitreous of rabbits after administration by various routes and presents pharmacokinetic parameters obtained from the time course.
Table I. General equations of pharmacokinetic models used in this study

<table>
<thead>
<tr>
<th>Model</th>
<th>Administration route</th>
<th>General equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open one-compartment*</td>
<td>Extravascular</td>
<td>[C = B e^{-k_{a}t} - A e^{-k_{a}t}]</td>
</tr>
<tr>
<td>Open two-compartment†</td>
<td>Intravascular</td>
<td>[C = B e^{-\beta t} + A e^{-\alpha t}]</td>
</tr>
<tr>
<td>Open two-compartment*</td>
<td>Extravascular</td>
<td>[C = B e^{-\beta t} + A e^{-\alpha t} - C_{0}e^{-k_{a}t}]</td>
</tr>
</tbody>
</table>

\(C = \) drug concentration; \(A\) and \(B = \) empirical constants; \(k_{a}\) and \(k_{el} = \) absorption and elimination rate constants; \(\alpha\) and \(\beta = \) hybrid rate constants that are functions of intercompartmental \((k_{12} \text{ and } k_{21})\) and elimination rate constants; \(\alpha \cdot \beta = k_{12} \cdot k_{10} \text{ and } \alpha + \beta = k_{12} + k_{10}\); \(C_{0} = \) intercept of \(k_{a}\) slope with ordinate.

*Absorption proceeding according to drug liberation and absorption mechanism.
†No absorption; all injected drug is in the systemic circulation.

Materials and methods

Reagents. BCNU was obtained from a commercial source. All other chemicals were reagent grade.

Animals. New Zealand albino rabbits of both sexes, weighing 3.5 to 4.5 kg, were maintained in standard laboratory animal cages and allowed food and water ad libitum. For general anesthesia before the experiments, the rabbits were given 5 mg of promazine hydrochloride per kilogram of body weight intramuscularly and 25 mg/kg pentobarbital sodium via a marginal ear vein by means of a catheter with a 25-gauge needle.

BCNU assay. The colorimetric assay of Loo and Dion was used. This procedure is based on the reaction of intact BCNU with hydrochloric acid to release nitrous acid, which makes a red substance with sulfonamide and Bratton-Marshall reagent.

Blood sampling and BCNU assay after intravenous injection. Four anesthetized rabbits were each injected with 250 USP units/kg heparin sodium via an ear vein. A shorter catheter with a 25-gauge needle was placed in an artery of the other ear for collecting blood. Each rabbit received 5 mg/kg BCNU in 10% ethanol through the heparin catheter over 1 min. Arterial blood (approximately 2.5 ml) was sampled at 3, 10, 20, 30, 40, 60, and 90 min after the termination of the BCNU injection. Two milliliters of the whole blood, measured exactly by pipette, were poured into a stoppered tube; 4 ml of chilled anhydrous ether were added, and the tube was immediately shaken well and placed in an ice bath. The concentration of BCNU in the ether extract was determined colorimetrically. Calibration standards (controls) for whole blood were prepared at the same time from a rabbit treated the same as the experimental animals except for the BCNU injection.

Aqueous sampling and BCNU assay after intravenous injection. Fourteen anesthetized rabbits were each injected with 250 USP units/kg heparin sodium, followed by 5 mg/kg BCNU in 10% ethanol through the ear vein over 1 min. Aqueous (approximately 300 µl) was aspirated by a 1 ml
disposable syringe with a 27-gauge needle 1.5, 3, 6, 10, 20, 40, and 60 min after the termination of the BCNU injection. (Four eyes were used at each time interval.) Then 200 μl of aqueous, measured exactly by pipette, were mixed in a stoppered tube with 0.5 ml of chilled anhydrous ether, and the tube was immediately shaken well and placed in an ice bath. The BCNU in the ether phase was assayed colorimetrically. Aqueous standards (controls) were prepared from bovine aqueous. It had been previously demonstrated that bovine aqueous gave the same results for BCNU standards as had rabbit aqueous.

**Aqueous sampling and BCNU assay after topical application.** Twelve anesthetized rabbits each received 0.2 mg of BCNU in 0.01 ml of sesame oil USP topically. The eyelids were held open with an ocular speculum, and a drop of the viscous oil solution was placed carefully on the apex of the cornea and spread slowly on the corneal surface. Aqueous was aspirated at 3, 6, 10, 20, 30, and 40 min after the BCNU was given. Immediately before each aqueous aspiration, the surface of the eye was washed quickly (about 5 to 10 sec) with about 6 ml of physiological saline. (Four eyes were used at each time interval.) The concentration of BCNU was determined by the procedure described above.

**Vitreous sampling and BCNU assay after intravenous injection.** Ten anesthetized rabbits were each injected with 250 USP units/kg heparin sodium and 5 mg/kg BCNU in 10% ethanol as described above. At 3, 20, 30, 60, and 90 min after the termination of the injection, animals were killed with an overdose of pentobarbital sodium and eyes were enucleated and immediately frozen in liquid nitrogen. (Four eyes were used at each time interval.) Care was taken to prevent eye rupture. The vitreous of the frozen eye was removed and transferred into a preweighed 20 ml test tube with a screw cap. After the wet weight of the vitreous was determined, 3 ml of chilled ether was added to the test tube, which was shaken well and placed in an ice bath. BCNU was assayed in 2 ml of this ether. Calibration standards for vitreous were prepared from five rabbits (10 samples) treated the same as the experimental animals except for the BCNU injection.

**Vitreous and aqueous sampling and BCNU assay after subconjunctival injection.** Both corneas of 10 anesthetized rabbits were washed continuously with normal saline at a flow rate of 10 ml/min to prevent BCNU from penetrating into the anterior chamber through the cornea during the experimental period. BCNU (2 mg in 0.3 ml of 20% ethanol) was injected subconjunctivally, near the superior rectus muscle, into both eyes with a 30-gauge needle in a 1 ml disposable syringe. At 6, 15, 30, 45, and 60 min after the termination of the injection, eyes were enucleated and frozen in liquid nitrogen. (Four eyes were used at each time interval.) The frozen vitreous and aqueous were dissected and transferred into preweighed 20 and 10 ml test tubes, respectively. The analytic procedure and controls were the same as those described above.

**Pharmacokinetic calculations.** After plotting the time-course data of BCNU on a semilogarithmic graph, an experimental curve was fitted to the results with the assistance of regression analysis using a programmable calculator, TI-59, and the Applied Statistics program library of Solid State Software module. According to the shape of the curve and the speculative penetration route, a
pharmacokinetic model was tentatively selected. Pharmacokinetic parameters were calculated from the regression coefficients with the selected model and substituted into the general equation for the simulation, which allowed verification of the model for further pharmacokinetic analysis. The pharmacokinetic models used in this study are presented in Fig. 1; their general equations are shown in Table I.

It is well known that a pharmacokinetic model is a hypothetical structure that can be used to characterize the behavior and the fate of a drug in a biological system when the drug is given by a certain administration route and in a particular dosage form. Therefore compartments used in pharmacokinetic analysis cannot be assumed to fit actual physical compartments. The half-life of BCNU in each experiment was calculated from the rate constant ($k_a$ or $\beta$, Table I) using the following equation:\textsuperscript{11} $t_{1/2} = 0.693/k_a$ (or $\beta$). The lag time ($t_{lag}$), the time interval between administration and first appearance, was calculated from the parameters (Table I) with the following equations\textsuperscript{11}:

\begin{equation}
 t_{lag} = \ln \frac{A}{B} / (k_a - k_{el}) \tag{for one-compartment model}
\end{equation}

\begin{equation}
 Ce^{-k_{el}t_{lag}} = Ae^{-\alpha t_{lag}} + Be^{-\beta t_{lag}} \tag{for two-compartment model}
\end{equation}

**Results**

**Blood, aqueous, and vitreous data after intravenous injection.** Figs. 2, 3, and 4 show the BCNU concentration in blood, aqueous, and vitreous, respectively, after intravenous injection. The open two-compartment model, intravascular administration (intra),\textsuperscript{11} was chosen for the pharmacokinetic analysis of blood, because the time course was biphasic. Penetration into the anterior chamber and the
Table II. Pharmacokinetic parameters after intravenous BCNU injection (5 mg/kg) in rabbits

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Blood</th>
<th>Aqueous</th>
<th>Vitreous</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ (min$^{-1}$)</td>
<td>0.136</td>
<td>0.218</td>
<td>0.0388</td>
</tr>
<tr>
<td>$\beta$ (min$^{-1}$)</td>
<td>0.00568</td>
<td>0.0304</td>
<td>0.00519</td>
</tr>
<tr>
<td>$A$ (µg/ml)</td>
<td>3.71</td>
<td>0.919</td>
<td>0.241</td>
</tr>
<tr>
<td>$B$ (µg/ml)</td>
<td>0.422</td>
<td>1.29</td>
<td>0.247</td>
</tr>
<tr>
<td>$C^*$ (µg/ml)</td>
<td>4.13</td>
<td>2.21</td>
<td>0.488</td>
</tr>
</tbody>
</table>

$C^*$ represents simulated initial BCNU concentration. See Table I footnote for explanation of other parameters.

Vitreous was too rapid to detect the penetration phase. The time courses in the aqueous and vitreous were found also to be biphasic; therefore the same model and equation as those used for blood were applied to their pharmacokinetic analysis. Pharmacokinetic parameters were calculated for each time course (Table II). The half-life of BCNU in blood was calculated to be 122 min; in aqueous, 22.8 min; and in vitreous, 134 min. The BCNU concentration in blood, aqueous, and vitreous can be simulated from the pharmacokinetic parameters using the general equation for the open two-compartment model (intra), $C = Be^{-\beta t} + Ae^{-\alpha t}$. The simulated time course is given in each figure.

**Aqueous data after subconjunctival injection.** Fig. 5 shows the BCNU concentration in aqueous after subconjunctival injection. The maximum concentration was 4.50 ± 3.04 µg/ml ($n = 4$) at 15 min after injection. We tried to fit several kinds of pharmacokinetic models to these results; the open two-compartment model, extravascular administration (extra),$^{11}$ was found to fit better than the open one-compartment (extra) model. The parameters $k_a$, $\alpha$, and $\beta$ were calculated to be 0.248 min$^{-1}$, 0.169 min$^{-1}$, and 0.0360 min$^{-1}$, respectively, and the half-life was 19.3 min. The BCNU concentration in aqueous ($C_a$) can be simulated from the following equation:

$$C_a = 4.14e^{-0.0360t} + 62.3e^{-0.169t} - 98.6e^{-0.248t}$$

The simulated time course is also given in Fig. 5. The simulated maximum concentration was 6.13 µg/ml at 10 min, and the lag time was 4.4 min.

**Vitreous data after subconjunctival injection.** Fig. 6 shows the BCNU concentration in vitreous after subconjunctival injection. The maximum concentration was 0.387 ± 0.102 µg/ml ($n = 4$) at 30 min after injection. The open one-compartment model (extra) was chosen for pharmacokinetic analysis. The absorption and elimination rate constants were calculated to be 0.0948 min$^{-1}$ and 0.0380 min$^{-1}$, respectively; the half-life was 18.2 min. The BCNU concentration in vitreous ($C_v$) can be simulated from the following equation:

$$C_v = 1.31e^{-0.00380t} - 1.44e^{-0.0948t}$$

The simulated time course is also given in Fig. 6. The simulated maximum concentration was 0.400 µg/ml at 18 min, and the lag time was 1.7 min.

**Aqueous data after topical application.** Fig. 7 shows the BCNU concentration in
aqueous after topical application. The maximum concentration was 9.44 ± 0.713 μg/ml (n = 4) at 10 min after application. The time course was fitted to the theoretical time course of the open one-compartment model (extra). The absorption and the elimination rate constants were calculated to be 0.203 min⁻¹ and 0.105 min⁻¹, respectively; the half-life was 6.60 min. The BCNU concentration in aqueous humor (Cₐ) can be simulated from the following equation:

\[ C_a = 45.7e^{-0.105t} - 50.1e^{-0.203t} \]

The simulated time course is also given in Fig. 7. The simulated maximum concentration was 9.87 μg/ml at 7.7 min, and the lag time was 0.94 min.

**Calculation of area under the time-course curve (AUC).** The experimental AUC (AUC_{exp}^{∞}) of each experimental time course, calculated using the trapezoidal rule, is presented in Table III. The simulated AUC (AUC_{sim}^{∞}), which is the total integration of the simulated equation from zero time to infinity, was also calculated and is summarized in Table III.

**Discussion**

Colorimetry, which measures only intact BCNU, was selected for this study instead of other analytic methods because, according to Woolley and Schein, drug effects must be correlated with the active form of the drug—measurements based on plasma concentrations of inactive species are of little or no value.

BCNU penetrates into the central nervous system through the blood-brain barrier (BBB). Two barrier systems are important in the intraocular penetration of drugs: the blood-aqueous barrier (BAB) and the blood-retina barrier (BRB) (or blood-vitreous barrier).
Table III. AUC after BCNU treatment of rabbits*

<table>
<thead>
<tr>
<th></th>
<th>Aqueous</th>
<th>Vitreous</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IV</td>
<td>SC</td>
<td>TA</td>
</tr>
<tr>
<td>AUC_{IV}^{0-10min}</td>
<td>46.6</td>
<td>120</td>
<td>199</td>
</tr>
<tr>
<td>AUC_{IV}^{0-24h}</td>
<td>46.6</td>
<td>140</td>
<td>190</td>
</tr>
<tr>
<td>AUC_{SC}^{0-10min}</td>
<td>33.3</td>
<td>139</td>
<td>187</td>
</tr>
<tr>
<td>AUC_{SC}^{0-24h}</td>
<td>45.0</td>
<td>129</td>
<td>190</td>
</tr>
</tbody>
</table>

* IV = intravenous injection; SC = subconjunctival injection; TA = topical application. IV dose, 5 mg/kg body weight (mean total dose, 12.5 mg/rabbit); SC dose, 2 mg/eye; TA dose, 0.2 mg/eye.

Table IV. Ocular anatomic dimensions and BCNU delivery after intravenous injection in rabbits

<table>
<thead>
<tr>
<th></th>
<th>Aqueous</th>
<th>Vitreous</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCNU concentration at t = 0 (µg/ml)</td>
<td>2.21</td>
<td>0.488</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>0.305^a</td>
<td>1.47^b</td>
</tr>
<tr>
<td>BCNU delivered at t = 0 (µg)</td>
<td>0.674</td>
<td>0.717</td>
</tr>
<tr>
<td>Surface area through which BCNU diffuses (mm^2)</td>
<td>620^a</td>
<td>680^b</td>
</tr>
<tr>
<td>BCNU delivered/surface area (µg/mm^2)</td>
<td>0.00109</td>
<td>0.00105</td>
</tr>
</tbody>
</table>

^a According to Kinsey and Reddy. 20
^b Calculated from the mean weight of vitreous measured in this study, on the basis that the density of the vitreous is 1.
^c Calculated from the mean diameter of the frozen eye measured in this study, using the schema of Kinsey and Reddy 20 as a model.

The penetration rate of nonelectrolytes across the BAB into the anterior chamber of rabbit is apparently related to their lipid solubility expressed as the ether/water partition coefficient. 18, 19 Our results show that BCNU penetrates rapidly not only into the anterior chamber through the BAB but also into the vitreous through the BRB. To interpret how BCNU penetrates into the eye across the BAB and BRB, the theoretical initial concentration in aqueous (C_{a}^{0}) and in vitreous (C_{v}^{0}) were related to the anatomic dimensions of the eye (Table IV). The C_{a}^{0} was found to be 4.5 times higher than the C_{v}^{0}, whereas the volume of the aqueous (V_{a}) is 1.8 times that of the vitreous (V_{v}). The theoretical delivered BCNU in aqueous (C_{a}^{0} \times V_{a}) was close to that in vitreous (Table IV). The theoretical delivered BCNU per unit of surface area through which BCNU is thought to diffuse, i.e., both sides of the iris, including the ciliary body, for the aqueous, and the retina for the vitreous, was also calculated (Table IV). These results indicate that BCNU penetration into rabbit aqueous and vitreous is related to the surface area through which the drug diffuses and is independent of the anatomic and physiologic differences between the BAB and BRB. Therefore it may be suggested that for BCNU the barrier systems (BAB and BRB) behave as though they are absent.

The simulated time course of BCNU in the aqueous and vitreous after subconjunctival injection does not fit the experimental results very well (Figs. 5 and 6). In our opinion, this disagreement is due to the variable distribution of the drug in the tissues after subconjunctival injection. The coefficient of variation of BCNU in the distribution phase was calculated for each experiment (Table V). Subconjunctival injection produced higher variation of BCNU concentration in both the aqueous and the vitreous than did the other administration modalities. All subconjunctival injections differ somewhat in exact location, amount of drug that leaks out through the injection site, 21 intratissue spreading of injection bolus, etc. Although it is relatively easy to reproduce intravenous and topical administration of drugs, it is more difficult to
Table V. Coefficient of variation of BCNU concentration in the distribution phase

<table>
<thead>
<tr>
<th>Analysis site</th>
<th>Administration method</th>
<th>Mean of coefficient of variation (%)</th>
<th>S.D. of the mean</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>SC</td>
<td>64.0*</td>
<td>24.8</td>
<td>3</td>
</tr>
<tr>
<td>Aqueous</td>
<td>IV</td>
<td>12.8</td>
<td>1.69</td>
<td>4</td>
</tr>
<tr>
<td>Aqueous</td>
<td>TA</td>
<td>18.9</td>
<td>8.94</td>
<td>4</td>
</tr>
<tr>
<td>Vitreous</td>
<td>SC</td>
<td>27.4</td>
<td>1.38</td>
<td>3</td>
</tr>
<tr>
<td>Vitreous</td>
<td>IV</td>
<td>21.5</td>
<td>1.86</td>
<td>3</td>
</tr>
</tbody>
</table>

1 = intravenous injection; SC = subconjunctival injection; TA = topical application.
*Significantly higher than other administration methods, p < 0.01 (determined by one-way analysis of variance).
†Significantly different from intravenous injection, 0.01 < p < 0.02.

be exact in administering subconjunctival injections.

The lag time can be observed only in the extravascular routes of administration.11 This is thought to be related to the complexity of the penetration route to reach the aqueous or the vitreous. The longest lag time was found in the aqueous after subconjunctival injection. The lag time in the vitreous after subconjunctival injection is shorter than that in the aqueous. The shortest lag time was in the aqueous after topical application. Because of the configuration of eye compartments and the site of administration (under the conjunctiva and on the cornea), we can assume that the most complex route for BCNU to reach the aqueous is after subconjunctival injection. The second most complex route for BCNU to reach the aqueous is after topical application.

We presume that the high variation of the results after subconjunctival injection is caused by the more complex penetration route for BCNU to reach the target site. Furthermore, the coefficient of variation was found to be related to the lag time (which is apparently related to the complexity of the route), according to the regression equation, y = 13.1x + 6.05, where y is the coefficient of variation and x is the lag time (relative coefficient r = 0.846 [n = 10], 0.001 < p < 0.01).

BCNU could not be detected in the vitreous after topical application to the cornea, even after administration of 1 mg/0.02 ml of sesame oil (five times the dose used in the aqueous experiment). This indicates that BCNU cannot diffuse into the vitreous through the barrier formed by the lens, the iris, and the countercurrent of the aqueous.

On the basis of the pharmacokinetic models (Fig. 1) and the experimental results, we propose the possible penetration routes of BCNU into the aqueous and vitreous from the various administration modalities, as shown in Fig. 8.

Several criteria are used to evaluate drug administration routes: peak level, biological half-life, and AUC. According to Woolley and Schein,16 AUC is the most important criterion because it provides an index of the total exposure of drug receptors over time and allows interspecies comparisons of drug levels. The therapeutic level of BCNU in plasma of patients who received the drug as part of their therapy for malignant brain tumor was observed to be in the range of 0.05 to 0.4 μg/ml.3 If the therapeutic level of BCNU in aqueous and vitreous is assumed to be the same as that in plasma, if the BCNU is distributed in an identical manner in rabbits and humans, and if the therapeutic level is the same as the minimum effective concentration (MEC) of BCNU against tumor cells in vivo, then the integration of the simulated time course from the time when the BCNU concentration reaches the MEC until it falls below the MEC (AUC|Hnt>MEC) can be calculated for the various routes of administration in the case that MEC = 0.05 and 0.4 μg/ml, respectively (Table III). From Table III we would predict that topical application would be the best and subconjunctival injection the second best route for the treatment of an iris tumor, and intravenous injection would be the best route for treatment of choroidal and retinal tumors.

To minimize the adverse effects of BCNU,1 the drug should not be administered to the whole body but only to the tumor. The ideal route would minimize the total dose but maximize the drug level at the tumor site and maintain this level as long as possible. From this point of view, our prediction concerning
the treatment of iris tumors holds true because the topical dose was 0.2 mg/eye; the subconjunctival dose, 2 mg/eye; and the intravitreal dose, 5 mg/kg body weight (mean total dose, 19.2 mg/rabbit). The prediction concerning choroidal and retinal tumors, that intravenous administration is better than subconjunctival, conflicts with the ideal. Minimizing the adverse effects of subconjunctival administration rather than those of intravenous administration is probably the better approach, since the difference in AUC^iv-auc between intravenous and subconjunctival injection was small compared with the difference in doses. It may be desirable to use a sustained-delivery system such as the silicone balloon5,6 to increase the AUC without increasing the total dose and the risk of adverse effects.

However, the BCNU partition ratios of aqueous, vitreous, and blood to intracellular space of ocular tumors may vary with stages of tumor growth, e.g., with tumor volume and vascularization.23,24 Therefore it is very difficult to extrapolate directly from pharmacokinetic data to clinical results. Further work is needed to improve chemotherapy for ocular malignancy.

REFERENCES
14. Weinkam RJ, Wen JHC, Furst DE, and Levin VA: Analysis for 1,3-bis(2-chloroethyl)-1-nitrosourea by