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RESEARCH ARTICLES

Pharmacokinetics and Metabolic Fate of Two Nitroxides Potentially Useful as Contrast Agents for Magnetic Resonance Imaging[5]

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Abstract: Paramagnetic nitroxyl-containing compounds have been useful as contrast agents in magnetic resonance imaging (MRI) experiments in animals. Preliminary information on the metabolic fate, pharmacokinetic behavior, stability in tissues, and chemical reduction of two prototypic nitroxides, PCA and TES, is presented. In the dog TES was eliminated more rapidly than PCA. More than 80 % of the dose of both nitroxides was recovered in urine within 6 hours. Nitroxides were reduced *in vivo* to their corresponding hydroxylamines. No other metabolite was observed. Measured reducing activity in tissue homogenates was greater in liver or kidney than in brain, lung or heart. In each tissue PCA was more stable than TES. PCA was also more resistant to reduction by ascorbic acid at physiologic pH. These preliminary results favor the use of PCA, a pyrrolidinyl nitroxide, over TES, a piperidinyl nitroxide, for MRI contrast enhancement.

Nitroxide spin labels have been shown, in animal studies, to have a potential diagnostic applicability as contrast enhancing agents for magnetic resonance imaging (MRI) (1). Applications include the identification of renal disease (2), demonstration of blood-brain-barrier anatomical defects (3), and enhancement of infarcted myocardium (4). Because of their paramagnetic properties nitroxides decrease the relaxation times (T_1 and T_2) of the hydrogen nuclei in the tissues into which they distribute. The contrast between various tissues depends on the relative distribution of nitroxides in these tissues. Knowledge of the metabolic fate and pharmacokinetics of these nitroxides is important in assessing their potential advantages and in optimizing the timing of imaging. Nitroxides are potentially reduced *in vivo* to diamagnetic molecules. However, there is limited information on the pharmacokinetic behavior of this class of compounds (2, 5). In this report, we present preliminary data on the metabolic fate and pharmacokinetics of the two water soluble nitroxides previously tested in animals as *in vivo* MRI contrast agents (2–4). Stability both *in vivo* and *in vitro* is considered.

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 (6). PCA and TES (structures given below) were stored as dry powders or as aqueous solutions buffered to pH 7.4 with 0.067 M phosphate buffer. Solutions were passed through a 0.2 μm filter (Acrodisc®, Gelman Sciences, Inc.) prior to intravenous administration. L-Ascorbic acid was obtained from Sigma Chemical Co.

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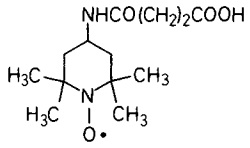
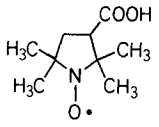


TES



PCA

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 nitroxide concentrations. The height of the first derivative of the low field peak of the nitroxyl triplet spectrum, where the EPR spectrum of ascorbate radical ion does not interfere (7), was used for these measurements.

A high performance liquid chromatograph (HPLC) (Model 6000 A, Waters Associates), equipped with an automatic injector (WISP 710 B, Waters Associates), variable wavelength UV detector (Model SF 770, Kratos Analytical Instruments), and integrator (Model 3390A, Hewlett Packard), was also used to measure the concentration of the nitroxides. A stainless steel column, 250 × 3.2 mm, packed with μBondapack Phenyl® material, 5 μm particle size (Waters Associates), and a 40 × 3.2 mm precolumn packed with C_{18} material, 30–38 μm particle size, were used at ambient temperature. The mobile phase was 25 % methanol in 20 mM phosphate buffer, pH 2.2, for the plasma assay; the concentration of methanol was 15 % in the same buffer for the urine assay. The flow rate was 2.6 ml/min and the column effluent was monitored at 245 nm. The retention times of PCA and TES were 4.7 and 4.8 minutes, respectively, for the plasma assay and 10.0 and 10.5 minutes for the urine assay.

In Vivo Experiments

Experimental design. A conditioned male mongrel dog weighing 30 kg was given 0.55 mmol/kg of PCA and TES on separate occasions two weeks apart. The dog was positioned in a sling and catheters were placed in a hindlimb vein and in the urinary bladder, respectively, for collection of blood and urine. Hydration was maintained with a continuous drip infusion of normal saline at a flow rate of 25–50 ml/h. After collection of blank samples, PCA or TES was administered intravenously over approximately one minute with an intermittent infusion set (Butterfly INT, Abbott Hospitals, Inc.) in a leg different from that of sampling. Following the injection, the line was flushed with 10 ml of normal saline. Blood (5 ml) was collected in tubes (Vacutainer®, #6428 Becton and Dickinson) containing oxalate/fluoride at 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360 minutes after the midpoint of the injection time. Urine was collected for 360 minutes. The bladder was flushed with 20 ml of normal saline at the end of the experiment, and the total volume was recorded.

Studies of both the pharmacokinetics and the metabolic fate of the two nitroxides used the same experimental design. However, the experiments were conducted on separate occasions, and the samples were handled differently as subsequently described. The dog was used here as the experimental animal, because 2 ml of plasma was necessary for the EPR measurement of each time sample.

Samples for pharmacokinetic studies. Blood was centrifuged for 1 min at 3000 g, plasma was directly injected into the EPR cavity within 5 minutes of sampling for measurement of the nitroxide concentration. After the last blood collection, 2 ml of each plasma sample was oxidized with 100 μl of a 30 % solution of hydrogen peroxide to determine the sum of the nitroxide and its corresponding hydroxylamine. These oxidized samples were measured 24 hours later. Samples kept for 48 hours showed no further increase in the concentration. The measurements on each day were compared to standards prepared in plasma and treated identically. Urine was collected and oxidized as above, before measurement. The urine was diluted so that the measured concentration was within the standard range.

Samples for metabolic studies. Blood was centrifuged immediately after collection, and plasma was stored at −20°C until analyzed. Urine was collected and frozen until analyzed. Plasma and urine samples were assayed within three days of each experiment, although storage for up to one month produced no detectable change in the nitroxide concentration. Samples were thawed and brought to room temperature on the day of analysis. Plasma samples were prepared by mixing 1 ml of plasma with 3 ml of methanol to precipitate proteins. After centrifugation, 20 μl of the supernate were directly injected onto the HPLC precolumn, and 2 ml were placed into the cavity of the EPR spectrometer. Plasma samples were also measured after the addition of 100 μl of a 30 % solution of hydrogen peroxide to 2 ml of plasma for 24 hours. Urine was only measured after oxidation and treatment with methanol.

The concentrations of both PCA and TES in methanolic supernates remained constant for at least 24 hours. The lowest measurable concentration of PCA or TES was 50 μM for the HPLC assay and at least 100 times lower by the EPR method. The standard curves were linear for both methods up to 1.5 mmol/l; the averaged coefficients of correlation were 0.985 and 0.998 for the HPLC and the EPR method, respectively.

Pharmacokinetic analysis of plasma data. Total body clearance (CLt) was calculated as CLt=Dose/AUC, where AUC is the total area under the plasma concentration versus time curve. The AUC 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. The rate constant, k, and its corresponding half-life ($T_{1/2}$) were estimated by linear least squares fit of data points (time, log concentration), in the terminal phase of the decline. The volume of distribution (V) was obtained from CLt/k.

Analysis of urine data. The total recovery was calculated as the product of the volume of urine collected and the urinary nitroxyl radical concentration after reoxidation.

In Vitro Reduction by Tissue Homogenates

Male Sprague-Dawley (Simonsen Laboratories) rats (200 to 250 g) were used in these experiments. After the animal was sacrificed, brain, heart, kidney, liver, lung or muscle were immediately removed, weighed, and homogenized (Potter-Elvehjem tissue grinder) with 3 parts of isotonic saline. Ten μl of a 50 mM concentrated solution of PCA or TES in water were added, at time zero, to 2 ml of fresh homogenate to achieve a final nitroxide concentration of 0.25 mM, corresponding to 1 mmol/kg of fresh tissue. After mixing 5 seconds with a vortex agitator, the sample was injected into the cell of the EPR spectrometer and was not removed until the end of the experiment. Measurements were made at ambient temperature (22–26°C) every 5 minutes for 60 minutes. To assure that

the homogenate was fresh, only one organ was used from each rat. The nitroxide was added to the homogenate less than 10 minutes after the animal was sacrificed, and the experiment was stopped 1 hour after addition of the nitroxide. At least 3 experiments were conducted with kidney and liver tissues to examine the interanimal variability in the reduction.

In Vitro Reduction by Ascorbic Acid

The time courses of the reduction of the nitroxide radicals by ascorbic acid were measured by EPR spectrometry at room temperature at different initial concentrations of nitroxide (0.1 mM, 1 mM and 10 mM) and of ascorbic acid (from 1 mM to 200 mM). Solutions of nitroxide and of ascorbic acid were prepared on the day of the experiment in 0.067 M phosphate buffer at pH 7.4. The pH was adjusted with sodium hydroxide (3M), when necessary. Solutions of the nitroxide and ascorbic acid were mixed at time zero so that the desired initial concentration for each was achieved. After mixing for 5 seconds with a vortex agitator, the sample was injected into the cell of the EPR spectrometer and was not removed until the end of the experiment. The peak height was recorded continuously for 8 minutes. Reoxidation studies were conducted by measuring the nitroxide concentration 24 hours after addition of hydrogen peroxide.

Results

Stability in Plasma and Urine

Initial observations indicated that PCA was stable, with no measurable change in the EPR signal, for at least one hour at room temperature and at an initial concentration of 0.1 μM or more upon addition to fresh dog plasma. Conversely, TES was unstable under the same conditions; for example, at initial concentrations of 1 μM and 1 mM, 20 % and 3 % respectively of the EPR signals disappeared in 15 minutes. Stability tests in fresh dog urine showed that both PCA and TES were more unstable in this medium than in plasma. Results obtained with urine collected at different occasions, showed greater variability than those observed with plasma. The disappearance of the EPR signal was always greater for TES than for PCA. As an example, with an initial urinary nitroxide concentration of 1 mM, 99 % of TES disappeared in 5 minutes while 35 % of PCA disappeared in 15 minutes.

The stability of the EPR signal in plasma and urine samples obtained after injection of the nitroxide into the dog was also tested. In plasma, an increase of the signal was observed with time for both TES and PCA. For example, a plasma sample collected 2 hours after administration of PCA to the dog, measured as soon as possible after blood was withdrawn, showed 20 % increase in the nitroxide concentration when the sample was retained 30 minutes in the EPR cell. Such spontaneous oxidation was observed for PCA and TES, in plasma and in urine.

In Vivo Experiments

Pharmacokinetic studies. Pharmacokinetic parameters calculated from plasma samples measured as soon as possible after sampling are presented in Table I. The decay half-lives of the signal measured in plasma after oxidation (total) were 85 and 44 minutes, respectively, for PCA and TES. The urinary recoveries after oxidation were 82 and 86 per cent of the dose administered for these respective compounds. The plot of PCA concentration versus time, before and after oxidation, is presented in Fig. 1.

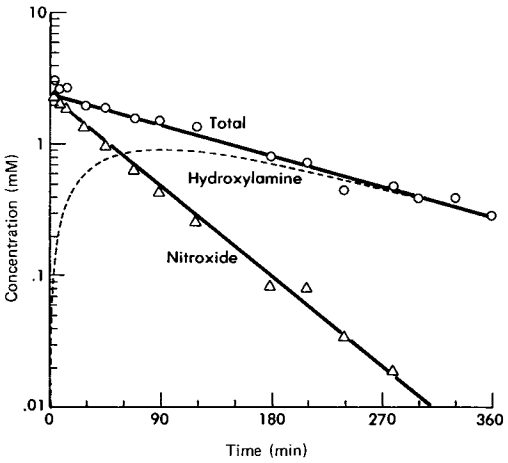


Fig. 1 Decline of the plasma PCA concentration with time in the dog, when the concentration is measured as soon as possible (nitroxide) after sampling (△) and after oxidation (total) with hydrogen peroxide (o). Linear least square fit of both sets of data are shown (solid lines). The arithmetic difference (dashed line), corresponding to the hydroxylamine form, is shown.

Table I. Preliminary values of plasma pharmacokinetic parameters in the dog

	Clearance[a] (ml/min)	Volume of Distribution[b] (l/kg)	Half-life[c] (min)
PCA	185	0.41	43.5
TES	214	0.21	18

[a] Determined from dose/AUC.
[b] V = CL/k, where k is the slope of terminal line of log (c) vs. time.
[c] From least squares fit of line after 30 min.

Metabolic studies. The HPLC chromatograms of methanolic plasma and urine supernates showed only one peak, corresponding to the nitroxide, compared to their respective blanks. The hyperfine spectra of plasma and urine, after intravenous administration of the nitroxide to the dog, were similar to those obtained by adding the nitroxide to plasma or urine blanks. The measurements, by both analytical methods, of the concentrations of PCA and TES in methanolic supernates were virtually the same, as shown for PCA in Fig. 2. PCA concentrations in methanolic supernates were not increased by the subsequent addition of hydrogen peroxide as was the case for TES. PCA concentrations obtained after reoxidation in the pharmacokinetic studies were close (± 20 %) to those measured in methanolic supernates, during metabolic studies.

In Vitro Reduction by Tissues Homogenates

The interanimal variability in the reduction of both nitroxides is shown in Table II. The loss of the nitroxides in various tissues is shown for PCA and TES in Fig. 3 and 4.

In Vitro Reduction by Ascorbic Acid

The results of these experiments are presented in Fig. 5.

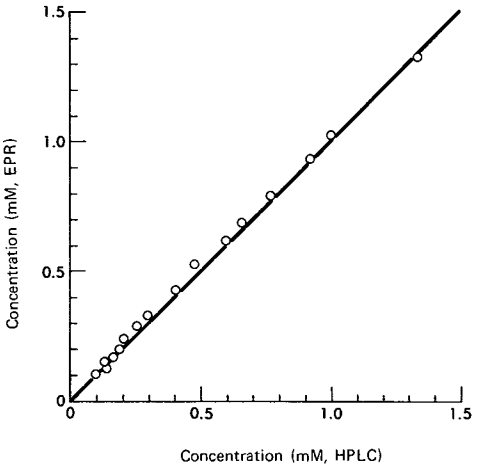


Fig. 2 The concentrations of PCA in methanolic supernates of dog plasma samples, measured by EPR, are plotted against the corresponding concentrations, determined by HPLC. The straight line shows the perfect relationship.

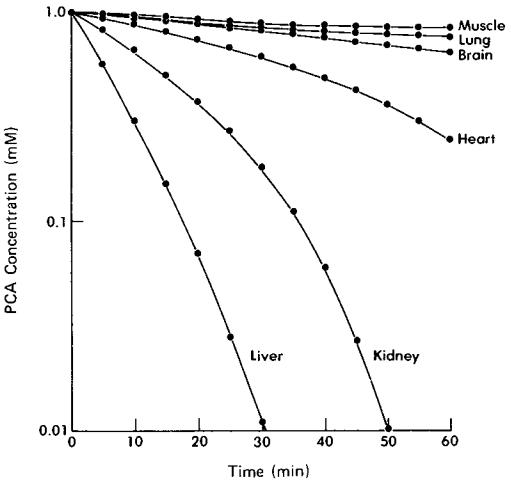
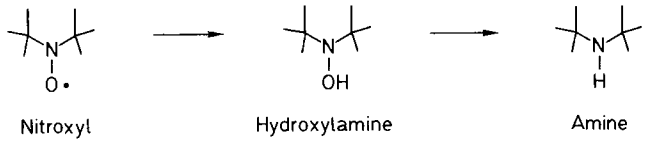


Fig. 3 Decline of PCA (initially 1 mmol/kg tissue) in rat tissue homogenates diluted with 3 parts of normal saline. The lines connect the points for identification purposes. Note the large differences in the reductive activity between liver or kidney and brain, lung or muscle homogenates.

Discussion

The nitroxyl moiety is capable of being reduced to the hydroxylamine, and under more extreme reducing conditions, to the amine (8), as follows:



We have observed the disposition of PCA and TES *in vivo*, and their reduction in tissue homogenates and in the presence of ascorbic acid. In all cases, PCA was more stable than TES, as discussed below.

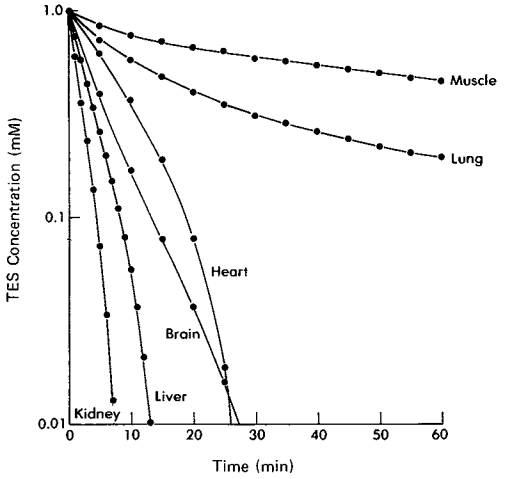


Fig. 4 Decline of TES (initially 1 mmol/kg tissue) in rat tissue homogenates diluted with 3 parts of normal saline. The lines connect the points for identification purposes. As with PCA (see Fig. 3), the reductive activity of liver and kidney homogenates greatly exceed those of lung and muscle, but there appears to be relatively more activity in brain for TES than observed for PCA (Fig. 3).

In Vivo Experiments

Nitroxides are known to be reduced in biologic systems (9). Our stability assays showed differences between PCA and TES, PCA being more resistant to reduction than TES. The decay of the EPR signal in biologic media can be attributed to reduction of the nitroxide. The observed increase in signal *in vitro*, when both nitroxide and its hydroxylamine are present, can be attributed to spontaneous oxidation of the reduced form (hydroxylamine) by oxygen in air (8). The spontaneous oxidation of plasma and urine samples, obtained after administration of either nitroxide to the dog, suggests the presence of the hydroxylamine. Thus, in pharmacokinetic studies, instability in plasma is a more important experimental problem for the reduced form than for the nitroxide itself. Concentrations measured in methanolic supernates can be explained by such a spontaneous oxidation. Oxidation of a hydroxylamine upon extraction with organic solvents has been previously reported (10, 11). It is interesting to note that on precipitating plasma proteins with methanol, the hydroxylamine form of PCA was more readily oxidized than that of TES. This was determined by the addition of hydrogen peroxide to the plasma 24 hours before adding methanol. For PCA, the concentration in methanolic supernates was the same with or without previous oxidation by peroxide, but for TES the oxidation produced an increase of the concentration.

Because of these stability problems, it can be concluded that the HPLC method, as well as any technique which requires protein precipitation or extraction into organic solvents, cannot be used for quantitative measurement of nitroxides *in vivo*. In contrast, plasma samples can be directly and quickly measured by EPR, making this technique opportune for pharmacokinetic studies of nitroxides.

The EPR technique is specific for paramagnetic substances. Therefore, distinction between a nitroxide and its biotransformed nitroxyl-containing products, e.g., a conjugate, can be difficult. HPLC was used to determine if such metabolites were formed *in vivo*. No new peak was observed in the chromatogram of either plasma or urine. Consequently, if a nitroxyl-

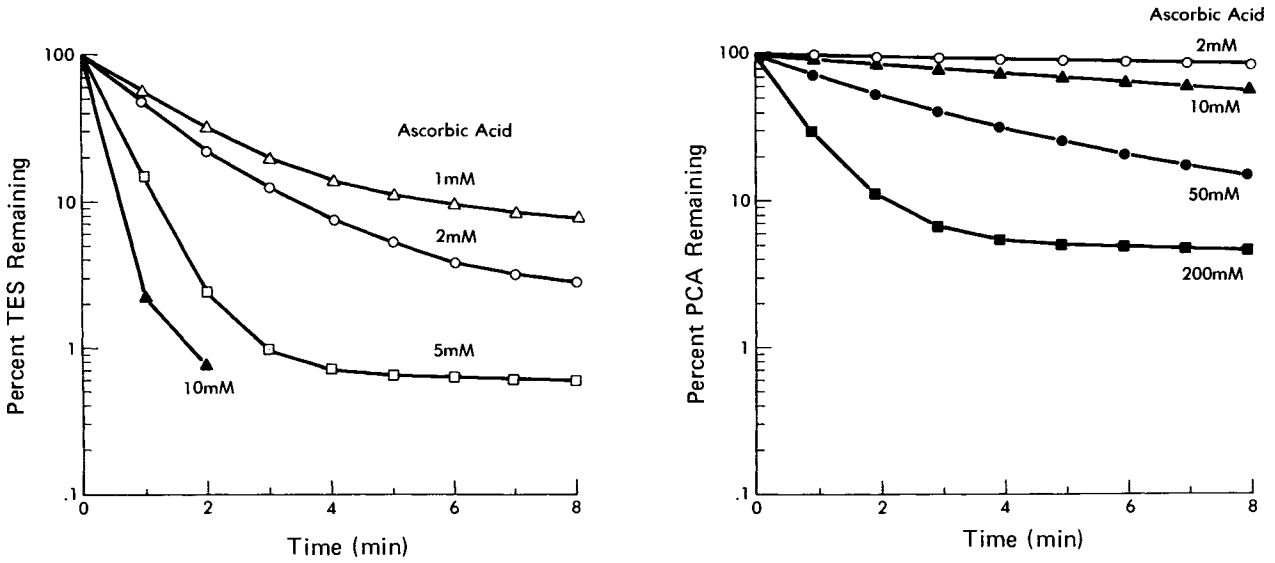


Fig. 5 The decline of the concentration of PCA (on left) and TES (on right) depends on the initial concentration of ascorbic acid added. The pH was 7.4 in all cases. The lines connect points for purpose of identification. Note that the rate of reduction of PCA is much slower than that of TES.

containing metabolite is formed, it would have to either cochromatograph with the parent compound or occur at concentrations below detection limits. A stable glucuronide conjugate is unlikely for two reasons. First, such a conjugate would be much more polar than the parent drug and would have been easily observed by HPLC. Second, the concentrations of the same methanolic samples measured by HPLC and by EPR were close at all times, as shown in Fig. 2. Consequently, we conclude that there is probably little or no metabolite containing the nitroxyl moiety formed *in vivo* after administration of either PCA or TES. For this additional reason, the EPR technique was the method of choice for pharmacokinetic studies of these two nitroxides.

Comparison of the plasma concentrations before and after oxidation indicates that both nitroxides were extensively reduced *in vivo*, as shown in Fig. 1 for PCA. Three hours after administration of an initial dose of 0.55 mmol/kg, the hydroxylamine was virtually the only form present *in vivo* for both PCA and TES. The elimination of the nitroxide is more rapid than the elimination of hydroxylamine at this dose level. From these experiments in a single dog, it is also clear that TES is more rapidly eliminated *in vivo* than PCA.

The pharmacokinetic parameters have been calculated from concentrations measured as soon as possible after sampling. Indeed, because of the propensity of the hydroxylamine to be oxidized to the nitroxide when exposed to air *in vitro*, it is possible that PCA and TES plasma concentrations may have been slightly overestimated.

The reversible conversion of a metabolite to its parent drug *in vivo* introduces complications to a pharmacokinetic analysis. In particular, the usual mode of evaluation of clearance and volume of distribution, cannot be used (12). Therefore, it was necessary to establish whether the hydroxylamine was reoxidized *in vivo* to the nitroxide, to validate the clearance and volume values calculated as previously described. The oxidation of the hydroxylamine is unlikely to be of importance *in vivo* since the terminal half-lives of the nitroxides and their corresponding hydroxylamines are different. From Fig. 1, the nitroxide concentration appears to decline to levels less than 1 % of the hydroxylamine without evidence of parallelism in the two curves, the same observation was made with TES. This indicates that the hydroxylamines of PCA and TES are not reoxidized to the nitroxide *in vivo.*

On the other hand, a complication does occur as a consequence of the instability of the nitroxides in urine. The nitroxides are reduced to the hydroxylamine after excretion in urine. Therefore, renal clearance of nitroxides is unknown, and it is consequently difficult to know whether metabolic reduction or renal excretion is mainly responsible for the *in vivo* elimination of the nitroxides. We previously concluded that renal excretion was the most important (3). This conclusion may be correct at the dose used in our first experiment, 3.7 mmol/kg, but spontaneous oxidation of the samples may have occurred as well.

The urinary recovery of PCA and TES indicated that most of the administered dose was eliminated in urine within 6 hours as the nitroxide and the hydroxylamine. This fairly complete and rapid elimination is compatible with the use of these compounds as contrast agents. The formation of the more completely reduced derivative, the amine, cannot be excluded. However, the amine probably does not contribute substantially to the total elimination of the nitroxide, as most of the dose was recovered in urine after oxidation in conditions that are inadequate to oxidize the amine form (8).

In Vitro Reduction by Tissue Homogenates

PCA and TES are reduced *in vivo* to their corresponding hydroxylamines. The purpose of this set of experiments was to determine which organs could be responsible for this reduction.

The ability of the tissues to reduce the nitroxides depended on the freshness of the homogenate. In the experimental conditions described previously, a small decrease of the reducing activity was observed for a tissue homogenate prepared 1 hour, instead of a few minutes, before addition of the nitroxide. Consequently, in all subsequent experiments the nitroxide

Table II. Percent nitroxide remaining in rat kidney homogenates[a]

PCA		TES	
Time (min)[b]	Remaining (%)[c]	Time (min)	Remaining (%)
5	82.7 ± 1.9	1	67.3 ± 7.4
10	65.9 ± 2.0	2	42.5 ± 5.7
15	50.3 ± 1.6	3	27.8 ± 3.9
20	37.5 ± 1.6	4	16.7 ± 2.8
25	26.3 ± 1.5	5	7.7 ± 3.1
30	17.4 ± 1.8	6	3.7 ± 1.9
35	9.9 ± 2.1	7	1.5 ± 1.0

[a] Mean ± SD of the homogenate from three different rats.
[b] Time after addition of nitroxide.
[c] The initial concentration corresponds to the EPR signal height extrapolated to zero time.

was added to the homogenate no more than 10 minutes after the rat was sacrificed and the experiment was stopped 1 hour after addition of the nitroxide.

The spontaneous oxidation of the hydroxylamine by contact with air was again observed in these experiments. To minimize air exposure, the homogenate was not removed from the EPR cell between each measurement.

Interanimal differences, depicted in Table II, were small compared to interorgan differences, shown in Fig. 3 and 4. The kidneys of different rats showed remarkable consistancy in their reductive ability. Liver showed more variability, but these variations were less than the interorgan differences presented in Fig. 3 and 4. Liver and kidney showed the greatest reductive activity, lung and muscle tissues had the least. In all tissues, TES was reduced more rapidly than PCA.

Potential reduction in homogenized brain was studied, because brain images showed localized accumulation of nitroxides in region of blood-brain-barrier disruption (3). In brain, the reducing activity was low with PCA but high with TES. These differences in stability favor the use of PCA for MRI contrast enhancement.

A previous study reported an enzymatic reduction of different lipophilic spin labels (13). This activity was associated with membrane sulfhydryl groups. It is therefore not surprising to observe the greatest reductive activity in liver and in kidney which are rich in enzymes. However, reducing agents present in body fluids under physiologic conditions could also be responsible for some *in vivo* reduction of nitroxides. In urine, the extensive reduction of PCA and TES can be better explained by the presence of chemicals, such as ascorbic acid, than by an enzymatic process. Ascorbic acid is an agent known to reduce nitroxides (14), and its concentration in urine is high in animals that synthesize it and in humans who take megadoses of vitamin C (15). Ascorbic acid is physiologically present in tissues, especially in liver and kidney (16). Therefore, ascorbic acid could, in addition to enzymes, participate in the observed reduction of nitroxides in tissue homogenates. In living animals, the ascorbic acid may be primarily responsible for reduction of nitroxides; enzymatic reduction may be more or less important, depending on cellular penetration.

In Vitro Reduction by Ascorbic Acid

The chemistry of nitroxides has been explored over the past 20 years and has been recently reviewed (14). However, the dynamic aspects of nitroxides reduction have received only limited attention (17, 18). In our experiments, the reduction of PCA was always slower than that of TES under the same conditions as shown in Fig. 5. From our results, it is evident that the chemical structures of the nitroxides, PCA and TES, have an effect on the stability of the nitroxyl moiety.

An increase in the ascorbic acid concentration or in the nitroxide concentration produced a proportionate increase in the initial rate of reduction. This reduction has been described as first-order in the radical concentration (17, 18). However, in our hands first-order decay did not always occur. An initial rapid exponential decay followed by a much slower process was observed, as shown in Fig. 5. Although the curves seem to indicate that the reaction approached an equilibrium, the reaction continued to completion when the sample was not removed from the cell and the signal was observed over a longer period of time (24 hours). When the sample was removed, enough air was introduced to oxidize some of the hydroxylamine. For both PCA and TES, the reduced form could be completely reoxidized to the nitroxide form at pH 7.4 by adding a 10-fold excess of hydrogen peroxide and letting stand for 24 hours.

The average concentrations of ascorbic acid in different tissues of different species, including human, have been reviewed (16). In the rat, ascorbic acid concentration is close to 0.1 mM in plasma and approximately 3 mM in liver and kidney. At these concentrations, with a nitroxide concentration of 1 mM, which is a reasonable estimate of the concentration required for MRI, the effect of ascorbic acid on PCA and TES is different. Therefore, measurement of the reduction of the nitroxide by ascorbic acid could be of predictive value for selecting a good contrast agent; a more detailed study of the factors affecting this reaction is currently in progress.

In summary, TES consistently showed a higher propensity for reduction than PCA. The major metabolite of reduction, the hydroxylamine form, is non-paramagnetic and thus ineffective for MRI contrast enhancement. Good enhancement of tissues such as kidney, brain, inflammatory masses and neoplasms, have been obtained with TES at doses of 0.07 to 3.30 mmol/kg (2–4). However, PCA, being more stable, may be a more effective MRI enhancing contrast agent.

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Radioreceptor Assay of Narcotic Analgesics in Serum

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Abstract: A sensitive radioreceptor assay (RRA) to determine the serum concentrations of fentanyl, pentazocine and morphine was developed on the basis of the drug's competition with a labeled tracer (^{3}H-naloxone) for the membrane bound opioid receptor in rat brain homogenates. The binding data were computer-fitted to a standard curve by means of nonlinear least square regression. Sensitivity of the assay applied directly to serum samples without extraction was limited to approximately 3, 5 and 25 ng/ml for fentanyl, morphine and pentazocine, respectively, because of endogenous plasma constituents that interfere with the opioid receptor binding. With the use of petrol-ether extraction the sensitivity was improved to 0.3 ng/ml fentanyl and 3 ng/ml pentazocine (0.3 ml serum samples). No RRA-active metabolites were detectable after HPLC separation of serum from a patient treated with fentanyl. The plasma concentration time course of fentanyl in a patient, measured by RRA, was similar to that obtained by a radioimmunoassay (RIA). The RRA represents a general procedure for the detection of clinically used opioid analgesics and their active metabolites.

Numerous assay procedures have been published for individual opioid drugs (1). For example the fast-acting narcotic analgesic fentanyl can be detected in serum with a sensitive gas chromatographic assay with a nitrogen/phosphorus-specific detector (2). Most pharmacokinetic studies of fentanyl, however, have employed a radioimmunoassay (RIA) (3). While these assays may be specific for fentanyl in the presence of its metabolites, they fail to answer the question whether any pharmacologically active metabolites are present. Indeed, fentanyl metabolites are known to accumulate in serum to concentrations exceeding those of the parent drug (4). This problem is not limited to fentanyl, but applies to most other opioids as well. An opioid radioreceptor assay (RRA) could be applied to detect active drug metabolites as well as provide a valuable general assay method for comparison with other techniques.

The lack of a significant literature on this subject may be caused by a series of difficulties that impede the application of the RRA to the measurement of opioid serum levels. In addition to interference from serum constituents, the heterogeneity of the opioid receptor system must be considered. The only previous report (5) describing an RRA of opioids in serum failed to adequately overcome these difficulties because of the lack of receptor type selectivity of the tracer employed (^{3}H-buprenorphine). The present report describes a reliable RRA technique to measure serum levels of fentanyl, pentazocine and morphine and their potential active metabolites in the subnanogram range.

Materials and Methods

Chemicals and equipment. [N-Allyl-2,3-^{3}H]naloxone (specific activity 203.5 x 10^{10} s^{-1} $mmol^{-1}$ ≙ 55 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Fentanyl citrate was supplied by Janssen Pharmaceuticals (Beerse, Belgium), pentazocine by Sterling-Winthrop (Rensselaer, NY), and morphine sulfate was purchased from Mallinckrodt (St. Louis). Control serum was purchased in freeze-dried form from Sigma (St. Louis, MO). Glass fiber filters type GF/B were from Whatman (England). The HPLC equipment consisted of: pump M600A from Waters Ass. (Milford, MA), UV detector LC-15 from Perkin-Elmer (Norwalk, CT), column C-18 250 mm x 4.6 mm from Alltech (Los Altos, CA.). The liquid scintillation cocktail was Aquasol, New England Nuclear (Gardena, CA). The scintillation counter (LS 7800) was from Beckman (Irvine, CA).

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