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# Decalin Analogs of Phenethylamines as Inhibitors of Amine Uptake by Rabbit Platelets II: Uptake of 5-Hydroxytryptamine

**JOUKO TUOMISTO *§, EDWARD J. WALASZEK *, EDWARD E. SMISSMAN ‡†, and THOMAS L. PAZDERNIK ‡ˣ**

**Abstract** □ The steric aspects of the uptake of 5-hydroxytryptamine by rabbit platelets were studied utilizing the conformationally rigid decalin analogs of ephedrine and amphetamine. These analogs or their parent compounds were added to platelet-rich plasma, and their influence on the uptake of $^{14}$C-5-hydroxytryptamine was determined along with the kinetics of the process. Among the β-phenethanolamine-type compounds, the isomer having an axial phenyl and equatorial amino function was the most effective inhibitor and the isomer in which the phenyl and amino functions were both axial was the weakest inhibitor of uptake. In the β-phenethylamine series, the same cisoid isomer [(a) phenyl, (e) $NH_2$] was the weakest inhibitor and the remaining three isomers were equipotent. The most effective cisoid decalin isomer of phenethanolamine displayed a mixed type of inhibition. The inhibition by amphetamine or β-phenethanolamine was also of a mixed type but was closer to competitive than to noncompetitive inhibition. Norepinephrine was a competitive inhibitor. The differences were suggested to be due to different binding of the aromatic ring. It was also suggested that lipid solubility causes the decalin derivatives to accumulate in the cell membrane and bind part of the carrier so that it is inaccessible to the substrate. Different lipid solubility would then explain the inconsistency of the most active conformation in different series. In essence, these results are in agreement with the inhibition of histamine uptake by rabbit platelets, which was earlier studied using a number of rigid and semirigid phenethylamine derivatives.

**Keyphrases** □ Phenethylamines, decalin analogs—conformationally rigid inhibitors of amine uptake by rabbit platelets, uptake and kinetics of 5-hydroxytryptamine □ Histamine uptake inhibitors—conformationally rigid inhibitors, decalin analogs of phenethylamines (ephedrine and amphetamine), uptake and kinetics of 5-hydroxytryptamine □ Decalin analogs of phenethylamines (ephedrine and amphetamine)—uptake and kinetics of 5-hydroxytryptamine by rabbit platelets, conformation effects □ Structure–activity relationships—decalin analogs of phenethylamines–histamine inhibition, kinetics and uptake of 5-hydroxytryptamine by rabbit platelets

Experiments concerning stereochemical aspects of the inhibition of histamine uptake by platelets have been reported from this laboratory (1). Several amines which were conformationally rigid substituted *trans*-decalins were studied. In general, a cisoid conformation in which the phenyl group is axial and the amine group is equatorial appeared to be more favorable for uptake inhibition. In three out of four cases, the most effective isomer had an axial phenyl and an equatorial amino group. Among β-phenethylamine-like decalin isomers, the axial phenyl and equatorial amino isomer was the least active; the most active had a diaxial structure.

To determine whether these findings were due to

secondary factors, such as effects on histamine metabolism during the long incubation, it was decided to utilize another substrate, 5-hydroxytryptamine. 5-Hydroxytryptamine is taken up rapidly, thus making it possible to use a very short incubation period which would minimize the effects of metabolism. A study of the kinetics of uptake is more facile with 5-hydroxytryptamine as compared to histamine because the active uptake is rapid, the saturation concentration is much sharper, and the role of diffusion is less than in the long incubations with histamine. Therefore, the information obtained as to competitiveness or noncompetitiveness of uptake inhibition is more reliable.

## EXPERIMENTAL

Platelet-rich plasma of male rabbits, 2.2–3.0 kg, was prepared as described earlier (2). It contained 522,000–1,100,000 platelets/mm$^3$. The substances to be tested were pipetted into polypropylene centrifuge tubes in 0.2 ml of saline or 3% dimethyl sulfoxide in saline. Then, $^{14}$C-5-hydroxytryptamine was added to a pool of ice-cold plasma to give the final concentration of $10^{-7}$ *M*. Two-milliliter plasma samples were rapidly added to the polypropylene tubes and incubated at 37° for 5 min. The incubation was terminated by placing the tubes in an ice bath, after which they were centrifuged for 30 min at 2000×*g*. The pellet was washed with 2 ml of saline and centrifuged again. The supernate was discarded, the tube was dried of excess saline with filter paper, and the pellet was solubilized[1]. The total radioactivity was counted[2] with a scintillation fluid containing 0.4% diphenyloxazole and 0.01% 1,4-bis(5-phenyloxazol-2-yl)benzene in toluene.

To study the reaction kinetics, a 1-min incubation period was used to represent the initial velocity of $^{14}$C-5-hydroxytryptamine ($10^{-7}$ *M*) and a necessary amount of cold carrier was used. The incubation in these experiments was terminated by adding 5 ml of ice-cold saline and centrifuging the tubes immediately for 5 min at 20,000×*g*. The pellet was washed with 2 ml of ice-cold saline, solubilized, and counted.

The noncommercial decalin derivatives and 2-amino-1-phenethanol were synthesized[3] as described earlier (3, 4). Dextroamphetamine sulfate[4], 5-hydroxytryptamine creatinine sulfate[4], β-phenethylamine hydrochloride[5], 5-hydroxytryptamine-3-$^{14}$C creatinine sulfate[6], and the scintillation chemicals[7] were obtained from commercial sources.

For calculating the reaction kinetics, Lineweaver–Burk (5) and Eadie–Hofstee (6, 7) plots were used. The standard error of the mean was calculated where necessary.

## RESULTS AND DISCUSSION

**Rate of Uptake**—5-Hydroxytryptamine uptake under the conditions described was extremely rapid (Fig. 1). Depending on the platelet count in plasma, around 40% of the added total amount of $^{14}$C-5-hydroxytryptamine was taken up during the 1st min, after which the rate decreased to give an uptake of 80% in the first 10 min. When the inhibitor was present, the rate was affected to the greatest extent at the start, with the uptake continuing for a longer time before reaching a plateau; thus the inhibition calculated after a 10-min incubation was very slight.

**Inhibition of Uptake**—All β-hydroxy-β-phenethylamine-like compounds inhibited 5-hydroxytryptamine uptake, and the most effective was the decalol derivative with an axial phenyl and equatorial amino group (Compound 3, Table I). The transoid isomer (Compound 4) was the weakest. All isomers except Compound 4 were more effective than β-hydroxy-β-phenethylamine itself.

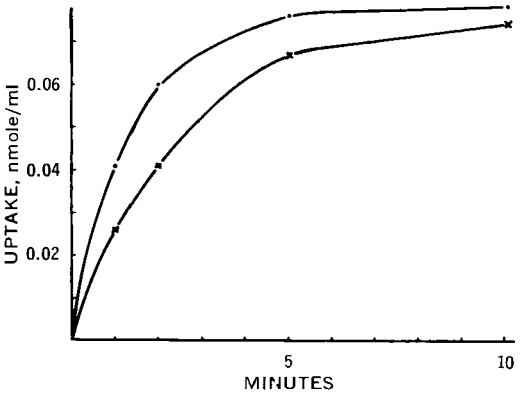



**Figure 1**—*Time course of 5-hydroxytryptamine uptake by rabbit platelets incubated in plasma. Added $^{14}$C-5-hydroxytryptamine concentration was $10^{-7}$ M, or 0.1 nmole/ml. The result is given as nanomoles taken up by platelets in the same volume. Upper curve is without an inhibitor. Lower curve is with $10^{-4}$ M 2(e)-amino-3(a)-phenyl-3-*trans*-decalol. (Mean of two duplicate virtually identical results.)*

Among β-phenethylamine-like compounds, the isomer with the same conformation as Compound 3 was the least active and about equal to the parent compound. All other compounds, two *gauche* and one *anti*, were equipotent.

**Reaction Kinetics**—When 1-min incubations were used to indicate the initial velocity and accumulation was plotted against 5-hydroxytryptamine concentration, a two-phase curve was obtained. At low concentrations, up to about $10^{-6}$ *M*, there appeared to be a saturable process; in high concentrations (from about $10^{-6}$ *M* to at least $10^{-4}$ *M*), a linear process existed (Fig. 2). The latter was interpreted to be due to diffusion of 5-hydroxytryptamine into the cells. The linear part of the curve was extrapolated to the ordi-

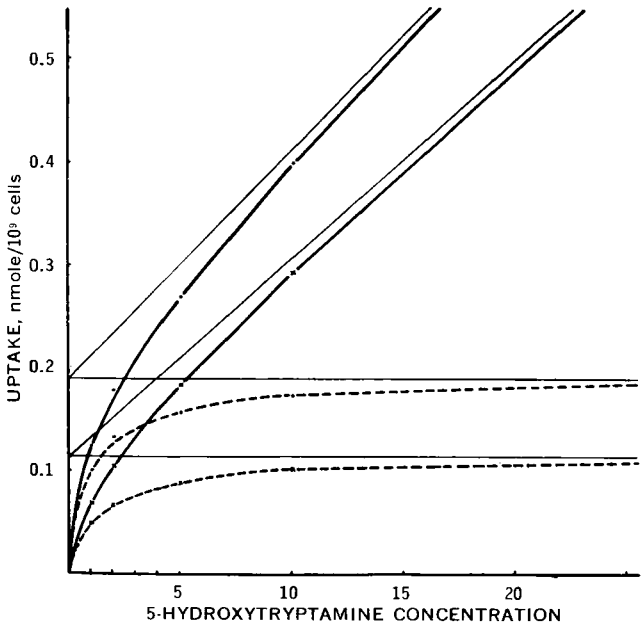
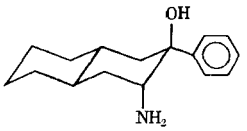
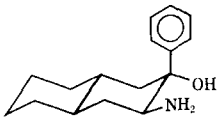
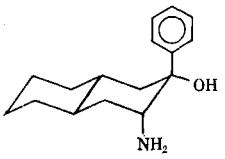
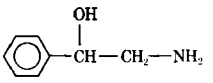
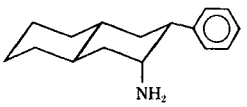
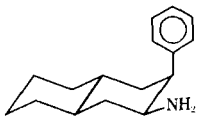
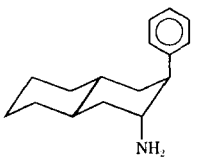
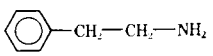



**Figure 2**—*Uptake of 5-hydroxytryptamine plotted against 5-hydroxytryptamine concentration. The thick solid lines represent the total uptake, the upper line without inhibitor and the lower line with $10^{-4}$ M 2(e)-amino-3(a)-phenyl-3-*trans*-decalol. The curves continued linear up to $10^{-4}$ M 5-hydroxytryptamine concentration, the highest concentration tested. The thin angles confine the linear part of uptake, which then is subtracted from the total, and the broken lines show the saturable part supposedly representing the active uptake without or with the inhibitor, respectively. The uptake is given per $10^9$ platelets. (Mean of four duplicate experiments.)*

[1] Soluene 100.
[2] Packard Tri-Carb scintillation counter.
[3] Department of Medicinal Chemistry, University of Kansas.
[4] Sigma Chemical Co.
[5] Calbiochem.
[6] Amersham/Searle.
[7] Packard Instrument Co.

**Table I**—Inhibition of $^{14}$C-5-Hydroxytryptamine Uptake in Percent by Some Conformationally Rigid Amino-*trans*-Decalins[a]

| Inhibitor | Structure | Inhibition, % |
|---|---|---|
| 1. 2(e)-Amino-3(e)-phenyl-3-*trans*-decalol |  | 26.0 ± 2.3 |
| 2. 2(a)-Amino-3(e)-phenyl-3-*trans*-decalol |  | 23.0 ± 2.9 |
| 3. 2(e)-Amino-3(a)-phenyl-3-*trans*-decalol |  | 39.8 ± 3.1 |
| 4. 2(a)-Amino-3(a)-phenyl-3-*trans*-decalol |  | 11.1 ± 3.4 |
| 5. β-Hydroxy-β-phenethylamine |  | 16.0 ± 4.0 |
| 6. 2(e)-Amino-3(e)-phenyl-*trans*-decalin |  | 58.5 ± 4.4 |
| 7. 2(a)-Amino-3(e)-phenyl-*trans*-decalin |  | 58.4 ± 6.8 |
| 8. 2(e)-Amino-3(a)-phenyl-*trans*-decalin |  | 25.3 ± 6.7 |
| 9. 2(a)-Amino-3(a)-phenyl-*trans*-decalin |  | 60.8 ± 4.4 |
| 10. β-Phenethylamine |  | 23.8 ± 8.7 |

[a] Rabbit blood platelets were incubated for 5 min at 37°. Values are means ± standard error of five experiments. Inhibitor concentration was $10^{-4}$ *M*.

nate, and this line was used for calculating the diffusion at different concentrations. The calculated diffusion was then subtracted from the total accumulation; as a result, a typical saturation curve was obtained (Fig. 2).

These new curves were used for Lineweaver–Burk and Eadie–Hofstee plots (Fig. 3) to determine the reaction kinetics. Without an inhibitor, both plots gave a $V_{max}$ of 0.18 nmole/$10^9$ platelets/min and $K_m$ of $0.85 \times 10^{-7}$ *M* when the results were corrected to the platelet count of $10^9$ platelets/ml plasma. With $10^{-4}$ *M* 2(e)-amino-3(a)-phenyl-3-*trans*-decalol (Compound 3), the $V_{max}$ was 0.11 nmole/$10^9$ platelets/min and $K_m$ was $1.4 \times 10^{-7}$ *M*. So there was a change both in $V_{max}$ and in $K_m$, indicating a mixed type of inhibition. On the other hand, norepinephrine under similar conditions showed competitive kinetics with no change in $V_{max}$ (Fig. 4). Amphetamine (Fig. 5) and β-hydroxy-β-phenethylamine (Fig. 6) both showed a mixed type of kinetics but with less change in $V_{max}$ than in $K_m$. Thus, they appear to be closer to competitive inhibitors than the decalin derivative.

The $V_{max}$ and $K_m$ values of the first series of experiments were lower than the others (compare Figs. 3 and 4–6, respectively). This finding is probably due to the use of a thicker tube in the first experiments, which caused a slower rise in temperature when the tubes were placed into the water bath; thus the actual incubation time was shorter than in the later experiments. (The reason for change of tubes was the purchase of a new centrifuge with slightly different tubes.)

The two series of decalin derivatives gave almost identical results for 5-hydroxytryptamine uptake compared with histamine uptake by platelets. Therefore, it can be assumed that the effect is on the uptake process itself and not on amine metabolism.

The noncompetitive component of uptake inhibition may mean that these compounds bind differently to the amine carrier compared with the substrate. However, they might also bind similarly, if it is assumed that their high lipid solubility causes them to accumulate on the cell membrane. If the freely moving receptor of the carrier were occupied by the excess decalin derivative inside the membrane so that it could not attract the polar substrate molecules outside the membrane in the incubation medium, the inhibition would evidently be partly noncompetitive regardless of the actual mode of binding.

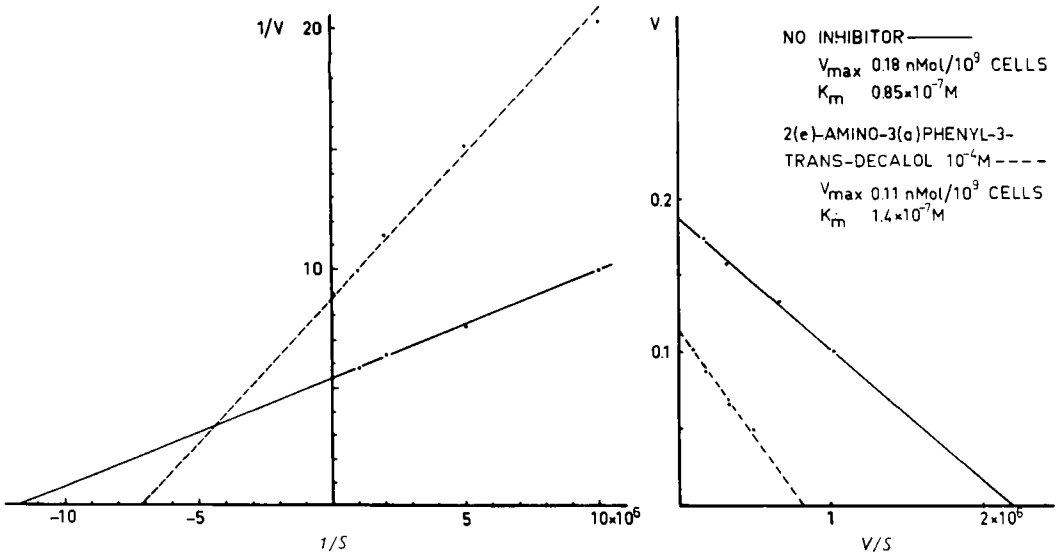



**Figure 3**—*Lineweaver–Burk plot (left) and Eadie–Hofstee plot (right) drawn on the basis of the saturable process of the same material as in Fig. 2.*

On the other hand, experiments with various amines inhibiting histamine uptake suggest that binding may be different. Heterocyclic amines might bind by ionic forces and van der Waals forces, but aliphatic amines may bind by ionic forces and hydrophobic forces (8). The saturated decalin structure would evidently then belong to the last category. The decalin derivatives of amines might thus have two possible ways of binding, one using the phenethylamine moiety and the other using the amino-*trans*-decalin moiety.

Since the suggested ionic binding through the amino nitrogen appears to be by far the most important mode of binding, it is possible that the amine function alone would be enough to inhibit uptake if the concentration is high enough. Thus, it may not be necessary that the secondary binding site, *i.e.*, the suggested hydrophobic site, be present in the carrier molecule. If this is the case, the results with highly lipid-soluble uptake inhibitors, such as the decalin derivatives, do not give a reliable indication of conformational preferences. Therefore, the differences in lipid solubility and other physicochemical properties could be the most important aspects of the activity. This would explain the inconsistency of the most effective conformation in different series of decalin derivatives as histamine uptake inhibitors (1) and with 5-hydroxytryptamine uptake inhibitors as shown in this study. The possibility of one-point inhibition is favored by the earlier finding that plain amino-*trans*-decalin is only a slightly weaker inhibitor than the 2-amino-3-phenyl-*trans*-decalins (1). Therefore, the presence of a phenyl group increases the potency but it is not necessary for activity and its influence is small enough that in some cases it may be negated by opposing factors.

It is necessary to incubate for a very short period to obtain a reliable initial velocity, and it is absolutely necessary to subtract the linear part of the uptake. If these steps are omitted, even results with decalin derivatives may show an apparently competitive type of inhibition, at least in Lineweaver–Burk plots. If the concentration is too high and/or incubation prolonged, one is dealing with a complex situation where inhibition on the outer membrane, metabolism of excess amine by mitochondrial monoamine oxidase, and inhibition at the granular level all have their own role. In these circumstances, one might have several zero-order and first-order processes taking place at the same time. Actually an incubation time of less than 1 min would be preferable, but using this technique it would not be possible to maintain the incubation time constant in different tubes.

In most early studies, amphetamine has been considered a purely competitive inhibitor. It would be important to know if this is due to a longer incubation time and/or to the higher concentrations used. If one accepts the model of binding suggested earlier (8), the differences among the amphetamines and the catecholamines or heterocyclic amines could be explained since the benzyl ring of amphetamine would bind in part through hydrophobic forces, in contrast to pure van der Waals-type binding of catechol, indole, or imidazole rings.

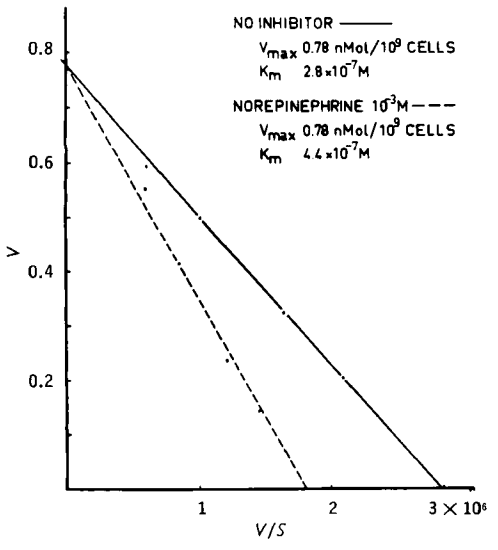



**Figure 4**—*Kinetics of 5-hydroxytryptamine uptake with norepinephrine as an inhibitor; Eadie–Hofstee plot according to the same principles as in Fig. 3. (Mean of four duplicate experiments.)*

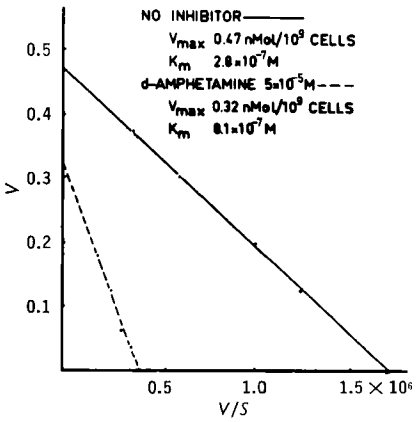



**Figure 5**—*Kinetics of 5-hydroxytryptamine uptake with dextroamphetamine as an inhibitor. (Mean of four duplicate experiments.)*

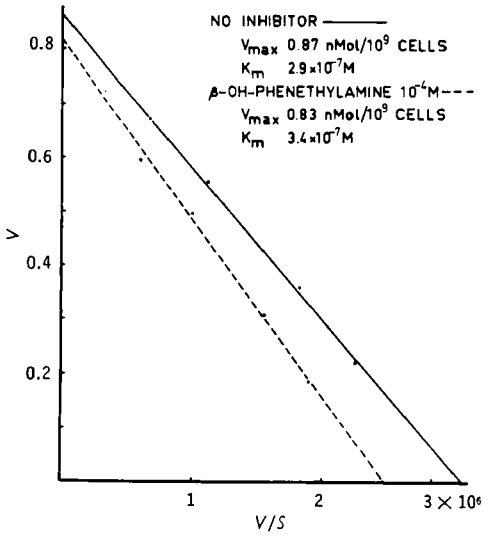



**Figure 6**—*Kinetics of 5-hydroxytryptamine uptake with β-hydroxy-β-phenethylamine as an inhibitor. (Mean of four duplicate experiments.)*

In conclusion, it is felt at this phase of the investigation that partly noncompetitive uptake inhibitors with large nonpolar groups may attach to the transport carrier in a relatively nonspecific manner and, in some cases, only through the amino group, which seems to be the only absolute requirement for the inhibition. The relatively high activity in the cases studied might be explained by the accumulation of the inhibitor in high concentrations at the cell membrane. To prove conclusively that one conformation of phenethylamine-type inhibitors is actually the most effective, relatively small molecular weight compounds that possess a lipid solubility not too different from the parent compound should be investigated.

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# Distribution of $^{14}$C-Lomustine ($^{14}$C-CCNU)-Derived Radioactivity following Intravenous Administration of Three Potential Clinical Formulations to Rabbits

**CHARLES L. LITTERST *x, EDWARD G. MIMNAUGH *, ALAN C. COWLES [‡], THEODORE E. GRAM *, and ANTHONY M. GUARINO ***

**Abstract** □ $^{14}$C-Lomustine (CCNU) was administered intravenously to rabbits in three potential clinically useful vehicles of propylene glycol–ethanol (4:1), fat emulsion, and vegetable oil emulsion; the organ distribution of the radioactivity was followed for 24 hr. Bile and fat were the only tissues showing a consistent vehicle-dependent change in the distribution of radioactivity, and in these two instances the fat emulsion and the vegetable oil emulsion both produced the same distribution pattern. Liver, kidney, and lung tissue consistently contained the highest amounts of radioactivity. By 12 hr after dosing, essentially all radioactivity had been eliminated by the animals. Binding of radioactive drug to plasma proteins was found to be twice as great *in vitro* (93%) as *in vivo* (51%).

**Keyphrases** □ Lomustine, radiolabeled—distribution of radioactivity, three vehicles tested, intravenous administration, rabbits □ 1-(2-Chloroethyl)-3-(cyclohexyl-1-$^{14}$C)-1-nitrosourea (lomustine)—distribution of radioactivity, three vehicles compared, intravenous administration, rabbits □ Antineoplastic agents—radiolabeled lomustine, effect of three vehicles on distribution of radioactivity, intravenous administration, rabbits

Lomustine, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU, I), is a potent antineoplastic drug utilized in the treatment of Hodgkins disease and other solid tumors (1). Although it shares the nitrosourea structure with several other drugs, I differs from other nitrosoureas in having no satisfactory parenteral dose formulation. In experimental animals and humans, I has been administered in propylene glycol–