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Short communication

High-performance liquid chromatographic determination of the silicon phthalocyanine Pc 4 in human blood

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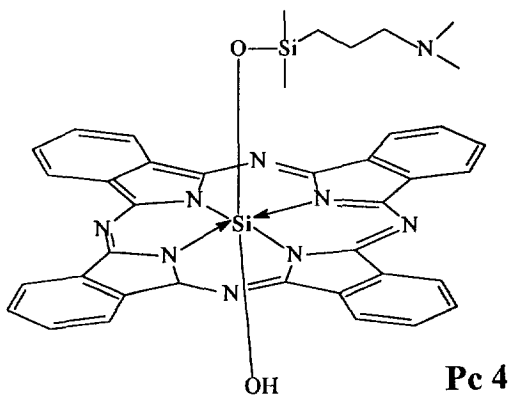
Abstract

A sensitive and reproducible method has been developed for the measurement of the silicon phthalocyanine Pc 4 in red blood cell concentrates (RBCC). The procedure involves extraction of the RBCC with acetonitrile, purification of the extracts with reversed-phase Sep-Pak C_{18} cartridges and determination of Pc 4 in the extracts by high-performance liquid chromatography (HPLC) using a reversed-phase C_{18} column. The detection limit with 1-ml RBCC samples is 2 ng. This method is applicable to monitoring Pc 4 during its use as a photosensitizer for the inactivation of viruses in RBCC prior to transfusion. It has the potential to be adapted for measuring Pc 4 in tissues during its use in photodynamic therapy of cancer.

1. Introduction

Blood transfusion is a highly safe medical procedure in general. However, the transfusion of blood involves certain risks. Among these is the transmission of human pathogenic viruses, most notably human immunodeficiency virus (HIV). Because of this, considerable work has been done on methods for the sterilization of blood products [1]. With the exception of cellular components, all blood products are currently available in virally inactivated forms. For virus inactivation of red blood cells (RBC) and platelet concentrates the use of photosensitizer-based methods has considerable potential [2,3]. Phthalocyanines, with their intense absorption band in the far red (650–700 nm), are particularly suitable for use in RBCC. Among these, the silicon phthalocyanine $HOSiPcOSi(CH_3)_2(CH_2)_3N(CH_3)_2$ (Pc 4) (Fig. 1) merits attention



Pc 4

Fig. 1. The molecular structure of Pc 4.

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because it is particularly effective in killing HIV and other lipid enveloped viruses as well as blood borne parasites [3,4].

Currently we are developing a practical procedure for sterilizing RBCC prior to transfusion using Pc 4 and red light. For this work, it is necessary to be able to follow the fate of the Pc 4 at various stages of the procedure. In this paper we describe an HPLC method that permits that to be done. This method is quite sensitive, allowing the detection of 2 ng/ml of Pc 4 in RBCC.

2. Experimental

2.1. Chemicals

All reagents were analytical or HPLC grade, as required. The Pc 4 was synthesized as previously described [5] and was purified by reversed-phase chromatography on a C_{18} column. Its purity was about 97%.

2.2. Formulation of Pc 4 in CRM

Pc 4 is water-insoluble and thus it was necessary to formulate it. It was formulated as an aqueous emulsion using the emulsifying agent Cremophor EL (CRM) (Sigma, St. Louis, MO, USA). To prepare this formulation, 3 mg of Pc 4 was dissolved in 2 ml of ethanol and then 1 ml of CRM prewarmed to 45°C was added dropwise to the ethanol solution with vigorous mixing. Mixing was continued overnight at room temperature to evaporate off the ethanol and the concentrate was diluted with 9 ml of saline to obtain a final concentration of 10% CRM and 0.4 m*M* Pc 4. The resulting clear emulsion was filter-sterilized using a 0.2-μm filter and stored at −20°C. The residual ethanol concentration in the emulsion was 5 ppm, determined by gas chromatography. The exact Pc 4 concentration was determined both by spectrophotometry and by HPLC.

2.3. Determination of Pc 4 in RBCC

To determine Pc 4 in RBCC, samples of 10 ml RBCC (70% hematocrit), obtained from the New York Blood Center, were spiked with 2 μM Pc 4 and a mixture of scavengers of reactive oxygen species (5 m*M* Trolox, 4 m*M* glutathione and 4 m*M* mannitol, obtained from Sigma). RBC were separated from plasma by centrifugation at 2500 rpm (1300 *g*) for 15 min. Aliquots of 0.6 ml plasma, RBC or RBCC were taken for analysis. The RBC were rinsed five times in phosphate buffered saline (PBS) prior to extraction.

In the determination procedure, the plasma, RBC or RBCC aliquots were mixed with 0.1 ml of 8 *M* guanidine-HCl (aminomethaneguanidine hydrochloride) (Sigma). After 15 min at room temperature samples were extracted with 2.5 ml acetonitrile (Sigma) by vortex-mixing for 1 min and ultrasonication for another 10 min. The extracts were centrifuged at 2500 rpm (1300 *g*) for 10 min. The acetonitrile layer was saved and the residue was extracted as above with 2.5 ml acetonitrile. The acetonitrile extracts were combined and evaporated to dryness at 45–50°C under a stream of nitrogen. The residue was dissolved in 0.1 ml of 5% Cetrimide (hexadecyltrimethylammonium bromide) (Sigma), and the solution was vortex-mixed, diluted with 3 ml H_2O, and transferred to a 1-g C_{18} Sep-Pak reversed-phase cartridge (Waters, Milford, MA, USA) that had been prewashed with 15 ml of methanol and 15 ml of water. The loaded cartridges were washed with 12 ml of water, and then dried under vacuum for 10 min. The Pc 4 was eluted with 18 ml of methanol and the eluant was evaporated to dryness under a stream of nitrogen at 45–50°C. The residue was dissolved in 1 ml methanol and filtered through a 0.45-μm Centrex filter (Schleicher and Schuell, Keene, NH, USA). Samples were kept at 4°C prior to HPLC analysis.

For Pc 4 analysis, an HPLC system (Thermo Separation Products, Fremont, CA, USA) equipped with a fluorescence detector (Model FL 2000) set at $\lambda_{ex} = 610$ nm and $\lambda_{em} = 670$ nm as well as an absorption detector (Spectra Focus)

set at 668 nm was used. The system consisted of autosampler (Model AS 3000), high pressure quartenary gradient pump (Model P4000), a controller (Model SN4000), and was operated with a proprietary software (Spectra System PC 1000, version 2.5). In the analysis of the solution, aliquots of 10–100 μl were injected into C_{18} analytical HPLC column, Dynamax 60A, 8 μm, 25 cm × 4.6 mm (Rainin Instrument, Woburn, MA, USA). The mobile phase consisted of 10% H_2O and 1% CH_3COOH in methanol isocratically pumped at a flow-rate of 1.5 ml/min. The retention time of Pc 4 was about 2–3 min. The importance of the mobile phase pH on the retention time was not determined. Increasing the water content increased the retention time. Quantitation of Pc 4 in analyzed samples was done using an external standard calculation method. Standard curves were constructed on each day of analysis. Under the conditions described 10 ng of authentic Pc 4 yielded a peak with an area of 15 000 (fluorescence detection) or 100 000 (focus detection). The detection limit is 2 ng/ml.

2.4. Determination of Pc 4 in RBC membranes

In order to determine the amount of Pc 4 bound to RBC membranes, RBCC were incubated with 2 μM Pc 4 for 1 h and the RBC separated from the plasma as described above. A 10-ml sample of RBC, suspended in saline at 50% hematocrit, was placed in a 50-ml centrifuge tube and 5 ml solution containing 10 mM tris-(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4 and 0.1 mM ethylene glycol-bis-(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA) (Sigma) was added dropwise and then was diluted with an additional 30 ml of it. The resulting RBC lysate was mixed gently for 5 min and centrifuged at 15 000 g for 10 min. The membrane pellet was resuspended in 35 ml of the lysing solution and centrifuged. This procedure was repeated 5–6 times until the membranes were white. The washed membranes were resuspended in 5 ml saline and 0.6 ml samples were extracted and analyzed for Pc 4 content, as described above.

3. Results

Fig. 2 shows an HPLC chromatogram of Pc 4. Evidently, no interfering peak is detected in the blank while Pc 4 extracted from RBC or plasma gives the same elution profile as does authentic Pc 4.

Table 1 summarizes the values for Pc 4 extracted from RBCC and plasma spiked with Pc 4

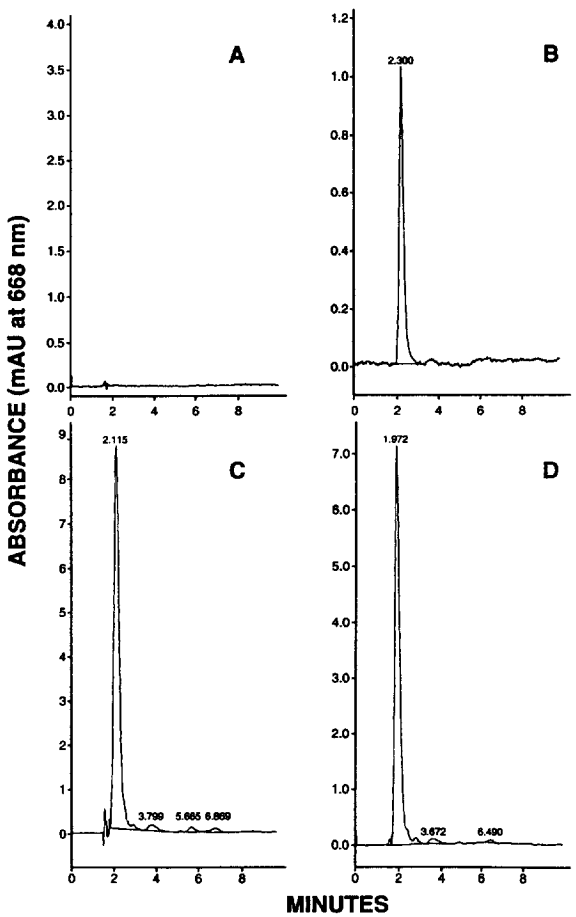

Fig. 2. HPLC chromatograms of Pc 4. (A) Blank, an extract of RBCC not spiked with Pc 4. (B) A standard of 1 ng Pc 4. (C) An extract of plasma spiked with 2 μM Pc 4. (D) An extract of RBCC spiked with 2 μM Pc 4.

Table 1
Analysis of Pc 4 in human RBCC and plasma[a]

Sample	Added		Measured (μg/ml)		Recovery (%)	C.V. (%)
	μg/ml	μM	Range	Mean ± S.D.		
RBCC	0.14	0.2	0.12–0.15	0.12 ± 0.01	87.3	5.5
	1.43	2.0	0.88–1.20	1.00 ± 0.10	72.2	9.6
	7.17	10.0	5.25–5.74	5.60 ± 0.11	78.1	3.2
Plasma	0.14	0.2	0.13–0.15	0.14 ± 0.01	99.5	4.0
	1.43	2.0	1.35–1.49	1.43 ± 0.01	100.2	3.4
	7.17	10.0	6.81–7.62	7.13 ± 0.29	99.5	3.9

[a] Samples of RBCC or plasma ($n = 10$) were treated with Pc 4 at the indicated concentrations and mixed for 30 min at room temperature. The Pc 4 was determined as described in Section 2. The correlation coefficient was >0.999. All determinations were made on the same day.

at a final concentration of 0.2–10 μM. The results show an excellent recovery of the photosensitizer from the plasma (essentially 100%) and a good recovery from RBCC (about 80%) is achieved. The value of the variation coefficient (about 4%) indicates that a very high reproducibility is also achieved. The accuracy and precision of the method were established intra-day.

When RBCC were separated into RBC and plasma fractions prior to analysis, it was found that Pc 4 was distributed almost equally between the two fractions. Thus, in RBCC containing 70% RBC and 30% plasma that had been treated with Pc 4 at a concentration of 2 μM (1.434 μg/ml), 58% of the photosensitizer was associated with RBC and 42% was associated with the plasma. This distribution was achieved within 5 min of adding Pc 4 to the blood and was independent of further incubation time.

In the case of RBC membranes that had been isolated from RBCC incubated with 2 μM Pc 4 for 1 h, 97 ± 1% of the dye in the RBC fraction was associated with the membranes.

4. Discussion

Work on the use of Pc 4 and red light for the sterilization of RBCC prior to their transfusion is now at preclinical stages of development at the New York Blood Center. For the further progress of this work, a reliable procedure for the determination of Pc 4 in the treated RBCC at various stages of the procedure is essential. Our results show that the combined use of extraction and clean-up prior to HPLC analysis allows an accurate, sensitive and a reproducible measurement of Pc 4 with very good recovery, and thus fulfills this need. This is to be compared with much lower recoveries of 20–50% usually encountered in the literature when analytes are measured in blood without prior extraction [6].

The procedure employed to obtain the data in Table 1 was optimized (see Section 2) after trying many variations. Thus, the choice of organic solvent for extraction was very important. Acetonitrile was better in this respect than dimethyl formamide, with ca. 20% higher recovery in plasma and ca. 5 times higher recovery in RBCC. This could be due to better solubility of Pc 4 in acetonitrile. Inclusion of guanidine-HCl during extraction was essential for a high, reproducible recovery. In its absence, recovery of Pc 4 was variable (46–86% in RBCC and 31–95% in plasma). Marked variability in the recovery of drugs from serum during HPLC assay has been observed and ascribed to the source of the serum [7]. Addition of Cetrimide prior to Sep-Pak clean-up also improved recovery of Pc 4 from 34% to 66% and reduced variability. Both of these additives presumably act as detergents

to dissociate Pc 4 from its binding to proteins [8,9]. A clean-up step using C_{18} Sep-Pak cartridge is not essential for a good, reproducible recovery. However, in its absence the retention time of Pc 4 on the C_{18} column becomes shorter and varied. Pc 4 could also be measured in plasma without solvent extraction, using a C_{18} Sep-Pak clean-up step only. However, recovery was reduced to about 65%. The use of a clean-up step with Sep-Pak C_{18} is a common procedure [6,10] and its use in the present work improved recovery of Pc 4 and particularly enhanced reproducibility of the measurement.

The use of reversed-phase HPLC with C_{18} column for separation and determination of sulfonated phthalocyanines has been reported [11–14]. However, this is the first report of such use to analyze a silicon phthalocyanine with an axial ligand. It underlines the non-fragile nature of compounds of this class. Even more important is the demonstration in this paper that a solvent extraction of plasma is essential to obtain complete and reproducible recovery. Our previous paper [12] where an extraction procedure was used to measure sulfonated aluminum phthalocyanine ($AlPcS_4$) in blood and tissues is not applicable to the present case. This is because $AlPcS_4$ is water-soluble and could be extracted with 0.2 *M* NaOH while Pc 4 is water insoluble and an appropriate organic solvent had to be identified.

This is also the first report demonstrating the distribution of Pc 4 between plasma and RBC and showing that essentially all of the dye bound to RBC is localized in the membrane. Such a distribution will be helpful in devising procedures to remove Pc 4 from the blood after treatment and prior to transfusion. It is reasonable to assume that dye bound to the RBC membrane will be easier to remove than dye molecules located inside the cell. Our results (unpublished), showing that about 90% of Pc 4 bound to RBC can be removed with an appropriate resin, support this conclusion.

In summary, the method described in this paper permits reproducible and sensitive detection of Pc 4 in RBCC and it should be useful in further developing medical applications of this dye, such as in the photodynamic treatment of cancer [15].

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