Pharmacokinetics of Two Prototype Nitroxide Spin Labels for Contrast Enhancement in Magnetic Resonance Imaging

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Abstract: Two nitroxide spin labels called PCA and TES have been used experimentally as contrast enhancing agents in magnetic resonance imaging. Pharmacokinetic data for these nitroxides, after intravenous administration to three dogs at two dose levels (0.1 and 2.5 mmole/kg), are presented. In this dose range, the clearance (13 ml/min-kg) and half-life (22 min) of PCA stayed almost constant while TES clearance decreased (28 to 12 ml/min-kg) and half-life increased (8 to 17 min) with dose. The urinary recovery, determined from the sum of the nitroxide and its corresponding hydroxylamine, was 85 to 90 % for both PCA and TES. PCA was investigated in more detail because of its lower clearance value and its lack of dose-dependence. The major pathways of elimination are renal excretion and metabolic reduction to the corresponding hydroxylamine which is eliminated by renal excretion. We estimated renal clearance of both PCA (5.5 ml/min-kg) and its hydroxylamine (3.7 ml/min-kg) to be close to glomerular filtration rate (4 ml/min-kg) in the dog, while the metabolic clearance (7.5 ml/min-kg) was slightly higher. Approximately 35 % of the administered dose of PCA was excreted unchanged and half was reduced in the body.

The relaxation times of hydrogen nuclei are decreased in the presence of paramagnetic molecules. This property of proton relaxation enhancement has permitted the successful application of nitroxide spin labels (subsequently often referred to as nitroxides) for contrast enhancement of proton magnetic resonance imaging (MRI) (1–5). A pyrrolidinic compound called PCA and a piperidinic compound called TES have been used experimentally in animals to enhance the MRI intensities of normal functioning kidneys (2), defects in the blood brain barrier caused by radiation damage or infection (3), myocardial perfusion abnormalities (4), and implanted tumors (5). Both PCA and TES are relatively strong proton relaxing agents reducing T_1 relaxation times of living tissues as much as 50 % (3). Our preliminary pharmacokinetic study showed that PCA and TES are extensively reduced *in vivo* (6). The reduction, presumably to the corresponding hydroxylamine, causes loss of the paramagnetic property and, consequently, disappearance of the contrast enhancing activity. In this report, we present pharmacokinetic data on these two nitroxide spin labels given at two dose levels in three dogs. An attempt is made to estimate the relative importance of the process of metabolic reduction and renal excretion in the overall elimination of the nitroxides after intravenous administration.

NH–CO–$(CH_2)_2$–COOH
H_3C CH_3 N H_3C CH_3 O•
TES

COOH
H_3C CH_3 N H_3C CH_3 O•
PCA

Materials and Methods

Chemicals

2,2,5,5-Tetramethylpyrrolidine-1-oxyl-3-carboxylic acid (PCA) was obtained from Eastman Kodak Company (Lot A10, Rochester, NY). *N*-Succinyl-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (TES) was synthesized and purified by thin-layer chromatography in our laboratory as previously described (7). PCA and TES (structures given below) were stored at 4°C as aqueous solutions buffered to pH 7.4 with 0.067 M phosphate buffer at concentrations of 0.85 M and 0.25 M, respectively. Solutions were passed through a 0.02 µm filter (Acrodisc*, Gelman Sciences, Inc.) prior to intravenous administration.

Analytical Instruments

An electron paramagnetic resonance (EPR) spectrometer (Model E-104A, Varian Instrument Division), with a 0.04 ml flat cell placed in the microwave cavity, was used to determine the concentration of the nitroxide spin labels. The height of the first derivative of the low field peak of the nitroxide triplet spectrum was used for the measurements.

Protein Binding

Protein binding of the nitroxide spin labels in plasma was determined by ultrafiltration. Fresh dog plasma was spiked with nitroxide at concentrations of 0.1 mM and 1 mM and incubated for 30 minutes at 37 °C. The spiked plasma sample (500 µl) was transferred to the sample reservoir of the ultrafiltration device (micropartition system MPS-1, Scientific Systems Division, Amicon Corporation), and centrifuged for 15 minutes at 2000 g and 37 °C. The concentration in the ultrafiltrate was measured in the EPR spectrometer and compared to that of a standard solution in water. Fraction unbound was calculated from the ratio of the ultrafiltrate and original nitroxide plasma concentrations.

In Vivo Experiments

Experimental design: Three conditioned male beagle dogs (Marshall Research Animals, North-Rose, NY) weighing 10.5 to 15.0 kg were used. Each dog was given 0.1 and 2.5 mmole/kg of PCA and TES on separate occasions at least two weeks apart. The dog was positioned in a sling and frame, catheters were placed in a limb vein (Angiocath No. 2818, The Deseret

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Company, Sandy UT) for venous blood sampling and in the urinary bladder (Swan-Ganz, Monitoring catheter 93–111–7F, Edwards Lab. Inc., Anasco, Puerto Rico 00610) for urine collection. The venous sampling catheter was kept patent throughout the study by infusing normal saline (20 to 50 ml/h) between blood collections. Blank blood and urine samples were collected before administration of the nitroxide.

Each nitroxide spin label was administered as a bolus over 1 min. or less into a limb vein different from that of the sampling site. An intermittent infusion set was used for the injection except for the high dose of TES which was infused (quadruple syringe infusion/withdrawal pump, Model 600–930, Harvard Appartus Co. Inc., Dover, MA) into two different veins due to the large volume (110 to 150 ml) injected. The line was flushed with 10 ml of normal saline after the injection.

Blood samples were collected for 6 h at time intervals of 5 to 10 min within the first 30 min, 15 to 30 min for the next 90 min and every 30 min for the last 4 h. After 360 min, the bladder was flushed with 20 ml of normal saline and allowed to empty. All the catheters were removed, and the dog was transferred to a metabolic cage where urine was collected for another 18 h. Urine volumes were recorded and a portion was frozen for subsequent analysis. In two particular studies, urine was collected in three separate fractions, 0 to 2, 2 to 6 and 6 to 24 h after injection.

Analysis of Samples

Blood was always assayed for nitroxide concentration within 5 minutes of sampling. A standard curve was prepared from spiked blood samples measured immediately after their preparation. For the dose of 0.1 mmole/kg, blood was collected in plastic syringes and directly injected into the EPR cavity. For the dose of 2.5 mmole/kg, blood was collected in plastic syringes, immediately transferred to tubes (Vacutainer* #6428, Becton and Dickinson) containing oxalate/fluoride, and diluted five times with distilled water before measurement. In one particular experiment (PCA, 2.5 mmole/kg), both plasma and blood concentrations were measured. Nitroxide concentrations in blood and plasma were measured by EPR spectrometry as previously described (6). The specificity, sensitivity, reproducibility and linearity of the assay in blood were similar to those in plasma (6).

Blood samples were oxidized for the determination of the concentration of the reduced form of PCA. After the last blood collection, 0.5 ml of each sample was mixed with 2 ml of methanol, vortexed for 10 sec and centrifuged 1 min at 3000 g. The methanolic supernate was transferred to a closed vial and kept 1 to 2 days before measurement. The hydroxylamine concentration was determined from the difference between the total (nitroxide plus hydroxylamine) and initial (direct and immediate assay of whole blood) nitroxide concentrations.

Urine was oxidized by addition of 50 μl of 30 % hydrogen peroxide to 100 μl of urine. After 24 h, the mixture was diluted 30 to 100 times with water and analyzed by EPR spectrometry for nitroxide concentration.

Pharmacokinetic Analysis of Data

The following abbreviations were used for selected pharmacokinetic parameters:

$CL_T(Nit)$ = Total body clearance of the nitroxide.
$CL_M(Nit)$ = Metabolic clearance of the nitroxide.
$CL_R(Nit)$ = Renal clearance of the nitroxide.
$CL_R(Hyd)$ = Renal clearance of the hydroxylamine.
$Ae_T(0\text{-}t_1)$ = Total amount of drug (after reoxidation) excreted in urine from time zero to time t_1.
$Ae_T(t_1\text{-}t_2)$ = Total amount of drug (after reoxidation) excreted in urine from time t_1 to time t_2.
$AUC(Nit,0\text{-}t_1)$ = Area under the nitroxide blood concentration versus time curve from time zero to time t_1.
$AUC(Nit,t_1\text{-}t_2)$ = Area under the nitroxide blood concentration versus time from time t_1 to time t_2.
$AUC(Hyd,0\text{-}t_1)$ = Area under the hydroxylamine blood concentration versus time curve from time zero to time t_1.
$AUC(Hyd,t_1\text{-}t_2)$ = Area under the hydroxylamine blood concentration versus time from time t_1 to time t_2.

The rate constant of the decay of the nitroxide blood concentration with time, k(Nit) and its corresponding half-life, $t_{1/2}$ (Nit), were estimated by linear least squares fit to the data points (time, log concentration) in the terminal phase.

The area under the nitroxide blood concentration versus time curve from time zero to infinity, AUC(Nit), was calculated using the trapezoidal rule. The area remaining under the curve after the last measured concentration, C(last), was determined from C(last)/k(Nit). Nitroxide total body clearance was calculated as the ratio of dose and AUC(Nit).

The renal clearances of the nitroxide spin label and of its hydroxylamine metabolite were calculated from the two following equations:

$$Ae_T(0\text{-}t_1) = CL_R(Nit) * AUC(Nit,0\text{-}t_1) + CL_R(Hyd) * AUC(Hyd,0\text{-}t_1) \quad (1)$$

$$Ae_T(t_1\text{-}t_2) = CL_R(Nit) * AUC(Nit,t_1\text{-}t_2) + CL_R(Hyd) * AUC(Hyd,t_1\text{-}t_2) \quad (2)$$

The metabolic clearance of the nitroxide was calculated from the difference between total and renal clearances. The fraction of nitroxide elimination that occurs by excretion of unchanged drug was calculated as the product of renal clearance and area under the curve (blood nitroxide concentration versus time from time 0 to infinity) divided by total amount of nitroxide recovered in urine after reoxidation.

The volume of distribution of the hydroxylamine was calculated as the ratio of total clearance and rate constant of the decay of the total blood concentration.

Results

The pharmacokinetic parameters calculated for PCA and TES are presented in Tables I and II, respectively. A typical example of a semilogarithmic plot of blood concentration versus time is shown in Figure 1 for PCA at two different doses in the same dog.

The blood concentrations and the ratio of the blood and plasma concentrations obtained during the same experiment are reported in Table III.

The average recoveries at 24 h for PCA and TES after reoxidation (nitroxide + hydroxylamine), at the two different doses are presented in Table IV. The per cent of the dose excreted in urine from 6 to 24 h after administration was always less than 10 % for both PCA and TES.

The fraction unbound was 1.0 ± 0.1 (Mean ±S.D.) for PCA at concentrations of both 0.1 mM and 1.0 mM.

The terminal half-lives of the decay of PCA concentrations measured after oxidative treatment were 96.5 ± 13.0 min and

Table I Pharmacokinetic Parameters of PCA in Dogs

	Clearance[b] (ml/min-kg)	Volume of Distribution[c] (ml/kg)	Half-life[d] (min)
		0.1 mmole/kg[a]	
Dog #1	13.6	459	23.4
Dog #2	13.5	401	20.6
Dog #3	12.4	410	22.8
M ± SD[e]	13.2 ± 0.7	423 ± 32	22.3±1.5
		2.5 mmole/kg[a]	
Dog #1	12.2	487	27.6
Dog #2	11.5	457	28.6
Dog #3	10.0	474	32.9
M±SD	11.2±1.2	479±8	29.7±2.9

[a] Drug administered intravenously.
[b] Determined from Dose/AUC.
[c] V = Cl/k, where k is the slope of the terminal line, after 10 min.
[d] From least square fit of line after 10 min.
[e] Mean ± standard deviation of the parameters of the 3 different dogs.

Table II. Pharmacokinetic Parameters of TES in Dogs

	Clearance[b] (ml/min-kg)	Volume of Distribution[c] (ml/kg)	Half-life[d] (min)
		0.1 mmole/kg[a]	
Dog #1	29.9	262	6.1
Dog #2	26.3	373	9.8
Dog #3	27.1	332	8.5
M±SD[e]	27.8 ± 1.9	322 ± 57	8.1 ± 1.9
		2.5 mmole/kg[a]	
Dog #1	12.3	-	-
Dog #2	11.2	300	16.2
Dog #3	12.2	320	18.1
M ± SD	11.9 ± 0.6	-	-

[a] Dose administered intravenously.
[b] Determined from Dose/AUC.
[c] V = Cl/k, where k is the slope of terminal life, after 10 min.
[d] From least square fit of line after 10 min.
[e] Mean ± standard deviation of the parameters of the 3 different dogs.

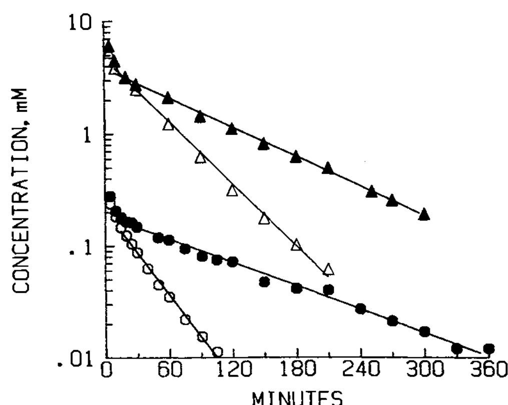

Fig. 1 Blood PCA concentration with time in Dog #3. The concentration was measured as soon as possible after sampling (open symbols) and after reoxidation (closed symbols). Circles represent the 0.1 mmole/kg dose and triangles the 2.5 mmole/kg dose. Linear least square fit of the different sets of data are shown.

Table III. PCA Blood Concentration and Blood to Plasma Concentration Ratio

Sampling time[a] (min)	Blood Concentration (mM)	B/P[b]
5	6.09	0.89
10	4.49	0.89
20	3.19	0.85
30	2.50	0.88
60	1.22	0.84
90	0.62	0.89
120	0.31	0.89
150	0.17	0.77
180	0.10	1.00

[a] Time after injection of PCA 2.5 mmole/kg in a dog.
[b] Ratio of PCA concentrations in blood and plasma.

Table IV. Urinary Recovery of PCA and TES[a]

	PCA		TES	
Dose (mmole/kg)[b]	0.1	2.5	0.1	2.5
M ± SD[c]	83.4 ± 8.6	89.8 ± 5.5	86.9 ± 11.7	88.1 ± 16.4

[a] Average values following oxidation of urine.
[b] Dose administered intravenously.
[c] Mean recovery ± standard deviation in 3 dogs.

74.3 ± 5.1 min, following the low and the high doses, respectively.

The estimates of the metabolic and renal clearance of PCA after administration of the 0.1 mmole/kg dose to two different dogs, are presented in Table V. The excretion of unchanged PCA, as a per cent of total drug recovered in urine, was estimated to be 56 % in Dog #1 and 45 % in Dog #3. The renal clearance and volume of distribution of the hydroxylamine were found to be equal to 4.0 ml/min-kg and 534 ml/kg in Dog #1 and to 3.4 ml/min-kg and 421 ml/kg in Dog #3.

Table V. Renal and Metabolic Clearances of PCA[a]

	Dog #1	Dog #3
Renal clearance (ml/min-kg)	5.8	5.2
Metabolic clearance (ml/min-kg)	7.8	7.2

[a] Calculated in 2 dogs after intravenous administration of PCA at a dose of 0.1 mmole/kg.

Discussion

In a previous study (6), we observed that PCA and TES were extensively reduced *in vivo* to their corresponding hydroxylamines. Neither the further reduction of the hydroxylamine to the corresponding amine nor the formation of other metabolites containing the nitroxyl moiety was observed. Thus, the EPR measurements were specific for the nitroxide administered. The spontaneous oxidation of the hydroxylamine to the nitroxide was noted previously (≥20 % in 30 min) during preparation of plasma samples (6). To reduce the risk of such oxidation, centrifugation was avoided by measuring nitroxide

concentration in blood rather than plasma and the time between sampling and measurement was limited as much as possible.

The pharmacokinetic parameters of PCA showed only minor differences with dose. The average value of clearance decreased 15 % while the average volume of distribution and half-life increased 13 % and 33 %, respectively, on increasing the dose. These changes are negligible compared to the 25-fold larger dose administered. With TES, clearance decreased more than 2-fold at the higher dose. The average values for volume of distribution and half-life, calculated from Dogs #1 and #3 after administration of TES at the higher dose, were 310 ml/kg and 17.1 min. In Dog #2 the half-life decreased with time, therefore no values for this parameter or the volume of distribution were obtained. The experiment was not repeated because of the expense of the large dose. No model for the dose dependency was proposed or tested.

The almost total absence of nonlinear kinetics, the greater stability in plasma (6), and the lower clearance value favored the use of PCA in our search for contrast agents in MRI. We therefore subsequently gave more attention to PCA.

PCA showed no appreciable binding to plasma proteins. This finding is consistent with the lack of broadening of the peaks in the EPR spectra of PCA between water and plasma solutions. Such spectral changes have been suggested as a method to estimate plasma protein binding (8).

An experiment was conducted in which both blood and plasma concentrations were measured after administration of PCA at the high dose. The observation of a practically constant ratio (0.88 ± 0.06) of blood and plasma PCA concentrations, even at early times, suggests that PCA penetrates easily and rapidly into red blood cells. Because the ratio is close to one, the pharmacokinetic parameters calculated from blood and plasma measurements are similar.

The hydroxylamine present in urine was oxidized to the nitroxide by the addition of hydrogen peroxide. The addition of this oxidant causes oxygen production in the sample which interferes with the measurements. However, because of the high concentration of total nitroxide (nitroxide plus hydroxylamine) present in urine, it was possible to eliminate this problem by diluting the sample in water before measurement. The large urinary recovery of the dose administered indicated that the reoxidation treatment was satisfactory and that the amine, which should not be reoxidized to the nitroxide simply by the action of hydrogen peroxide (9), or any other metabolite must account for 15 % or less of the dose.

Hydrogen peroxide could not be used as an oxidant for blood samples, because of foam formation. The addition of methanol to the blood samples precipitated the proteins, and a spontaneous oxidation of the hydroxylamine present in the supernate was observed (6). When such a methanolic supernate was kept for 1 to 2 days before measurement, the oxidation of the hydroxylamine appeared to be complete since the nitroxide concentration was stable. Addition of hydrogen peroxide did not further increase the measured concentration but caused a larger variation in the measurements because of oxygen production.

Analysis of blood nitroxide concentrations after reoxidation were conducted with PCA to assess the pharmacokinetics of the corresponding hydroxylamine. Because PCA was eliminated faster than the hydroxylamine, the average terminal half-life measured after oxidative treatment of blood was just that of the hydroxylamine. The values of this half-life, 96.5 ± 13.0 and 74.3 ± 5.1 min for the low and the high doses, respectively, are in good agreement with that (85 min) previously observed in plasma (6) for a 0.55 mmole/kg dose.

The relative importance of the reduction process in the overall elimination of PCA was a question of great interest in our search for nitroxides more resistant than PCA to reduction *in vivo*. Because PCA is reduced in urine (6), it was difficult to know the amount of PCA excreted in the unchanged and reduced forms, and to calculate renal clearance. However, by collecting urine in two different fractions and measuring the area under the blood concentration-time curves for both the nitroxide and its hydroxylamine in each collection period, we could estimate the renal clearance of the two substances, as shown in equations *(1)* and *(2)*. This calculation shows that approximately 35 % of the administered dose of PCA is excreted unchanged and half is reduced in the body.

The renal clearances of both the nitroxide and the hydroxylamine were close to the glomerular filtration rate (4 ml/min-kg) (10) in both dogs. The metabolic clearance for reduction of the nitroxide to the hydroxylamine was slightly higher than the renal clearance.

We previously concluded (6) that the hydroxylamine form of PCA is neither oxidized to the nitroxide *in vivo* nor transformed to another metabolite. The hydroxylamine should be exclusively eliminated by renal excretion, that is, its renal clearance should be equal to its total clearance. From this we estimate the volume of distribution of the hydroxylamine to be close to that of PCA. The pharmacokinetic properties and imaging capabilities of PCA have now been studied in some detail, and it appears to be the more promising nitroxide at this time. It is a prototype for the development of better contrast agents for MRI.

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