Pharmacokinetics of Aminolevulinic Acid After Intravesical Administration to Dogs

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Purpose. To examine the stability and systemic absorption of aminolevulinic acid (ALA) in dogs during intravesical administration.
Methods. Nine dogs received an intravesical dose of ALA either with no prior treatment, after receiving ammonium chloride for urinary acidification, or after receiving sodium bicarbonate for urinary alkalinization. Urine and blood samples collected during and after administration were monitored for ALA using an HPLC assay developed in our laboratories. Concentrations of pyrazine 2,5-dipropionic acid, the major ALA degradation product, and radiolabeled inulin, a nonabsorbable marker for urine volume, were also determined.
Results. Less than 0.6% of intravesical ALA doses was absorbed into plasma. Urine concentrations decreased to 37% of the initial concentration during the 2 hour instillation. Decreases in urinary ALA and radiolabeled inulin concentrations were significantly correlated, indicating that urine dilution accounted for over 80% of observed decreases in urinary ALA. ALA conversion to pyrazine 2,5-dipropionic acid was negligible.
Conclusions. These studies demonstrate that ALA is stable and poorly absorbed into the systemic circulation during intravesical instillation. Future studies utilizing intravesical ALA for photodiagnosis of bladder cancer should include measures to restrict fluid intake as a means to limit dilution and maximize ALA concentrations during instillation.

KEY WORDS: aminolevulinic acid; intravesical; pharmacokinetics; photodiagnosis; bladder; cancer.

INTRODUCTION

Aminolevulinic acid (ALA) is an endogenous metabolite synthesized during heme biosynthesis. Exogenous administration of ALA results in the rapid accumulation of protoporphyrin IX (PpIX) in both normal and neoplastic tissues (1–3). PpIX is a potent photosensitizer and can be used in conjunction with light to diagnose or treat cancer (4). Recently, Kriegmair *et al.* (5,6) showed that intravesical administration of a pH neutral solution of 3% ALA followed by monitoring of PpIX fluorescence in the urothelium could be used to identify malignant and pre-cancerous lesions in the urinary bladder. This suggests that intravesical ALA may be a valuable clinical tool for the detection of early stage and/or recurrent urinary bladder cancer.

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ALA degradation to pyrazine 2,5-dipropionic acid occurs in aqueous solutions with pH values slightly lower or greater than pH 7, but is minimal under more acidic conditions (7–9). The pH of the urine in adult humans during intravesical instillation may range from pH 5 to 8 (10). Thus, the stability of ALA may be compromised during intravesical instillation. The purpose of these studies was to examine the stability and systemic absorption of ALA during intravesical administration. The importance of urinary pH to ALA stability, bladder ALA exposure, and systemic ALA absorption during intravesical instillation was determined using established pharmacokinetic and statistical data analysis techniques. Figure 1 illustrates the experimental approach used in these studies to examine each of three potential factors (i.e., urine production, systemic absorption, and degradation) which may contribute to decreases in ALA concentrations during intravesical dosing.

MATERIALS AND METHODS

Animal Procurement

Male beagle dogs were used as an animal model, similar to previous intravesical instillation studies (11) and other preclinical ALA studies we have performed (12). All animal procedures utilized in this research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985) and were approved by the University of Tennessee Animal Care and Use Committee. Nine male beagle dogs weighing 9.8 to 11.2 kg were purchased from Harlan Laboratories (Indianapolis, IN).

Experimental Design

A three-way crossover design was used to assess the effects of urinary pH on intravesical ALA treatment and to provide an initial assessment of potential sequence-related effects. Animals were divided into three groups (n = 3 per group). On separate occasions, animals received either no pre-treatment and an intravesical ALA dose, pre-treatment with oral ammonium chloride and an intravesical ALA dose, or pre-treatment with oral sodium bicarbonate and an intravesical ALA dose. A washout period of two weeks was used between consecutive treatments to minimize the potential for sequence-related effects.

Modulation of Urinary pH

Urinary acidification and alkalinization were accomplished via oral pre-treatment with ammonium chloride or sodium bicarbonate, respectively. One day prior to each intravesical treatment, animals began oral therapy with the appropriate modifier of urinary pH. For acidification, one gram of ammonium chloride dissolved in 48 ml of water was administered orally every three hours starting at 8 AM the day before treatment (Day 0) (i.e., 8 AM, 11 AM, 2 PM, 5 PM, 8 PM, 11 PM, 2 AM, 5 AM, and 8 AM). Intravesical doses of ALA were administered between 9:27 AM and 11:37 AM the following day (Day 1), and remained in the urinary bladder for two hours. Oral ammonium chloride therapy continued every three hours throughout the intravesical instillation period and until the final blood and urine samples were collected. For alkalinization, four

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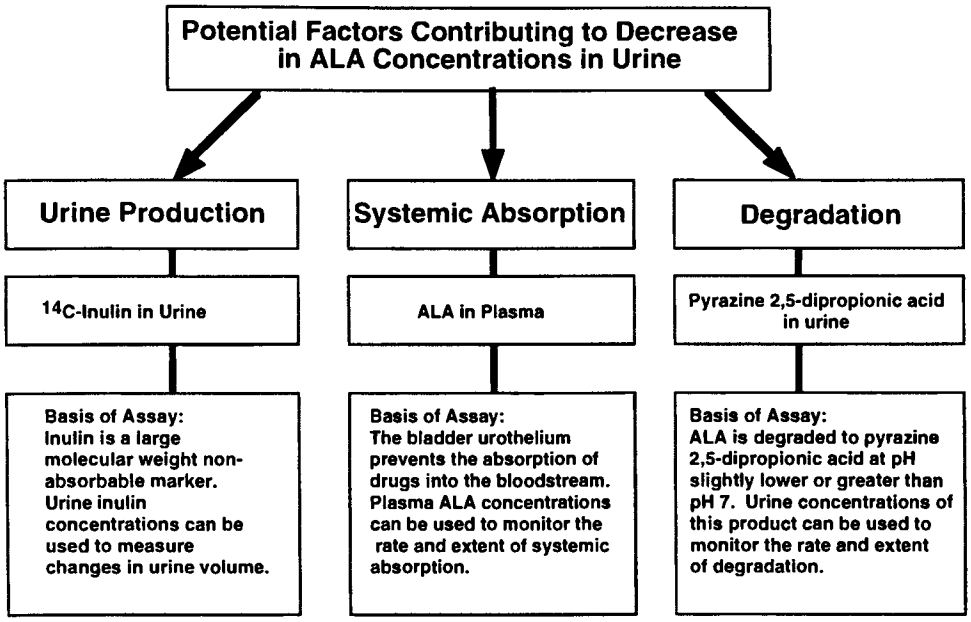

Fig. 1. Experimental design and basis.

grams of sodium bicarbonate were administered in an oral solution (48 ml of an 84 mg/ml solution) on an identical schedule to that described for ammonium chloride. Solutions were administered in 10 ml increments to prevent choking or gagging of the animals.

Animal Preparation

Animals were allowed free access to water and dog chow (Agway Prolab Canine 2000) prior to and between intravesical ALA treatments. However, water and food intake of animals was restricted twelve hours prior to each intravesical dose (i.e., at 10 PM the night before the experiment). Animals were not permitted free access to food and water until immediately after the last blood sample had been obtained. On the morning of the experiment (Day 1), angiocatheters with heparin well were placed in the jugular (7 inch, 18 gauge) and/or a saphenous vein (1.5 inch, 18 gauge) for blood sampling. Animals received a sedative, acepromazine (0.5 mg/kg), intravenously immediately prior to urethral catheterization. Animals were placed in a supine position on the examination table, and a size 8 or 10 French Foley catheter was inserted into the urinary bladder. Urine was drained from the bladder via the urethral catheter and collected in pre-labeled containers. The volume and pH of urine were determined and recorded. Serum samples obtained immediately before treatment 1 and immediately after treatment 3 were submitted for routine clinical analysis including electrolytes, cholesterol, creatinine, bilirubin, alkaline phosphatase, SGPT, and CPK.

Intravesical Dose Preparation

ALA hydrochloride (Levulan®) was provided by DUSA Pharmaceuticals, Inc. (Valhalla, NY). ALA hydrochloride (1.5 grams) was dissolved in 50 ml of 0.2 M phosphate buffer, pH 7.3, to yield a final dosing solution pH of 5.0. The solution was then spiked with 1 μCi of ^{14}C-labeled inulin (DuPont NEN, Lot #2978181, 2.27 mCi/gram, Boston, MA). The intravesical ALA dosing solution was withdrawn into a 60 ml syringe, and then passed through a 0.22 μm syringe filter for sterilization. A small aliquot (1.0 ml) was removed for HPLC and scintillation analysis, and the remainder of the dosing solution was then divided into three 15 ml aliquots for administration to individual dogs as described below. The final concentration of the dosing solution was 30 mg/ml (3% w/v) of ALA hydrochloride (corresponding to 23.6 mg/ml of ALA base). The final concentration of ^{14}C-labeled inulin in the dosing solution was 0.02 μCi/ml. With the exception of ^{14}C-inulin, dose preparation procedures utilized in these studies were chosen to result in a buffered solution of ALA identical to that proposed for human clinical trials of intravesical ALA.

Intravesical Dose Instillation

Each dog was placed in a canine restraint jacket and returned to a standing position to completely drain urine from the bladder. The intravesical ALA dosing solution (15 ml, equivalent to 450 mg of ALA hydrochloride) was placed in a 60 ml catheter-tip syringe, and the syringe connected to the urethral catheter. The intravesical ALA dose was instilled into the bladder over a one minute period, and the time of instillation noted. To ensure that the entire dose was instilled, the urinary catheter was flushed with 2.5 ml of air. Previous studies in our laboratory showed that this corresponds to the "dead" volume of a size 8 French Foley catheter. The catheter was then clamped shut with a stainless steel surgical clamp, and the intravesical ALA dose maintained in the bladder for 2 hours. At the end of the two hour instillation, the intravesical ALA dose was removed via the urethral catheter. The volume and pH were recorded, and an aliquot of the solution diluted and stored at 4°C until analyzed as described below. The urinary bladder was then rinsed with 25 ml of sterile saline using a new 60 ml catheter-tip syringe to ensure removal of any residual radioactivity before returning the animals to their cages.

Blood and Urine Collection

Serial blood and urine samples were collected before, during, and after the intravesical instillation period. Ten 8 ml blood samples were withdrawn from the jugular or saphenous vein catheter, including one immediately prior to the ALA dose and 9 additional samples at 30, 60, 90, 120, 125, 130, 140, 150 and 180 minutes after the ALA dose. Blood samples were collected in 10 ml vacuum tubes containing heparin and stored on ice. Blood samples were centrifuged at 500g for 10 min, and the plasma layer removed and stored frozen until analysis. Urine samples were obtained from the urethral catheter using methods previously described by one of us (9). Briefly, a clean 60 ml syringe was connected to the outlet of the Foley catheter. The air and 5 ml of the bladder contents were withdrawn to fill the catheter and the tip of the syringe. The latex catheter was then pierced with a 1 ml syringe (30 gauge needle), and samples (approximately 0.2 ml each) were withdrawn. The contents of the catheter were returned to the bladder, and the catheter flushed with air. By this method, the collection of urine samples was completed within less than 2 min, minimizing loss of urine or instillate volume and the time of the ALA instillate outside the bladder. A total of seven urine samples (0.2 ml each) were obtained, including one prior to the dose and six additional samples at 5, 15, 30, 60, 90, and 120 minutes after instillation. The bladder wash solution was also collected. Aliquots (0.05 ml) of each sample were immediately diluted 101-fold in 5 ml of 0.2 M sodium phosphate buffer, pH 5.0, and stored at 4°C until analysis. An additional 0.05 ml aliquot was utilized for determination of ^{14}C-inulin radioactivity as described below.

HPLC Analysis of ALA Concentrations in Plasma

ALA concentrations in plasma were determined using an HPLC method described earlier (11), except for the use of 0.5 ml of plasma, 0.25 ml of 1 N hydrochloric acid, and 0.1 ml of internal standard. Derivatized ALA and the internal standard were separated by isocratic reversed-phase HPLC as descibed previously (11). Standard curves were prepared in duplicate over a plasma concentration range of 0.0097 to 2.428 μg/ml. In addition, plasma control samples were prepared at concentrations of 0.0, 0.021, 0.415 and 2.076 μg/ml. Control samples were stored frozen with the dog samples from the pharmacokinetic studies, and assayed in triplicate at the same time the dog samples were assayed.

HPLC Analysis of ALA Concentrations in Urine

ALA concentrations in urine were also determined using an HPLC method developed in our laboratories. ALA concentrations in samples from the bladder instillate were several thousand-fold higher than those found in plasma (i.e., the initial concentration of the intravesical dosing solution was 30 mg/ml of ALA hydrochloride or 23.6 mg/ml of ALA). Samples were diluted 101-fold in 0.2 M sodium phosphate buffer, pH 5.0, immediately after collection to ensure stability during storage. Dilution was performed by adding an aliquot (0.05 ml) of each sample to 5.00 ml of buffer. These diluted samples were then used to determine ALA and pyrazine 2,5-dipropionic acid concentrations in urine. Immediately prior to HPLC analyses, an aliquot (0.05 ml) of the diluted samples was added to 0.95 ml of 0.2 M sodium phosphate buffer, pH 5.0, resulting in an additional 20-fold dilution to fall within the limits of the standard curve. Diluted urine samples were then treated in a similar manner to plasma samples.

Briefly, an aliquot of diluted urine (0.1 ml) was placed in a 15 ml glass culture tube, along with solutions containing the internal standard, acetylacetone and formaldehyde. The tubes were then capped and placed in boiling water bath for 15 minutes. After cooling, the derivatized solution was poured directly into a 1 ml glass autosampler vial and used for HPLC analysis. Standard curves were prepared in duplicate over a concentration range of 0.1 to 25 μg/ml. In addition, urine control samples were prepared at concentrations of 0.0, 0.12, 1.2 and 12.0 μg/ml. Control samples were stored at 4°C with the dog samples from the studies, and assayed in triplicate at the same time the dog samples were assayed.

HPLC Analysis of Pyrazine 2,5-Dipropionic Acid Concentrations in Urine

Concentrations of pyrazine 2,5-dipropionic acid in the bladder instillate were determined using methods developed in our laboratories. Briefly, samples from the bladder instillate were diluted 101-fold in 0.2 M sodium phosphate buffer, pH 5.0, as described above. Immediately prior to HPLC analyses, an aliquot (0.1 ml) of each diluted sample and 0.05 ml of an internal standard solution (7-β-hydroxypropyl theophylline, 50 μg/ml in methanol, Sigma Chemical Company, St. Louis, MO) were added to 0.85 ml of the HPLC mobile phase, resulting in an additional 10-fold dilution. The mobile phase consisted of 7% acetonitrile in a buffer containing formic acid, triethylamine, and potassium phosphate adjusted to pH 2.9. The flow rate was 1 ml/min. The stationary phase for these analyses was a Nova-Pak C_{18} column (3.9 × 150 mm, 3 micron particle size, Waters Associates, Milford, MA). Pyrazine 2,5-dipropionic acid and the internal standard were detected using an absorbance detector set at 265 nm. Standard curves were prepared in triplicate over a final concentration range of 0.1 to 50 μg/ml and analyzed with each set of samples.

Scintillation Analysis of ^{14}C-Inulin Concentrations

Concentrations of ^{14}C-inulin in the bladder instillate were determined using liquid scintillation counting. Briefly, an aliquot of each sample (50 μL) was placed in a 7 ml borosilicate scintillation vial containing 5.0 ml of EcoLite Plus scintillation cocktail (ICN Biomedical, Costa Mesa, CA). Vials were shaken vigorously for 10 seconds and then placed in a Beckman LS6800 scintillation counter (Beckman Instruments, Fullerton, CA). ^{14}C-inulin radioactivity was determined in a zero to 670 counting window for a period of 5 minutes.

Data Analysis

Statistical comparisons for treatment and sequence-related effects were performed using analysis of variance (ANOVA, general linear models procedure, SAS, SAS Institute Inc., Cary, NC) at a 5% level of significance, unless otherwise indicated. To correct for endogenous ALA, the pre-dose plasma concentration of ALA (0.005 ± 0.005 μg/ml, mean ± S.D.; Range 0 to 0.02 μg/ml) was subtracted from plasma ALA concentrations determined during the pharmacokinetic studies. The plasma concentration-time profiles were analyzed using established

non-compartmental methods. The area under the plasma concentration-time curve ($AUC_{plasma,\ 0-180}$) from time zero to the last sampling time (180 min) was calculated by the linear trapezoid rule.

Decreases in ALA urine concentrations during intravesical instillation may be due to three factors: (1) systemic absorption of ALA, (2) urine production, and/or (3) ALA degradation. Our studies were designed to examine the contribution of each of these processes to ALA disappearance. The area under the urine concentration-time curve ($AUC_{urine,\ 0-120}$) from time zero to the last sampling time (120 min) was calculated by the linear trapezoid rule. This value was used to estimate the overall exposure of the urinary bladder to intravesically administered ALA in individual treatments. The factors potentially contributing to changes in urine ALA concentrations were examined as follows:

Systemic Absorption of ALA

The fraction of the intravesical dose absorbed into the plasma (F) was estimated using equation 1, where $AUC_{plasma,\ 0-180}$ is the area under the plasma concentration-time curve for the intravesical dose ($Dose_{ive}$) in these studies and CL is the mean total body clearance of ALA (6.79 ± 1.77 ml/min/kg) determined in previous intravenous dosing studies of ALA in dogs (12).

$$F = \frac{AUC_{plasma,\ 0-180}}{Dose_{ive}} \cdot CL \qquad (1)$$

Urine Production

Changes in instillate volume due to the production of urine were estimated using ^{14}C-inulin. Inulin is a large molecular weight (ca. 5,000) non-absorbable marker which can be used to monitor urothelial integrity (13). Since inulin is too large to be absorbed, observed decreases in inulin concentration in the urine are due solely to dilution by urine. The decrease in ALA and ^{14}C-inulin concentrations during instillation were calculated as a percentage of the initial concentration in the intravesical dosing solution:

$$\%\ \text{of Initial Concentration} = \frac{[\text{analyte}]_{120\ min}}{[\text{analyte}]_{dose}} * 100\% \qquad (2)$$

where $[analyte]_{dose}$ and $[analyte]_{120\ min}$ were the concentrations of ALA or ^{14}C-inulin in the intravesical dosing solution before instillation and the concentrations of ALA or ^{14}C-inulin in the bladder instillate removed at the end of the instillation (120 min), respectively. Percent decreases in ALA and ^{14}C-inulin concentrations were compared to determine the contribution of urine production to decreases in ALA urine concentrations.

We also used ^{14}C-inulin data to calculate the volume of fluid in the bladder at various times during instillation, as described in equation 3:

$$\text{Volume In Bladder} = (\text{Initial volume}) \left\{ \frac{\text{Inulin concentration at time zero}}{\text{Inulin concentration at time, t}} \right\} \qquad (3)$$

where initial volume was the volume of the ALA dosing solution instilled (i.e., 15 ml), and inulin concentrations in the numerator and denominator were those determined by liquid scintillation counting in the initial dosing solution (time zero) and at various time points after instillation, respectively.

ALA Degradation

ALA degrades to pyrazine 2,5-dipropionic acid in neutral to basic aqueous solutions. The extent of ALA degradation in urine during the instillation period was assessed by comparison of the maximal pyrazine 2,5-dipropionic acid concentrations and $AUC_{urine,\ 0-120}$ values for pyrazine 2,5-dipropionic acid observed in each treatment.

Urinary Bladder Histopathology

Dogs were sacrificed via an overdose of pentobarbital eighteen to twenty-four hours after removal of the third intravesical ALA treatment. Necropsies were performed on each animal, including an examination of the external body surface, all orifices, thoracic cavity, abdominal cavity, and pelvic cavity and viscera with special attention afforded to the urinary bladder. The urinary bladder, with segments of the ureters and urethra attached, was removed and placed in a petri dish containing ice-cold saline. The urinary bladder was cut transversely to expose the urothelium, and the urothelium examined visually for abnormalities. Urinary bladders were then immersed in a ten-fold or greater volume of 10% neutral buffered formalin and stored in the refrigerator until tissue processing and microscopic histopathologic examination (Covance Laboratories Inc., Madison, WI).

RESULTS AND DISCUSSION

Plasma Concentrations and Systemic Bioavailability of ALA

Twenty-five intravesical ALA doses were administered to nine dogs on separate occasions. Two additional studies (Dog 1, treatment 2 and Dog 9, treatment 1) were not completed due to technical difficulties encountered during urethral catheterization. Mean concentration-time profiles of ALA in plasma for studies with no pre-treatment, ammonium chloride pre-treatment, and sodium bicarbonate pre-treatment are shown in Fig. 2. Plasma concentrations of ALA after intravesical dosing were low. Peak concentrations of ALA in plasma (C_{max}) averaged 0.135 ± 0.116 μ/ml (Range, 0.022 to 0.515 μg/ml) and occurred (t_{max}) at 53.5 ± 38.3 min (Range, 28.9 to 129.5 min) after administration of the intravesical dose (Table I). $AUC_{plasma,\ 0-180}$ values averaged 9.76 ± 6.96 μg*min/ml (Range, 1.75 to 24.9 μg*min/ml). Less than 0.6% of the intravesical ALA dose was absorbed into the plasma in all treatments, with the bioavailable fraction averaging 0.0021 ± 0.0015 (Range, 0.0004 to 0.0058). Thus, systemic absorption of ALA contributed little to the observed decreases in urine ALA concentrations. No statistically significant differences between pre-treatment groups were observed in the plasma pharmacokinetic parameters ($P > 0.1$).

Urine Concentrations of ALA

Mean concentration-time profiles of ALA in urine for studies with no pre-treatment, ammonium chloride pre-treatment, and sodium bicarbonate pre-treatment are shown in

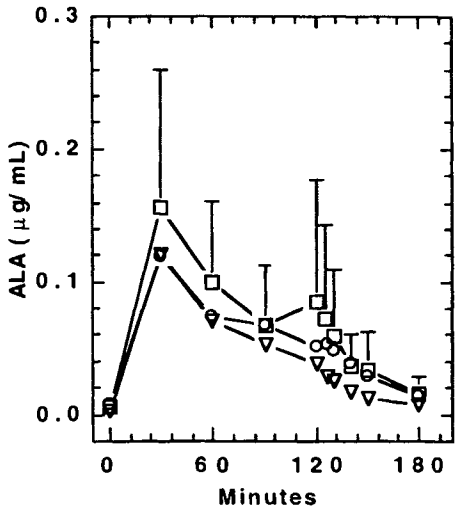

Fig. 2. Mean concentration-time profiles of ALA in plasma for studies with no pre-treatment (squares, n = 7), oral ammonium chloride pre-treatment (circles, n = 9), and oral sodium bicarbonate pre-treatment (triangles, n = 9). Error bars representing the standard deviation of the mean are presented only for studies with no pre-treatment, and are representative of the variation observed in the other studies.

Fig. 3A. Pre-treatment with ammonium chloride or sodium bicarbonate successfully modified urinary pH to the desired ranges (Table II). $AUC_{urine,\ 0-120}$ values for ALA in the urine (Table I) averaged 1366 ± 462 mg*min/ml (Range, 663 to 2443 mg*min/ml) and were on average about 140,000-fold greater than plasma AUC values, indicating that the urinary bladder serves as a significant barrier to ALA absorption. However, urine concentrations of ALA decreased to 36.6 ± 15.7% (Mean ± S.D.; Range, 10.6 to 68.9%) of the initial concentration in the dosing solution during the 120 minute instillation period (Table I), indicating that factors other than systemic absorption contributed significantly to the observed decreases in urine ALA concentrations and bladder exposure.

Contribution of Urine Production to the Observed Decreases in Urine ALA Concentrations

Mean concentration-time profiles of ^{14}C-inulin in urine for studies with no pre-treatment, ammonium chloride pre-treatment, and sodium bicarbonate pre-treatment are shown in Fig. 3B. ^{14}C-Inulin concentrations in urine decreased rapidly during the intravesical instillation period to 30.8 ± 12.7% (Range, 8.9 to 57.1%, Table I) of the initial concentration in the dosing solution during the 120 minute instillation period, whereas ALA concentrations decreased to 36.6 ± 15.7% of the initial concentration in the dosing solution. The difference in percentage of ALA and ^{14}C-inulin concentration decreases was not significantly different ($P > 0.1$). Further, the percent decreases in urinary ALA concentrations and ^{14}C-inulin concentrations were positively correlated (Fig. 4, $r^2 = 0.49$, $P < 0.0005$). These data indicate that dilution by urine accounted for an average of 84% (30.8/36.6) of the observed decrease in urine ALA concentrations during our studies.

The contribution of urine dilution to the observed decreases in urine ALA concentrations is corroborated by estimates of the volume of urine present in the bladder (equation 3) at various times during the intravesical instillation period. Actual urine volumes withdrawn at the end of the instillation period ranged from 18 to 169.5 ml, and were not statistically different between treatment groups ($P > 0.05$). Calculated urine volumes at the end of the 2 hr intravesical instillation period (see equation 3) ranged from 26.3 to 167.6 ml, and were highly correlated with the actual volumes of urine withdrawn and measured at the end of the instillation period (Fig. 5, $r^2 = 0.98$, $P < 0.0005$). Importantly, the slope of this line was 0.9372 (i.e., approximately one), with an intercept value of 10.4 ml. These data demonstrate the validity of using ^{14}C-inulin for determination of urine volume during instillation (i. e., calculated volumes closely approximated the actual volumes present in the bladder during instillation). This data, in combination with the observed correlation between the observed decreases in urine ALA and ^{14}C-inulin concentrations, strengthen the assertion that urine dilution was the most significant factor leading to the observed decreases in urine ALA concentration.

Contribution of ALA Degradation to the Observed Decreases in Urine ALA Concentrations

Pyrazine 2,5-dipropionic acid was not detected in the urine of any animals after pre-treatment with ammonium chloride, and was detected in only two of the seven animals when no pre-treatment was administered. In each of these two instances, the urine withdrawn at the end of the instillation period was approximately pH 7 or greater, while in the remaining five

Table I. Average (Mean ± S.D.) Plasma and Urinary ALA Pharmacokinetic Data

Parameter n	None 7	Ammonium chloride 9	Sodium bicarbonate 9	All 25
		Plasma data		
Cmax (μg/ml)	0.165 ± 0.104	0.124 ± 0.077	0.121 ± 0.159	0.135 ± 0.116
tmax (min)	69.9 ± 49.7	44.1 ± 32.7	50.2 ± 33.5	53.5 ± 38.3
AUC (μg*min/ml)	12.2 ± 8.08	9.19 ± 5.77	8.44 ± 7.46	9.76 ± 6.96
F	0.0026 ± 0.0017	0.0020 ± 0.0012	0.0019 ± 0.0017	0.0021 ± 0.0015
		Urinary data		
% of initial [ALA]	41.7 ± 18.1	32.8 ± 13.9	36.5 ± 16.1	36.6 ± 15.7
% of initial [Inulin]	37.3 ± 14.1	30.1 ± 16.1	26.5 ± 4.28	30.8 ± 12.7
AUC (mg*min/ml)	1656 ± 576[a]	1390 ± 411	1119 ± 281[a]	1366 ± 463

[a] Significantly different, P = 0.0037.

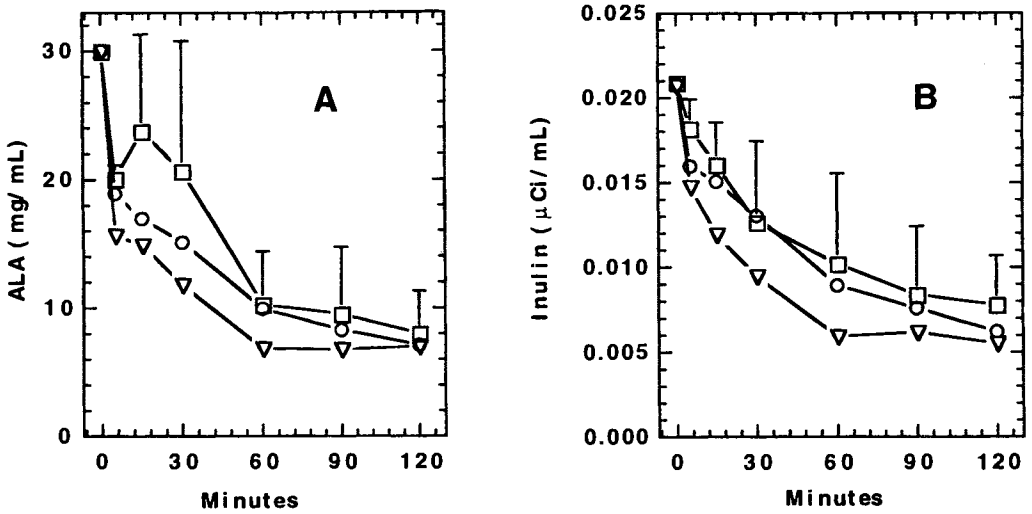

Fig. 3. Mean concentration-time profiles of ALA (panel A) and ^{14}C-inulin (panel B) in urine for studies with no pre-treatment (squares, n = 7), oral ammonium chloride pre-treatment (circles, n = 9), and oral sodium bicarbonate pre-treatment (triangles, n = 9). Error bars representing the standard deviation of the mean are presented only for studies with no pre-treatment, and are representative of the variation observed in the other studies.

treatments urine pH was less than 6.0. It is important to note that our limit of quantitation (LOQ) for pyrazine 2,5-dipropionic acid using our assay procedures (i.e., a 1,000-fold dilution prior to HPLC analyses) was 0.1 μg/ml. This LOQ would correspond to a pyrazine 2,5-dipropionic acid concentration of 0.1 mg/ml in an undiluted urine sample. Thus, we were able to detect as little as 0.33% conversion of ALA (initial concentration, 30 mg/ml) to pyrazine 2,5-dipropionic acid using these methods. Pyrazine 2,5-dipropionic acid was detected in the urine of all animals after pre-treatment with sodium bicarbonate. However, concentrations of pyrazine 2,5-dipropionic acid in urine were significantly lower than concentrations of ALA in urine. $AUC_{urine,\ 0-120}$ values for ALA during studies using sodium bicarbonate pre-treatment were significantly less (P = 0.0037) than those observed in studies using no pre-treatment (Table I). Mean urine pH at the end of instillation during these studies was 7.81 ± 0.20 (Table II). $AUC_{urine,\ 0-120}$ values for ALA in the urine during studies using ammonium chloride pre-treatment were not significantly different than those observed in studies with no pre-treatment. Mean urine pH at the end of instillation during these studies was 5.24 ± 0.20 (Table II). These data suggest that degradation of ALA to pyrazine 2,5-dipropionic acid in urine of basic pH might have contributed significantly to the observed decreases in urine ALA concentrations. However, $AUC_{urine,\ 0-120}$ values of pyrazine 2,5-dipropionic acid averaged only 11.3 ± 2.8 mg*min/ml, or less than 1% (calculated on a molar basis) of the $AUC_{urine,\ 0-120}$ values for ALA in the urine (Table I, mean 1366 ± 463 mg*min/ml). These data indicate that chemical degradation of ALA to pyrazine 2,5-dipropionic acid does indeed occur at urine pH values near or greater than pH 7, but that this process contributed little to the decrease in urinary ALA concentrations.

Table II. Modulation of Urinary pH by Pre-Treatment

Pre-treatment	pH (Mean ± S.D.)	pH (range)
Urine collected before instillation of ALA		
None	6.59 ± 1.24	5.23 to 8.55
Ammonium chloride	5.72 ± 0.31	5.38 to 6.00
Sodium bicarbonate	8.50 ± 0.12	8.31 to 8.69
Urine collected at the end of ALA instillation		
None	5.96 ± 0.82	5.18 to 7.24
Ammonium chloride	5.24 ± 0.20	5.02 to 5.67
Sodium bicarbonate	7.81 ± 0.20	7.52 to 8.13

Clinical Chemistries and Histopathology

Serum samples collected immediately before the first ALA instillation and immediately after the third ALA instillation

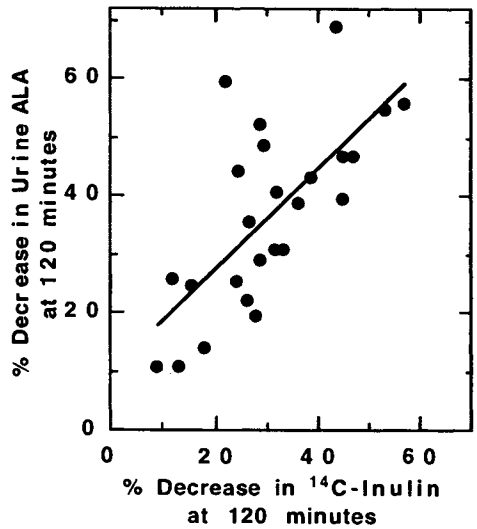

Fig. 4. Correlation between percent decreases in urinary ALA concentrations at 120 minutes and percent decrease in urinary ^{14}C-inulin concentrations at 120 minutes (n = 25, r^2 = 0.49, P < 0.0005). Dots represent actual experimental data, and the solid line represents best-fit linear regression.

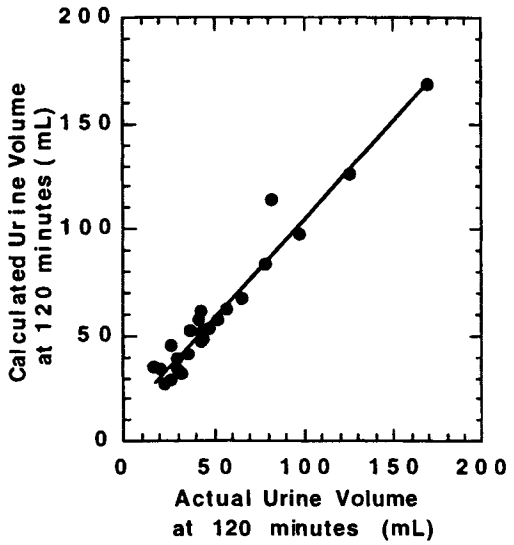

Fig. 5. Correlation of calculated urine volumes at the end of the 120 minute intravesical instillation period with the actual volumes of urine withdrawn and measured at the end of the instillation period ($n = 25$, $r^2 = 0.98$, $P < 0.0005$). Dots represent actual experimental data, and the solid line represents best-fit linear regression.

were submitted for routine analysis. Minor deviations from established ranges for adult dogs were noted in each animal, including elevated phosphorus, elevated alkaline phosphatase, and reduced total protein concentrations. However, animals used in this study were approximately six months of age and higher values of phosphorus and alkaline phosphatase, and lower total protein concentrations are commonly encountered in younger animals (14). It is also important to note that, although the intravesical ALA hydrochloride dose was dissolved in 0.2 M phosphate buffer, serum phosphorus levels before (7.2 ± 0.9 mg/dL) and after intravesical ALA treatment (7.4 ± 1.2 mg/dL) were not significantly different ($P > 0.1$). Further, no treatment-related changes in any other serum clinical parameters were noted, indicating that repeated intravesical ALA instillation was well-tolerated in these animals.

Small areas of focalized urothelial damage were observed in eight of nine animals, with damage most often noted near the trigone area. This urothelial damage was minor and most likely due to acute injury during urethral catheterization, as suggested by its prevalence near the opening of the urethra in the bladder (i.e., the trigone area) and the absence of blood in urine samples obtained during our studies. No abnormalities or irritation of the urothelial surface of the bladder were observed, indicating that repeated intravesical instillation of 3% ALA in phosphate buffer (final pH 5.0) was well-tolerated in the bladder of these animals. Microscopic histopathology revealed minimal to moderate hemorrhage and submucosal edema and minimal to slight acute inflammation in most of the urinary bladders. These findings were most prominent in the middle and posterior/trigone region of the bladder, and were most likely the result of physical trauma associated with catheterization and not from the presence of ALA. However, urinary bladders from catheterized dogs not treated with ALA were not available for comparison.

CONCLUSIONS

These studies demonstrate that high concentrations of ALA (i.e., up to 30 mg/ml of ALA hydrochloride) can be safely administered to the urinary bladder of dogs for periods of up to 2 hr. No local or systemic abnormalities were noted in these animals after administration of three separate intravesical ALA doses over a period of 5 weeks. No differences in serum phosphorus concentrations or any other clinical parameters were observed. Our studies were designed to examine the contributions of systemic absorption, urine production, and nonenzymatic degradation to the observed decreases in urine ALA concentrations during instillation. ALA is stable during intravesical instillation and is poorly absorbed into the systemic circulation. ALA conversion to pyrazine 2,5-dipropionic acid was negligible, accounting for less than 1% of the decrease in urinary ALA concentrations during the instillation period. Pyrazine 2,5-dipropionic acid concentrations were only noted in intravesical treatments during which the urine pH approached or exceeded pH 7.0. Less than 0.6% of the intravesical ALA dose was absorbed into the systemic circulation. Urine produced during the 2 hr intravesical instillation period caused ALA concentrations to decline to approximately 30% of the concentration in the original dosing solution. In summary, the primary factor contributing to observed decreases in urinary ALA concentrations was urine production. Future studies utilizing intravesical ALA instillation should include measures to restrict fluid intake prior to and during intravesical dosing as a means to limit dilution of the dosing solution and maximize bladder ALA concentrations during instillation.

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