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Note

Reversed-phase liquid chromatographic determination of chloroaluminum phthalocyanine tetrasulfonate in canine tissues and fluids

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Photodynamic therapy (PDT) of cancer is an experimental modality in which a photosensitizer that localizes in tumor tissue is administered and, subsequently, activated by the appropriate wavelength of light, resulting in tumor necrosis [1]. Of the second-generation photosensitizers that are being studied for use in PDT, phthalocyanines appear to be promising candidates [2]. Chloroaluminum phthalocyanine tetrasulfonate (AlPCS) (Fig. 1) was shown to be a highly effi-

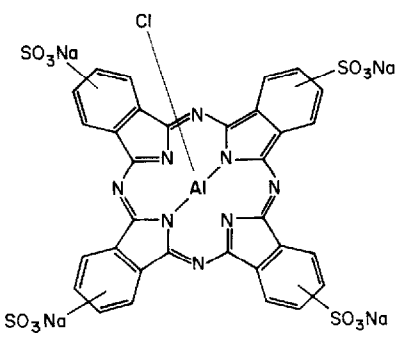

Fig. 1. Molecular structure of chloroaluminum phthalocyanine tetrasulfonate, sodium salt.

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cient photosensitizer for cultured mammalian cells [3,4] and in PDT of experimental animal tumors [5,6].

Information about tissue distribution and pharmacokinetics of AlPCS is a prerequisite to its rational use in PDT. The only two reports in which such studies were attempted [6,7] used spectrofluorometric measurement for the determination of AlPCS in total tissue extract. Analysis of AlPCS by high-performance liquid chromatographic (HPLC) procedures has not been reported.

This paper describes a sensitive HPLC method for the determination of AlPCS in fortified canine serum, muscle and urine. The applicability of this method for the measurement of AlPCS distribution in canine tissues following intravenous administration of a therapeutic dose is demonstrated.

EXPERIMENTAL

Chemicals

All reagents were of analytical grade, obtained from Fisher Scientific (Raleigh, NC, U.S.A.). AlPCS was synthesized by condensation of 4-sulfophthalic acid [8]. The final product was purified by reversed-phase column chromatography.

Serum and urine sample preparation

Samples (0.5 ml) were diluted with 0.5 ml of 0.2 *M* sodium hydroxide in a microseparation system (Centricon 30 molecular mass cut-off filter at 30 000 daltons, Amicon, Danvers, MA, U.S.A.) and vortex-mixed for 20 s. Samples were incubated for 1 h (serum) or 1.5 h (urine) at 60°C (vortex-mixed every 20 min) and centrifuged for 30 min at 4000 *g*. The filtrates were used for HPLC analysis.

Tissue sample preparation

Tissue samples were minced and carefully weighed. A 0.2 *M* sodium hydroxide solution was added to 0.1–0.3 g of tissue to obtain a total volume of 1 ml and this was sonicated for 5 min in an ultrasonic bath (Model 450, E/MC, Hauppage, NY, U.S.A.). Samples were incubated for 1 h at 60°C with sonication every 20 min. The extract was then transferred to a microseparation system and centrifuged for 30 min at 4000 *g*. The colorless filtrate was used for HPLC analysis.

Liquid chromatographic determination

The HPLC system consisted of a Model 501 HPLC pump and Model U6K injector (Waters Chromatography Division, Milford, MA, U.S.A.), a Model C-R3A Chromatopac integrator (Shimadzu, Kyoto, Japan), a Model FL-749 spectrofluorometric detector with 150-W xenon short ARC lamp and power supply (S.L. McPherson, Acton, MA, U.S.A.) and an Ultrasphere^{TM}–nitrile (CN) Spherisorb (5 μm) analytical column, 15 cm$\times$4.6 mm I.D. (Phenomenex, Rancho Palos Verdes, CA, U.S.A.). The mobile phase was a mixture of methanol–0.05 *M* sodium dihydrogenphosphate–glacial acetic acid (60:39:1, v/v) deaerated under vacuum prior to use. The flow-rate was 1.4 ml/min. The spectrofluorometric detector was set at a sensitivity of 0.01 for low concentrations of AlPCS in serum and 0.03–0.1 for tissues and urine samples and serum with high concen-

trations of AlPCS. Excitation and emission wavelengths were 610 and 685 nm, respectively, with a time constant of 5.0. Column temperature was maintained at room temperature. Standard curves were based on peak area. Samples of 10 μl were injected and quantitated using a calibration curve.

RESULTS AND DISCUSSION

Previous reports on the determination of AlPCS in plasma and tissues [6,7] were based on homogenization of specimens in 0.1 *M* sodium hydroxide and spectrofluorometric measurements of supernatant cleared by centrifugation. Because extracts of urine and some tissues, especially liver and spleen, are colored, a purification step was employed to avoid quencing of AlPCS fluorescence. This step involves a microseparation system with a 30 000 dalton cut-off filter. The usefulness of the microseparation system in removing macromolecules from serum and tissue extracts for β-lactam and tetracycline analysis was shown recently [9–11]. Because AlPCS binds tightly to serum proteins [3,12], the purification procedures employed would lead to a poor recovery, unless the dye was first released from the binding proteins. For this purpose, purification was preceded by incubation in hot sodium hydroxide. Preliminary attempts using incubation of spiked canine serum in 0.1 *M* sodium hydroxide for 4 h at 50 °C resulted in a recovery of AlPCS of 33.8% ($n=30$).

To improve the recovery of AlPCS from biological fluids and tissues, the following reagents were tested for their ability to release the bound dye: aqueous 0.1 *M* and 0.2 *M* sodium hydroxide, 50% ethanolic 0.1 *M* sodium hydroxide, 10% triethylamine and 1% dodecylotriethylammonium phosphate. Recovery of AlPCS after treatment with these solutions for 4 h at 50 °C varied from 15 to 50%. Increasing the incubation temperature to 60 °C in 0.1 *M* sodium hydroxide did not improve significantly the recovery of AlPCS from serum or tissues ($\sim$39%). However, when the sodium hydroxide concentration was increased to 0.2 *M*, recovery of AlPCS from serum and muscle tissue was over 90% already after 1 h

TABLE I

STATISTICAL ANALYSIS OF AlPCS RECOVERY FROM CANINE SERUM, MUSCLE AND URINE

Tissues were spiked with the indicated amount of AlPCS and extracted with 0.2 *M* sodium hydroxide for 1 h at 60 °C (serum and muscle) or 1.5 h at 65 °C (urine) prior to HPLC analysis.

Sample	Added (μg/ml)	n	Found (μg/ml)		Coefficient of variation (%)	Recovery (%)
			Range	Mean ± S.D.		
Serum	0.1	10	0.076–0.096	0.085 ± 0.0061	7.18	84.9
	1.0	10	0.88 –1.05	0.99 ± 0.0474	4.82	98.5
	5.0	10	4.18 –4.97	4.52 ± 0.2585	5.72	90.4
Muscle*	1	7	0.83 –0.98	0.90 ± 0.0518	5.75	90.0
Urine	5	7	2.88 –3.21	3.05 ± 0.1220	4.00	61.0

*Each extract contained 0.1–0.3 g tissue.

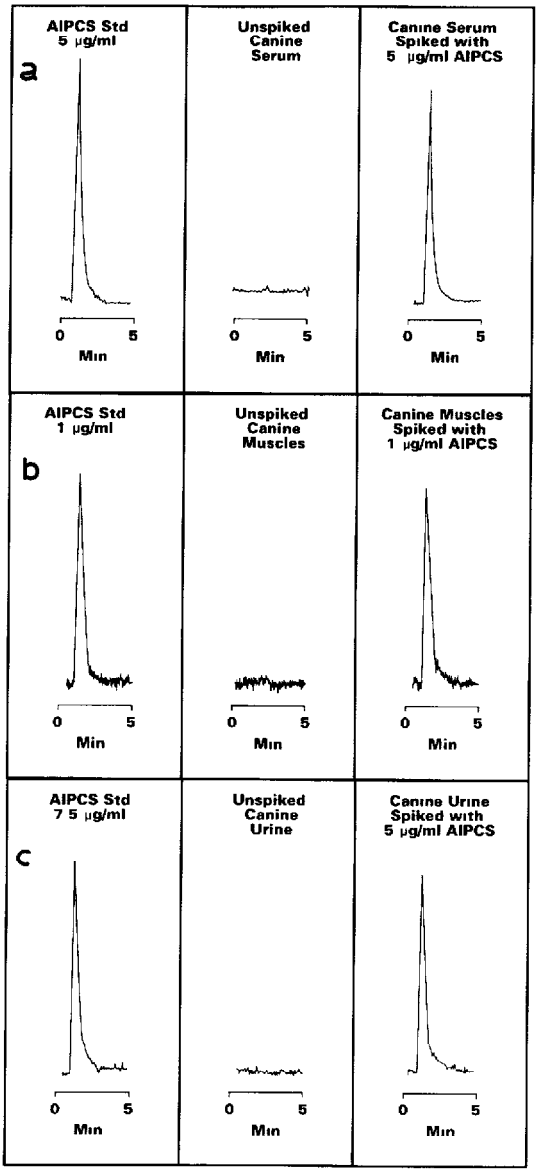

Fig. 2. Liquid chromatograms of: (a) 50 ng AlPCS in a standard solution, serum extract and extract from serum spiked with 5 μg/ml AlPCS, as indicated; (b) 10 ng AlPCS in a standard solution, muscle extract and extract from muscle spiked with 1 μg/ml AlPCS, as indicated; and (c) 75 ng AlPCS in a standard solution, urine extract and a urine extract spiked with 5 μg/ml AlPCS, as indicated.

incubation at 60°C (Table I). Maximum recovery from urine was 61% after 1.5 h incubation at 65°C (Table I).

Various stationary phases [Spheri-5 phenyl and nitrile (CN), 3 and 5 μm] and mobile phases (different combinations of methanol, water, sodium phosphate and acetic acid) were investigated. Optimal results were obtained using a re-

TABLE II

DISTRIBUTION OF AlPCS IN CANINE TISSUES

AlPCS was administered intravenously into a 19-kg beagle dog (1 mg/kg). Tissues samples (0.1 g) were taken for analysis 20 h after administration.

Tissue	AlPCS (μg/g)
Liver	9.02
Spleen	2.01
Urine	1.79 μg/ml
Lung	0.66
Serum	0.63 μg/ml
Large intestine	0.61
Small intestine	0.54
Kidney	0.38
Oral gingiva	0.34
Skin	0.24
Testes	0.12
Muscle	0.07
Brain	0.01

versed-phase Ultrasphere–nitrile, 5-μm column and a mobile phase consisting of methanol–0.05 *M* sodium dihydrogenphosphate–acetic acid (60:39:1, v/v). Under these conditions, the retention time of AlPCS was ca. 1.5 min.

Linearity of the spectrofluorometric detector response was studied by injecting increasing amounts of a standard AlPCS solution (0.1–10 μg/ml; injection volume 10 μl). Each measurement was repeated twice. The relationship between peak area and amount of AlPCS injected was linear with a correlation coefficient of 0.9981 ($n=6$).

Fig. 2a shows a chromatogram of 50 ng AlPCS in a standard solution, a serum extract (blank) and an extract from serum spiked with 5 μg/ml AlPCS. Analysis of the blank serum reveals no peaks at 685 nm. The retention time of AlPCS in both standard solution and spiked serum was the same.

Fig. 2b shows a chromatogram of 10 ng AlPCS in a standard solution, a muscle extract (blank) and an extract from muscle spiked with 1 μg/ml AlPCS. Again no peaks were observed in the chromatogram of the blank extract.

Results of HPLC analysis of 75 ng AlPCS standard, blank urine and urine spiked with 5 μg/ml AlPCS are shown in Fig. 2c. Even when incubation in 0.2 *M* sodium hydroxide at 65°C was longer than 1.5 h, the filtrate was slightly yellow. Recovery of AlPCS from urine was 61% with a coefficient of variation of 4.0% (Table I).

The reproducibility of the method was studied by analyzing thirty serum samples at AlPCS concentrations of 0.1, 1.0 and 5.0 μg/ml (Table I). The coefficient of variation ranged form 4.8 to 7.2% and the recovery was 84.9–98.5%. The limit of detection, defined as a peak at least three times the height of the baseline noise, was ca. 0.5 ng/ml.

The distribution of AlPCS in canine tissues 20 h after intravenous administra-

tion is shown in Table II. The dose administered is in the therapeutic range and was well above the detection limit in all tissues. AlPCS was eluted as the single peak with a retention time of 1.5 min from all tissues analysed. Thus, no metabolites have been seen under the experimental conditions used. The reticuloendothelial (liver, spleen) are clearly the major sites of AlPCS accumulation. Detailed pharmacokinetic and tissue distribution studies are now in progress using a large group of dogs.

CONCLUSION

A sensitive and selective HPLC assay was developed to quantitate AlPCS concentrations in canine tissues and body fluids. Due to its ability to detect low concentrations, the method is appropriate for the description of residues depletion profiles.

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