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Photosensitizers for tumor therapy: determination of bis(di-isobutyl octadecylsiloxy)silicon 2,3-naphthalocyanine (isoBOSINC) in rat tissue and serum by high-performance liquid chromatography

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ABSTRACT

Bis(di-isobutyl octadecylsiloxy)silicon 2,3-naphthalocyanine (isoBOSINC) is a synthetic potential photosensitizer for tumor therapy. A new method, which combines solvent extraction and several purification steps, has been developed to determine its presence in tissues. Separation and quantitation of isoBOSINC is done by high-performance liquid chromatography on a silica column with toluene as a mobile phase and using fluorescence detection (λ_{ex} = 365 nm, λ_{em} = 750 nm). For recovery studies, isoBOSINC was added to muscles at levels of 0.067 and 0.67 μg/g; the mean recoveries were 100%, with coefficients of variation of 6.1 and 6.4%, respectively. For liver samples, the amounts added were 0.67 and 6.7 μg/g and for serum 0.67 and 6.70 μg/ml. The mean recoveries for liver were 86 and 93%, with coefficients of variation of 7.7 and 4.4%, respectively. For serum, the mean recoveries were 99 and 96%, with coefficients of variation of 2.6 and 6.9%, respectively. Due to its low detection limit and selectivity, the method is appropriate for pharmacokinetic as well as tumor uptake studies following *in vivo* exposure to isoBOSINC. Preliminary data on tissue distribution of the photosensitizer in normal rats are also presented.

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INTRODUCTION

Photodynamic therapy (PDT) is an experimental treatment modality for tumors [1]. The technique involves the systemic administration of tumor-localizing photosensitizers and their subsequent excitation with visible light (600–800 nm) to produce tumor necrosis. Additional applications of this treatment modality relate to extracorporeal destruction of malignant cells with photosensitizers and light [2].

The most widely used sensitizers in PDT are hematoporphyrin derivative, HpD, and the purified product Photofrin II [3–6]. However, these sensitizers have substantial limitations [7]; these limitations have led to a search for additional sensitizers. Among the sensitizers that have been investigated are various phthalocyanines [8,9] and naphthalocynanines [10–13].

One of the interesting features of the naphthalocyanines is the position of their major long wavelength band, typically ≈780 nm. This feature makes them attractive because the penetration of human tissue by light in this region is better than that usually used with HpD and Photofrin II (≈630 nm, the orange–red region).

One of these naphthalocyanines, bis(di-isobutyl octadecylsiloxy)silicon 2,3-naphthalocyanine (isoBOSINC, Fig. 1), has physical and chemical properties that make it particularly attractive for PDT. Because of these properties, *in vivo* studies of the tissue and tumor distribution of this sensitizer and studies of its pharmacokinetics are desirable.

This paper describes a very sensitive high-performance liquid chromatographic (HPLC) procedure for isoBOSINC determination and offers some preliminary data on tissue distribution of the dye in normal rats.

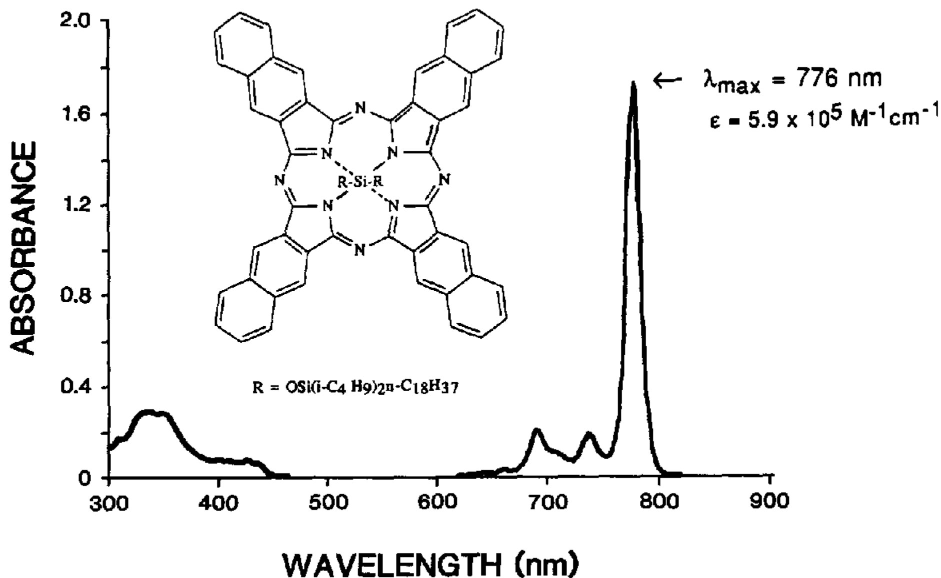

Fig. 1. Absorption spectrum and chemical structure of isoBOSINC in benzene.

EXPERIMENTAL

Chemicals

All reagents used in the analytical work were of reagent grade. They were obtained from a commercial source (Aldrich, Milwaukee, WI, USA). The isoBOSINC was synthesized by treating $SiNc(O\text{-}n\text{-}C_8H_{17})_2$ with $(iso\text{-}C_4H_9)_2C_{18}H_{37}SiOH$ (Nc is the ring system shown in Fig. 1). The crude isoBOSINC was purified by recrystallization [14].

Procedures

Animals. The animals used were Fischer 344 rats. They were obtained from a commercial source (Charles River Labs., Boston, MA, USA) and were housed at the Bowling Green State University Animal Facility. [This facility is operated in compliance with the Regulations of the Office for Protection from Research Risk (OPRR) and is registered under the Animal Welfare Act. Reg. No. 31-R-026.] The rats were kept in groups of three to four in cages held at 21°C and were allowed food and water *ad libitum.*

Spiked tissue samples. The rats were sacrificed by carbon dioxide inhalation, and tissue samples were taken for recovery studies. Tissues (10 g) were cut into small pieces and minced. Increasing amounts of isoBOSINC (ranging from 0.067 to 6.7 μg/g wet weight) were added. Aliquots of 0.3 g of minced tissue were weighed in glass scintillation vials and homogenized at $3 \cdot 10^4$ rpm in a high-speed homogenizer (Virtis Company, Gardiner, NY, USA) with 4 ml of tetrahydrofuran (THF) for 4 min. The homogenates were centrifuged for 30 min at 1600 *g*, and the resulting supernatants were evaporated to dryness at 60°C under a stream of nitrogen. After the residues had been dissolved in 2 ml of benzene, the solutions were vortexed at high speed for 1 min and centrifuged for 30 min at 1600 *g*. The supernatants obtained were transferred to 0.2-μm Centrex (cellulose acetate) filters (Schleicher & Schuell, Keene, NH, USA) and centrifuged at 400 *g* for 15 min. Aliquots of the filtrates were then analyzed by HPLC.

In vivo tissue samples. For pharmacokinetic studies, rats were anesthetized with sodium pentobarbital and injected intravenously with isoBOSINC suspended in Tween 80 in saline. Rats were sacrificed 48 h later as decribed above, and tissue samples were harvested. The samples were frozen (−20°C) immediately and kept frozen until analysis. When thawed, the samples were cut into small pieces and minced. About 0.30–0.35 g portions of the samples were weighed in glass scintillation vials (20 ml). All these portions except those from skin tissue were homogenized at $3 \cdot 10^4$ rpm in a high-speed homogenizer with 4 ml of THF for 4 min. The skin samples were homogenized with 5 ml of THF for 5 min. The homogenates were centrifuged for 30 min at 1600 *g*, and the resulting supernatants were evaporated to dryness at 60°C under a stream of nitrogen. The rest of the extraction procedure was as described above.

Serum samples. Blood was collected from rats in 15-ml sterile plastic vials and

was centrifuged at 400 *g*. Samples of serum (1 ml) were mixed with THF in a mechanical shaker for 1 h and vortexed at high speed for 1 min. The extracts were then processed in the same way as the tissue extracts.

Liquid chromatographic determination

HPLC analysis was performed on a Gilson 42 high-performance liquid chromatograph (Gilson Medical Electronics, Middleton, WI, USA) equipped with a 728 autosampler (MicroMeritics, Norcross, GA, USA). The detection system consisted of a McPherson FL 745 fluorometric detector (S.I. McPherson, Acton, MA, USA) equipped with an R1104 phototube and an SP 4270 integrator (Spectra Physics, San Jose, CA, USA). The excitation light used was the 365-nm line (filtered) from a medium-pressure mercury lamp (isoBOSINC absorbs at ≈350 nm and fluoresces at ≈800 nm, Fig. 1). A 750-nm cut-off filter was interposed in the emission pathway so as to allow only naphthalocyanine fluorescence to reach the detector. This prevented the detection of emissions from adventitious porphyrin contaminants. For the analysis of samples with low concentrations of isoBOSINC, the sensitivity of the detector was set at 0.01, and the time constant was set at 1.0 s. For samples with high concentrations, the sensitivity was set at 0.03–0.3, and the time constant was again set at 1.0 s. The analytical column used was a Spherisorb silica column (5 μm; 25 cm x 4.6 mm I.D.; Phenomenex, Rancho Palos Verdes, CA, USA). The column was used at room temperature (21°C). The mobile phase was benzene that had been deaerated under vacuum just prior to use; the flow-rate was 1.5 ml/min, the temperature was room temperature (21°C), and the sample size used was 50 μl. Because of the known carcinogenicity of benzene, an alternative solvent, toluene, was used both for dissolving the samples and as a mobile phase.

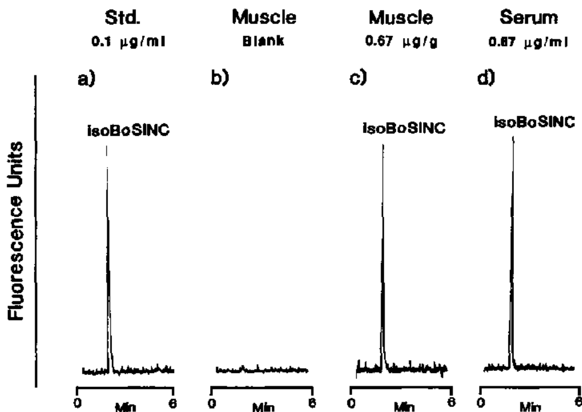

Fig. 2. HPLC profiles of extracts of muscle and serum. (a) Standard; (b) muscle blank (no photosensitizer added); (c) muscle and (d) serum to which isoBOSINC had been added.

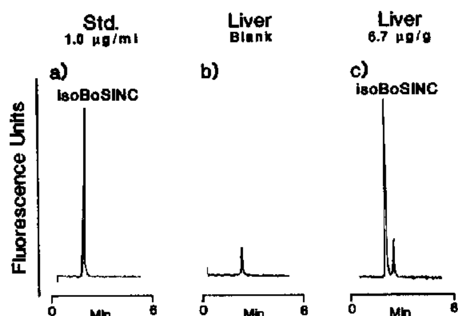

Fig. 3. HPLC profiles of liver extracts. (a) Standard; (b) liver blank (no photosensitizer added); (c) liver to which isoBOSINC had been added.

RESULTS AND DISCUSSION

Linearity of detector response

isoBOSINC standard solutions ranging from 0.01 to 1.0 μg/ml were utilized to determine the linearity of the detector's response. The solutions were injected in 50-μl amounts in triplicate. The relationship between the area under the peak and the amount of isoBOSINC was linear, with a correlation coefficient of 0.9988 (n = 10).

HPLC profiles

Fig. 2 shows chromatographic profiles of extracts from muscle and serum after isoBOSINC had been added to the tissues. Fig. 3 shows chromatographic profiles of spiked liver extracts. Fig. 4 shows chromatographic profiles of extracts from brain, spleen and liver after *in vivo* administration of isoBOSINC.

Extraneous peak

The extra peak in the profile in Fig. 3c is genuine. Since it is observed in the

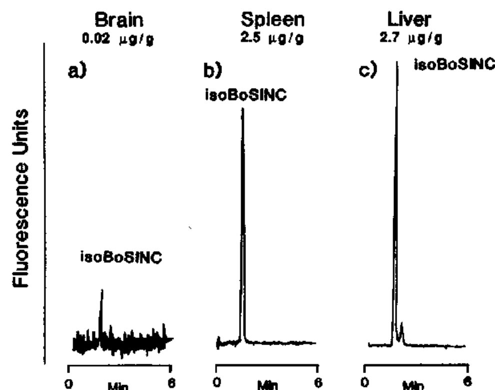

Fig. 4. HPLC profiles of tissue extracts from rats injected with isoBOSINC *in vivo*. (a) Brain; (b) spleen; (c) liver.

TABLE I

RECOVERY OF isoBOSINC FROM TISSUES AND SERUM OF RATS TO WHICH isoBOSINC HAD BEEN ADDED

Tissue	isoBOSINC added (μg/g)	isoBOSINC recovered (μg/g)		Mean recovery (%)	Coefficient of variation (%)
		Range	Mean ± S.D. (n = 10)		
Muscle	0.067	0.060 - 0.072	0.067 ± 0.004	100	6.1
	0.67	0.58 - 0.69	0.67 ± 0.042	100	6.4
Liver	0.67	0.52 - 0.62	0.58 ± 0.045	86	7.7
	6.70	5.55 - 6.44	6.19 ± 0.272	93	4.4
Serum	0.67[a]	0.64 - 0.68	0.66 ± 0.017	99	2.6
	6.70[a]	5.56 - 6.94	6.40 ± 0.442	96	6.9

[a] μg/ml.

blank profile (Fig. 3b), it is not due to a drug metabolite. Studies by Weagle *et al.* [15] have shown that normal mouse skin extracts give a prominent fluorescence emission at 674 nm. These workers concluded that this emission was due to one or more degradation products of chlorophyll that were in the mouse food pellets (either pheophorbide *a* or pheophytin *a* or both). *In vivo* studies may help clarify the origin of the peak observed here. We would like to caution that quantitative data obtained from fluorometric measurements alone, without HPLC separation, may include an additional compound and therefore lead to errors.

Recoveries of isoBOSINC

A summary of recovery studies of isoBOSINC found in spiked rat tissues and serum is given in Table I. The assay seems accurate, with mean recoveries ranging

TABLE II

isoBOSINC CONCENTRATIONS IN TISSUES AND SERUM OF RATS 48 h AFTER INTRAVENOUS INJECTION OF THE PHOTOSENSITIZER

Tissue	isoBOSINC found (mean ± S.D., n = 4) (μg/g)	
	0.25 mg/kg administered	0.50 mg/kg administered
Spleen	5.31 ± 0.85	6.77 ± 0.95
Liver	1.78 ± 0.20	3.38 ± 0.92
Serum	0.29 ± 0.05[a]	0.31 ± 0.08[a]
Muscle	0.02 ± 0.002	0.05 ± 0.01
Brain	< 0.02	< 0.02

[a] μg/ml.

from 86 to 100%, and coefficients of variation from 2.6 to 7.7%. From the mean recovery obtained for muscles spiked with 0.067 μg/g (Table I) and from the even lower levels detected in muscles and brain from *in vivo* studies (Table II and Fig. 4), we concluded that the method is very sensitive. The limit of detection under the described analytical conditions (the amount required to give a peak at least three times the height of the baseline noise) is 0.067 μg/g. By varying the final volume and the initial sample size, one can further improve the detection limit to 0.02 μg/g (Table II, muscle, Fig. 4, brain). Because of the lipophilic nature of isoBOSINC, very high levels of the dye were expected to be accumulated in certain organs, such as liver and spleen; consequently, the lowest spike levels were 0.67 μg/g. For serum, the lowest spike level was 0.67 μg/ml, but from samples taken from *in vivo* studies (data not shown) levels as low as 0.02 μg/ml were easily detected. The same recoveries, essentially, were obtained when toluene was used as a solvent.

Other analytical conditions

In addition to using benzene or toluene as the mobile phase, Spherisorb silica columns were also used with a mobile phase made of toluene in *n*-heptane (1:4, v/v). Although the HPLC profiles were essentially unchanged, the retention times were increased close to three-fold. Further confirmation of the high purity of the synthetic isoBOSINC was obtained by running HPLC on a reversed-phase column of Partisil 5 C_8 and a mobile phase composed of methanol–*n*-heptane–toluene (2:1:1, v/v). For purposes of simplicity, the very extensive *in vivo* tissue studies were conducted with a single solvent on silica columns.

Preliminary tissue distribution data

In preliminary studies, isoBOSINC was administered to rats intravenously at two doses, and the distribution of the photosensitizer in selected tissues and serum was determined 48 h later. A summary of relevant data is given in Table II. The data show that some tissues take up much more isoBOSINC than others and that the amounts of isoBOSINC taken up by the tissues are dose-dependent. The high concentrations of isoBOSINC in spleen and liver confirm that the reticuloendothelial system is heavily involved in the uptake of the sensitizer from the circulation. The low concentration of isoBOSINC in the brain indicates that isoBOSINC apparently does not penetrate the blood–brain barrier to any appreciable extent.

A number of studies of sensitizers in tissues and cells have been published in recent years (for reviews see refs. 16 and 17). Most of them involved solvent extraction followed by either spectrophotometric or spectrofluorometric quantitation [18,19], but only a few involved extraction and HPLC [20,21].

CONCLUSIONS

An analytical method to isolate and quantitate isoBOSINC, a novel photosensitizer with potential for PDT, has been developed. The method takes advantage of the hydrophobicity and the long-wavelength fluorescence of this compound. With appropriate filters, this method permits the detection and quantitation of small amounts of isoBOSINC without apparent interferences. Pharmacokinetic studies on normal and tumor-bearing rats using the extraction–HPLC method are in progress and will be reported elsewhere.

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REFERENCES

1 T. J. Dougherty, *Oncology*, 3 (1989) 67.
2 F. Sieber and G. J. Krueger, *Semin. Hematol.*, 26 (1989) 35.
3 C. J. Gomer and T. J. Dougherty, *Cancer Res.*, 39 (1979) 146.
4 M. L. Eckhauser, J. Persky, A. Bonaminio, J. Crespin, A. L. Imbembo and S. Holt, *Lasers Med. Sci.*, 2 (1987) 101.
5 R. Herrera-Ornelas, N. J. Petrelli, A. Mittelman, T. J. Dougherty and D. G. Boyle, *Cancer*, 57 (1986) 677.
6 Y. Hayata, H. Kato, C. Konaka, R. Amamiya, J. Ono, J. Ogawa, K. Kinoshita, H. Sakai and H. Takahaski, *Chest*, 86 (1984) 169.
7 M. Kreimer-Birnbaum, *Semin. Hematol.*, 26 (1989) 157.
8 E. Ben-Hur and I. Rosenthal, *Photomedicine*, Vol. 3, CRC Press, Boca Raton, FL, 1987, p. 1.
9 C. J. Tralau, H. Barr, D. R. Sanderman, T. Barton, M. R. Lewin and S. G. Bown, *Photochem. Photobiol.*, 46 (1987) 777.
10 P. A. Firey and M. A. J. Rodgers, *Photochem. Photobiol.*, 45 (1987) 535.
11 N. C. Yates, J. Moan and A. Western, *J. Photochem. Photobiol., B, Biol.*, 4 (1990) 379.
12 B. Paquette, H. Ali, R. Langlois and J. E. van Lier, *Photochem. Photobiol.*, 51 (1990) 313.
13 V. Cuomo, G. Jori, B. Rihter, M. E. Kenney and M. A. J. Rodgers, *Br. J. Cancer*, 62 (1990) 966.
14 J. R. Sounik, L. A. Schechtman, B. D. Rihter, W. E. Ford, M. A. J. Rodgers and M. E. Kenney, *SPIE 1203 Photodynamic Therapy: Mechanisms*, Vol. II, 1990, p. 224.
15 G. Weagle, P. E. Paterson, J. Kennedy and R. Pottier, *J. Photochem. Photobiol., B, Biol.*, 2 (1988) 313.
16 D. Kessel, *Int. J. Rad. Biol.*, 49 (1986) 901.
17 C. J. Gomer, *Semin. Hematol.*, 26 (1989) 27.
18 J. Moan, Q. Peng, J. F. Evensen, K. Berg, A. Western and C. Rimington, *Photochem. Photobiol.*, 46 (1987) 713.
19 M. L. Pantelides, J. V. Moore and N. J. Blacklock, *Photochem. Photobiol.*, 49 (1989) 67.
20 M. M. Zuk, K. Tyczkowska, E. Ben-Hur, H. Newman, I. Rosenthal and S. W. Crane, *J. Chromatogr.*, 433 (1988) 367.
21 D. A. Bellnier, Y.-K. Ho, R. K. Pandey, J. R. Missert and T. J. Dougherty, *Photochem. Photobiol.*, 50 (1989) 221.