Evaluation of fluorescence measurement techniques for tumour detection in vivo
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CHAPTER 8

Correlation between fluorescence in vivo and photosensitiser concentration in rat liver and tumour

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Abstract
Two techniques for photosensitiser pharmacokinetics measurements, fluorescence intensity at one wavelength and a fluorescence intensity ratio, were evaluated in vivo. Fluorescence spectra of mTHPC and SC102 in a rat liver tumour model were measured at different times after administration, and the concentration of PS in tissue was determined after extraction. A mathematical model predicted a linear relation between the fluorescence ratio and PS concentration, but due to a large spread in the data this was difficult to confirm. Pharmacokinetics behaviour of both techniques agreed reasonably with mTHPC concentration. Tumour to normal ratios could be determined using the fluorescence ratio.
Introduction

Fluorescence of a tumour-selective photosensitiser (PS) is used in photodynamic diagnosis and photodynamic therapy to determine relative concentrations of the administered drug in tumour and healthy tissue (Lam et al. 1990, Braichotte et al. 1995a, Kriegmair et al. 1996). Tissue is illuminated with a wavelength suitable for excitation of PS fluorescence and fluorescence is detected at a PS emission wavelength using imaging devices or optical fibres. Diagnosis or treatment is performed when maximum tumour to normal ratio in fluorescence is reached, based on the assumption that fluorescence is proportional to the PS concentration.

The shallow penetration depth of the excitation light, usually in the blue or green region of the spectrum, makes fluorescence detection in vivo suitable only for superficial lesions. The advantage of fluorescence detection, compared to other techniques that determine PS concentrations in tissue, is that it is non-invasive and can be performed in vivo. In techniques such as high performance liquid chromatography (HPLC) and radiolabelling, tissue has to be removed from the body (Brown and Vernon 1990).

A linear relation between the fluorescence intensity and concentration of meta-tetra(hydroxyphenyl)chlorin, mTHPC, has been reported by Braichotte et al. (1995b) using radiolabelling and Grahn et al. (1997) using extraction and HPLC. Different tissues, including liver and tumour tissue (Grahn et al. 1997) were compared. The proportionality constant between fluorescence and PS concentration was dependent on tissue type. However, using a mouse model, Ansell et al. (1995) observed that the time of maximum mTHPC concentration after injection did not coincide with maximum fluorescence, which would be expected if PS fluorescence was proportional to PS concentration.

Fluorescence measurements in vivo are complicated by tissue constituents. Absorption by haemoglobin strongly influences the distribution of the blue excitation light. In addition, variations in the geometry of the excitation light device and detection system cause artefacts in the measured fluorescence signal. Several techniques have been developed to correct the measured fluorescence for these artefacts, such as ratio fluorometry (Profio et al. 1983, Sinaasappel and Sterenborg 1993, Saarnak et al. 1998 (Chapter 6)).

Previous work using a rat liver tumour model with mTHPC (Chapter 7) indicated that the fluorescence ratio in vivo could be used to determine a tumour to normal ratio (T/N-ratio) which was reasonably in agreement with the T/N-ratio based on tissue concentrations. In the present study we extended the investigation of using fluorescence in vivo as a measure of PS concentration, using the same animal and tumour model. The PS used were mTHPC and SC102, a poly(ethylene glycol) conjugate of mTHPC. These two compounds have similar excitation and emission characteristics. SC102, in contrast to mTHPC, is a hydrophilic drug and is expected to show different pharmacokinetics behaviour.

In this paper spectrally resolved fluorescence measurements in vivo were compared with PS concentrations derived by tissue extractions. Two fluorescence techniques were used: fluorescence measured at a red PS emission wavelength band and a fluorescence ratio between red PS emission and green autofluorescence, normalised by the autofluorescence ratio measured before injection.
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The first method is the most straightforward way to determine whether PS is present in tissue. However, it is influenced by the differences in optical properties of the tissues and movements during the measurement. The second method eliminates the influence of these properties (Profio et al. 1983).

The aim of this study was to investigate fluorescence techniques for non invasive pharmacokinetics and semi-quantitative fluorophore measurements. The techniques were evaluated on a tumour model chosen for its obvious difference in optical properties with the surrounding normal tissue.

Materials and methods

Animals and tumour

All experiments were performed on male Wag/rij rats weighing 200-250 g. Tumours in the liver were induced by injecting 5x10⁵ tumour cells from the CC531 adenocarcinoma cell line (Marquet et al. 1984) subcapsular into the left lateral lobe, the upper right lobe and the lower right lobe of the liver after laparotomy. Tumour cell injection and other surgical procedures were performed under general anaesthesia using 1.5% halothane in 0.05 l/min nitrous oxide and 0.4 l/min oxygen. Measurements were performed eight to ten days after injection when the tumours had reached a diameter of 0.5-0.7 cm.

Chemicals

Meta-tetra(hydroxyphenyl)chlorin (mTHPC) and the tetra-pegylated (PEG-2000) derivative of mTHPC, SC102, were donated by Scotia Pharmaceuticals Ltd. (Guildford, UK). mTHPC was dissolved in 30% polyethylene glycol (PEG-400), 20% ethanol (96%) and 50% water. SC102 was dissolved in distilled water.

Fluorescence set-up

Fluorescence was measured with a set-up schematically described in Fig. 1. A 100 W mercury-lamp was used as light source and for excitation the Hg-emission line at 405 nm was selected with an interference filter (Oriel 56541). Excitation light was delivered to the tissue through a 600 µm fibre put in contact with the tissue. Fluorescence was detected through a second 600 µm fibre positioned parallel to the excitation fibre. Both fibres were inserted in metal tubes at the tip and had a black coating to prevent light coupling between the fibres. The detection fibre was connected to an
imaging spectrograph (Oriel MS257) with a 16 bit 256x1024 pixel CCD camera (Oriel Instaspec IV) cooled to -35°C. Reflections of the excitation light were avoided by placing a Schott KV 500 high-pass filter at the entrance of the spectrograph. The integration time was 0.2-0.5 s and spectra were measured between 500 and 830 nm. The spectrograph and the CCD camera were controlled by the program Instaspec 1.1 (Oriel). Data were collected on a PC and the fluorescence ratios were calculated with a Pascal-program.

Measurement procedure
mTHPC and SC102 were injected intravenously at a concentration of 0.3 mg/kg and 60 mg/kg, respectively, at 4, 24, 48, 72 and 120 (only SC102) h before measurements. Each measurement group contained five rats. Two control rats with tumours did not receive any PS. After general anaesthesia a laparotomy exposing liver and tumours was performed. Fluorescence was measured on three tumours and three adjacent normal spots on the liver per animal. Three measurements were performed on each site with the fibre slightly displaced between every measurement. After the measurements the rat was killed and liver and tumours were excised, frozen in liquid nitrogen and stored at -20 °C. After thawing of the tissue samples, mTHPC or SC102 extraction (see below) was performed.

Photosensitiser extraction
The extraction method was based on a solubilization technique reported by Lilge et al. (1997). Tissue (50-100 mg) was dissolved in 2 ml Solvable™ (Packard, Groningen, The Netherlands) for 2 h in a waterbath at 55 °C. The dissolved samples were diluted 1:21 to obtain an optical density < 0.1 at the excitation wavelength. Fluorescence of the samples was measured in a fluorescence spectrophotometer and the concentration was determined using a standard curve with known concentrations of mTHPC and SC102. The standard curve was determined from a solution of PS which had been prepared under the same circumstances as the samples, i.e. dissolved in Solvable™ in the waterbath at 55 °C for 2 h. Fluorescence intensity of the standard curve was linear as a function of the concentration. Optimal measurement conditions were obtained by detecting fluorescence at 652 nm after excitation with 405 nm (SC102) or 418 nm (mTHPC). Baseline fluorescence caused by autofluorescence and fluorescence of the cuvette was subtracted before the PS concentration was determined. Correction for the dilution factor and the weight of the sample was performed and the concentration was expressed in ng/mg (mTHPC) or µg/mg (SC102) wet tissue.

Mathematical fluorescence model
The fluorescence techniques used were investigated with a mathematical model used by Sinaasappel and Sterenborg (1993). Using this model, fluorescence and the fluorescence ratio could be analytically expressed as a function of tissue parameters.

The model assumes a semi-infinite medium illuminated by an infinitely broad beam of excitation light at wavelength λ_{exc}. The excitation light at a certain location r in the medium is described with the fluence rate \( \Phi(r) [W/m^2] \). Fluorescence at emission wavelength \( \lambda_{em} \) generated at tissue depth z and leaving the tissue surface at location x is described by the escape function \( \xi(r,x) \). The \( \Phi \) and \( \xi \) depend on absorption and scattering of tissue at the wavelengths \( \lambda_{exc} \) and \( \lambda_{em} \), respectively.
A fluorescence yield \( Y_{p \lambda_{\text{exc}}, \lambda_{\text{em}}} [1/\text{kg}] \) and \( Y_{a \lambda_{\text{exc}}, \lambda_{\text{em}}} [1/\text{kg}] \) of the PS and autofluorophores, respectively, is defined, which is the product of fluorophore absorption, the ratio of the excitation and fluorescence photon energies and the fluorescence quantum yield. The concentration of fluorophores is denoted \( C_p \) [kg/kg tissue] and \( C_a \) [kg/kg tissue] of the PS and autofluorophores, respectively.

**Fluorescence (F)**

If we assume that \( C_a \) as well as \( C_p \) are homogeneously distributed in the tissue, fluorescence \( F \) [W/m\(^2\)], detected at \( \lambda_{\text{em1}} \) after excitation with \( \lambda_{\text{exc}} \), measured at \( z=0 \) is, described as (Sinaasappel and Sterenborg 1993)

\[
F_{\lambda_{\text{exc}}, \lambda_{\text{em1}}} (x) = \left( C_a Y_{a \lambda_{\text{exc}}, \lambda_{\text{em}}} + C_p Y_{p \lambda_{\text{exc}}, \lambda_{\text{em}}} \right) \int \Phi(r) \xi(r,x) \, dr
\]

In the measurements, \( \lambda_{\text{em1}} \) was the wavelength band 640-740 nm for mTHPC and 640-760 nm for SC102 and \( \lambda_{\text{exc}} \) was 405 nm.

Tumour and normal tissue are assumed to have certain concentrations of autofluorophores \( C_{aT} \) and \( C_{aN} \), respectively, as well as PS concentration \( C_{pT} \) and \( C_{pN} \). A tumour to normal ratio, T/N-ratio, can be expressed as

\[
\frac{T}{N} = \frac{F_{T \lambda_{\text{exc}}, \lambda_{\text{em1}}} (x)}{F_{N \lambda_{\text{exc}}, \lambda_{\text{em1}}} (x)} = \left( \frac{C_{aT} Y_{a \lambda_{\text{exc}}, \lambda_{\text{em1}}} + C_{pT} Y_{p \lambda_{\text{exc}}, \lambda_{\text{em1}}} }{C_{aN} Y_{a \lambda_{\text{exc}}, \lambda_{\text{em1}}} + C_{pN} Y_{p \lambda_{\text{exc}}, \lambda_{\text{em1}}} } \right) \int \Phi_T (r) \xi_T (r,x) \, dr
\]

**Normalised fluorescence ratio (NFR)**

The fluorescence ratio \( FR \) [-] between fluorescence at wavelength \( \lambda_{\text{em1}} \) and \( \lambda_{\text{em2}} \) is expressed as

\[
FR_{\lambda_{\text{exc}}, \lambda_{\text{em1}}, \lambda_{\text{em2}}} (x) = \frac{\left( C_a Y_{a \lambda_{\text{exc}}, \lambda_{\text{em1}}} + C_p Y_{p \lambda_{\text{exc}}, \lambda_{\text{em1}}} \right) \int \Phi(r) \xi_{\lambda_{\text{em1}}} (r,x) \, dr}{C_a Y_{a \lambda_{\text{exc}}, \lambda_{\text{em2}}} \int \Phi(r) \xi_{\lambda_{\text{em2}}} (r,x) \, dr}
\]

where \( \lambda_{\text{em2}} \) is chosen at a wavelength band where no PS fluorescence is generated.

In the measurements the autofluorescence wavelength band, \( \lambda_{\text{em2}} \), was 510±9 nm.

By dividing the FR with the \( FR_{C_p=0} \), the FR of autofluorescence with no PS present, a normalised fluorescence ratio (NFR) is obtained which is independent of the fluence rate distribution and escape function, assuming these parameters do not change after PS administration:

\[
\text{NFR} = \frac{\text{FR}}{\text{FR}_{C_p=0}} = \frac{C_a Y_{a \lambda_{\text{exc}}, \lambda_{\text{em1}}} + C_p Y_{p \lambda_{\text{exc}}, \lambda_{\text{em1}}} }{C_a Y_{a \lambda_{\text{exc}}, \lambda_{\text{em1}}}} = 1 + C_p \alpha_{\lambda_{\text{exc}}, \lambda_{\text{em1}}}
\]

According to Eq. 4 the NFR is a simple linear function of the PS concentration \( C_p \) with proportionality constant
\[ \alpha_{\lambda \text{exc}, \lambda \text{em}1} = \frac{Y_{\lambda \text{exc}, \lambda \text{em}1}}{C_a Y_{\lambda \text{exc}, \lambda \text{em}1}} \] (5)

Tumour to normal concentration ratios (T/N-ratios) can be determined from Eq. 4 if the \( \alpha \) for tumour, \( \alpha_T \), and normal tissue, \( \alpha_N \), are known:

\[ \frac{T}{N} = \frac{C_{pT}}{C_{pN}} = \frac{\alpha_N}{\alpha_T} \frac{(\text{NFR}_T - 1)}{(\text{NFR}_N - 1)} \] (6)

**Data analysis**

The wavelength bands chosen to determine fluorescence (F) were 640-740 nm for mTHPC and 640-760 nm for SC102. For calculation of the NFR the integrated fluorescence from these ranges were divided by an autofluorescence wavelength range which in all cases was 510±9 nm. The NFR was calculated by dividing the measured FR with the FR of the control animals which received no PS (time \( t=0 \) h in the figures).

In order to compare the pharmacokinetics behaviour of the PS found by the three methods (extraction, red fluorescence F, and the normalised fluorescence ratio NFR) the concentration of the extracted photosensitiser, as well as the F and the NFR in liver and tumour were plotted against time after administration. Pharmacokinetics expressions of the form

\[ A(t) = A_0 (1 - \exp(-t \cdot r_1)) \] (7)

\[ A(t) = A_0 (1 - \exp(-t \cdot r_1)) \exp(-t \cdot r_2) \] or (8)

\[ A(t) = A_0 \exp(-t \cdot r_1) + A_0 (1 - \exp(-t \cdot r_1)) \exp(-t \cdot r_2) \] (9)

were used to fit the experimental data, using the mean and standard error of the mean (s.e.m.) of each animal. \( A(t) \) represents the PS concentration as a function of time and \( r_1 \) and \( r_2 \) rate constants for pharmacokinetic phases. These equations could be fitted on the F and NFR since they were assumed to be proportional to the PS concentration. The fit used a least-squares algorithm and a \( \chi^2 \) goodness of fit test was performed.

The mathematical fluorescence model was compared with the measurements by plotting the F and NFR against the extracted PS concentration. The linear model (Eq. 4) was fitted on tumour and liver using the NFR data. A least-squares algorithm was used and a \( \chi^2 \) goodness of fit test was performed. T/N-ratios were calculated of the tumours and adjacent liver sites using the PS concentration, F and the NFR. The mean and s.e.m. were determined of the T/N-ratio at each time of measurement after administration. In addition, the T/N-ratio found by Eq. 6 was calculated, using the \( \alpha_T \) and \( \alpha_N \) found by the fits with Eq. 4.
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Results

In Fig. 2 spectra of mTHPC and SC102 measured in tumour and liver at 48 h after administration are shown. The spectra have been scaled to fit in one graph. The photosensitisers show two emission bands, mTHPC at 653 and around 726 nm and SC102 at 663 and around 726 nm.

Figure 2. Fluorescence spectra of mTHPC and SC102 measured in tumour and liver at 48 h after administration. The spectra have been scaled to fit in one graph.

Figure 3 shows in vivo autofluorescence spectra in liver and in tumour in a control rat that received no PS. Dips in the spectra at 542 and 577 nm are most likely due to blood absorption. Influences of strong blood absorption was avoided by choosing the autofluorescence wavelength range around 510 nm. Fluorescence at 633 and around 700 nm is probably due to porphyrins produced in the tissue. Fluorescence intensity of this unknown component was less than that of mTHPC and SC102. The decrease at 500 nm is due to the cut off filter.

Figure 3. Fluorescence spectra in vivo of liver and tumour in a control rat that received no photosensitiser. Fluorescence at 633 and around 700 nm is probably due to porphyrins produced in tissue.

Photosensitiser concentration kinetics

In Fig. 4a, extracted mTHPC concentrations in liver and tumour are shown as a function of time following administration. Each point represents the mean and s.e.m. of all samples in five rats (n=15). The pharmacokinetic fits (Eq. 7, 8 or 9) are shown. Highest mTHPC concentration in liver was found at 4 h, decreasing back to zero at 72 h. In the tumour an increase was seen with a
maximum at 24 and 48 h. The concentration mTHPC in liver was initially higher than in the
tumour. At 48 h the two tissues had approximately the same amount of mTHPC and at 72 h the
tumour tissue contained slightly more photosensitiser. Extracted SC102 (Fig. 4b) in tumour and
liver initially followed the same increasing curve with a slightly higher concentration in tumour than
liver until 72 h after administration. At 120 h, the SC102 concentration in liver was still increasing,
while SC102 in tumour was decreasing.

Figure 4a

![mTHPC concentrations](image)

![SC102 concentrations](image)

Figure 4. mTHPC (a) and SC102 (b) concentration in liver and tumour shown as mean and s.e.m. (n=15) per animal as a function of time following administration. The line shows values found by pharmacokinetics fit.

Table 1 shows the rate constants $r_1$ and $r_2$ for mTHPC and SC102 together with the p-value found from the $\chi^2$ goodness of fit test. A p-value > 0.05 was considered as no significant difference between measured and expected values.

Table 1. Rate constants $r_1$ and $r_2$ found by the fits with pharmacokinetics models. The standard error is given within brackets. The p-value is found by a $\chi^2$ goodness of fit test.

<table>
<thead>
<tr>
<th></th>
<th>Conc.</th>
<th>$r_1$ [h$^{-1}$]</th>
<th>$r_2$ [h$^{-1}$]</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTHPC</td>
<td>Liver</td>
<td>0.5 (0.4)</td>
<td>0.036 (0.008)</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 (0.4)</td>
<td>0.034 (0.003)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NFR**</td>
<td>0.2 (6.7)</td>
<td>0.05 (0.04)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tumour</td>
<td>Conc.</td>
<td>0.12 (0.06)</td>
<td>0.019 (0.007)</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td></td>
<td>$F^*$</td>
<td>0.2 (0.1)</td>
<td>0.004 (0.004)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NFR*</td>
<td>0.4 (0.1)</td>
<td>0.006 (0.002)</td>
<td>0.025</td>
</tr>
<tr>
<td>SC102</td>
<td>Liver</td>
<td>0.07 (0.02)</td>
<td>-</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td></td>
<td>$F^*$</td>
<td>0.4 (0.1)</td>
<td>0.007 (0.002)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NFR**</td>
<td>0.1 (0.5)</td>
<td>0.011 (0.004)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tumour</td>
<td>Conc.</td>
<td>0.3 (0.1)</td>
<td>0.005 (0.002)</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td></td>
<td>$F^*$</td>
<td>0.3 (0.1)</td>
<td>0.009 (0.003)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NFR*</td>
<td>0.6 (0.4)</td>
<td>0.007 (0.002)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*$A_0(1-exp(-r_1 t))exp(-r_2 t)$

$**A_0exp(-r_1 t)+A_0(1-exp(-r_1 t))exp(-r_2 t)$

*** $A_0(1-exp(-r_1 t))$
Photosensitiser fluorescence kinetics

Fluorescence (F), presented as number of counts on the CCD-camera, of mTHPC and SC102 in liver and tumour are shown in Fig. 5a and b, respectively. Each point represents the mean and s.e.m. of measurements in five rats (n=15). Fluorescence in the tumour was at all times higher than in the liver.

Figure 5a

Figure 5. Fluorescence F of mTHPC (a) and SC102 (b) in liver and tumour shown as mean and s.e.m. (n=15) per animal as a function of time following administration. The line shows values found by pharmacokinetics fit.

The NFR of mTHPC in the liver (Fig. 6a) was found highest at 4 h and decreased thereafter. In the tumour the NFR increased at 4 h and remained virtually constant up to 72 h. Except at 4 h, the NFR in the tumour was higher than in the liver. The NFR of SC102 (Fig. 6b) in the liver is initially higher than in tumour, showing a large spread between the animals. From 48 h the two tissues show no difference between the NFR values.

Figure 6a

Figure 6. NFR of mTHPC (a) and SC102 (b) in liver and tumour shown as mean and s.e.m. (n=15) per animal as a function of time following administration. The lines shows values found by pharmacokinetics fit.

Looking at the general shape of the curves, the kinetics behaviour in mTHPC comparing the three methods are similar. For SC102, the kinetics in the liver is quite different comparing the three methods. The p-values in Table 1 indicate that Eq. 7, 8 or 9 fitted better on the extracted PS concentration behaviour than the F and NFR (p>0.05). The mTHPC concentration shows a fast
build-up phase and a slower decreasing phase. In liver the initial build-up goes faster
\( (t_{1/2} = \ln 2/0.5 = 1.4 \text{h}) \) than in tumour \( (t_{1/2} = 5.8 \text{h}) \). The F and NFR in liver and in tumour show a longer
half-life on this phase, \( (t_{1/2} = 1.7-3.5 \text{h}) \). Summarising the three methods, the second phase shows that
mTHPC has a slower clearance from tumour \( (t_{1/2} = 36-173 \text{h}) \) than liver \( (t_{1/2} = 13-29 \text{h}) \).
SC102 concentration in liver has a slow build-up phase \( (t_{1/2} = 9.9 \text{h}) \). Our experiment did not include
enough measurements at later times to determine the clearance rate of SC102 in liver using
extraction. The F and NFR gave half-lives 1.7 and 6.9h, respectively, and a slow clearance phase
with \( t_{1/2} = 99 \) and 63h, respectively. In tumour the build-up phase in SC102 concentration and F
showed good agreement \( (t_{1/2} = 2.3 \text{h}) \) while NFR showed a shorter half-life of 1.2h. This was followed
by a slow decrease \( (t_{1/2} = 77-139 \text{h}) \).

**Correlation between fluorescence and extracted PS**

Figure 7 shows the F and NFR of mTHPC (Fig. 7a,b) and SC102 (Fig. 7c,d) plotted against the PS
concentration found by extraction. Each datapoint represents one liver or tumour sample. The
experimental error of the fluorescence measurements was determined by calculating the standard
deviation of the three measurements performed on each sample. For the F, one standard deviation
was on average about 20-30% of the mean of these three measurements. The NFR had a standard
deviation that was about 17% of the mean. The error in the extracted PS data was estimated in
previously performed measurements (unpublished data) where mTHPC extraction was performed
on seven liver samples divided into five pieces each. The average standard deviation of the five
samples was 24% of the mean mTHPC concentration found in the livers.

**Figure 7a**

![mTHPC concentration vs. fluorescence](image1)

**Figure 7a and b.** F (a) and NFR (b) of mTHPC plotted against mTHPC concentration. The linear model is shown for
NFR of mTHPC (b) and p-values found by a \( \chi^2 \) goodness of fit test.

Fluorescence F of mTHPC in the liver (Fig. 7a) shows the expected linear relation as a function of
PS concentration. However, for tumour the data scatter strongly. Influence of high absorption in the
liver is seen in Fig. 7a where the points of tumour and liver are clearly separated and the liver points
show lower fluorescence than tumour even if the measured PS concentration was higher. The
negative PS concentration found in some samples is due to that an offset determined from control
animals was subtracted. This offset was the signal measured in the fluorescence spectrophotometer
of liver or tumour only, measured in control rats. A mean of tumour and liver offset signal was
calculated from samples of the control rats. Due to the experimental error, a sample containing very
little PS could be assessed as a negative PS concentration after subtraction of the offset.
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The NFR of mTHPC (Fig. 7b) pushes the tumour and liver points towards each other due to the correction of optical properties in the tissue but a difference is still seen. In liver as well as tumour the data show strong scatter and the expected linear relation (Eq. 4) is vaguely present.

**Figure 7c**

![Figure 7c and d. F (c) and NFR (d) of SC102 plotted against SC102 concentration.](image)

The F as a function of SC102 concentration (Fig. 7c) shows large scatter in tumour and higher values than in liver. In the NFR the difference between tumour and liver is less (Fig. 7d), however the liver data show large scatter.

Using the data in Fig. 7b, $\alpha_T$ and $\alpha_N$ (Eq. 6) were determined by fitting the data with the mathematical model (Eq. 4). The fits are shown in Fig. 7b with p-values found by a $\chi^2$ goodness of fit test. At time $t=0$ the straight line was forced to intercept the y-axis at NFR=1, according to the model. For mTHPC the $\alpha_T$ and $\alpha_N$ found by the fit were 25 and 11 (ng/mg)$^{-1}$, respectively. The kinetics measurements of SC102 in liver had shown that the NFR decreased with increasing concentration of SC102. The linear relation predicted by the mathematical model was not valid experimentally and no fit was performed (Fig. 7d).

**T/N-ratios**

T/N-ratios of the extracted PS, F, NFR and the $C_{pt}/C_{pn}$, calculated using Eq. 6 and the $\alpha_T$ and $\alpha_N$ reported above, are shown in Table 2 as the mean and s.e.m. of the ratio between all tumours and adjacent liver sites. The extracted mTHPC suggests tumour-selectivity only at 72 h after administration with a T/N-ratio of 10±5. In comparison to PS concentration, the F shows highly overestimated T/N-ratios. The NFR shows a T/N-ratio about 2-3 times higher than extracted mTHPC, except at 72 h. The T/N-ratio corrected for the $\alpha_N$ and $\alpha_T$, $C_{pt}/C_{pn}$, shows the best agreement with the extracted mTHPC, however it is 1.5-2 times higher at 24 and 48 h and about 3 times lower at 72 h.

The extracted SC102 shows an initial T/N-ratio of 2 at 4 h, thereafter decreasing to 0.46 at 120 h. Also in this case the F gave higher T/N-ratios, at all times higher than 3. The T/N-ratio of the NFR showed a reasonable agreement with extracted SC102, and reached a maximum of 1.6 at 48 h.
Table 2. T/N-ratio of mTHPC and SC102 calculated from measurements of extracted PS concentration as well as F, NFR and $C_{\text{PT}}/C_{\text{PN}}$ (Eq. 6). The mean and s.e.m. (in brackets) of all tumours and adjacent liver sites are given.

<table>
<thead>
<tr>
<th>T/N-ratio</th>
<th>time (h)</th>
<th>Extracted</th>
<th>F</th>
<th>NFR</th>
<th>$C_{\text{PT}}/C_{\text{PN}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTHPC</td>
<td>4</td>
<td>0.25 (0.04)</td>
<td>1.9 (0.1)</td>
<td>0.56 (0.03)</td>
<td>0.26 (0.01)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.61 (0.03)</td>
<td>6.4 (0.7)</td>
<td>1.9 (0.1)</td>
<td>0.91 (0.04)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.1 (0.1)</td>
<td>14 (2)</td>
<td>3.8 (0.3)</td>
<td>2.0 (0.2)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>10 (5)</td>
<td>19 (2)</td>
<td>5.9 (0.4)</td>
<td>3.5 (0.3)</td>
</tr>
<tr>
<td>SC102</td>
<td>4</td>
<td>2.0 (0.2)</td>
<td>4.4 (0.7)</td>
<td>0.8 (0.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.4 (0.1)</td>
<td>4.2 (0.5)</td>
<td>1.0 (0.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.1 (0.1)</td>
<td>4.7 (0.9)</td>
<td>1.6 (0.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1.1 (0.2)</td>
<td>4.4 (0.9)</td>
<td>1.0 (0.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.46 (0.02)</td>
<td>3.2 (0.3)</td>
<td>0.73 (0.07)</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

In other studies (Whelpton et al. 1995, Whelpton et al. 1996), three phases were found in mTHPC kinetics in the liver of mice, with half-lives of about 2.5 h, 13.3 h and 20 days. It was hypothesised that in the short-term phase mTHPC was taken up by tissue from plasma, metabolism occurred in the liver in the middle phase and clearance of mTHPC from the body in the third phase. In our study, one or two phases could be fitted but the pharmacokinetics was not followed long enough to determine the rate constant of the third phase. The half-lives of mTHPC concentration in liver were 1.3 h for the first phase and 19 h for the second which agree well with the results of Whelpton et al. (1995).

Pharmacokinetics of SC102 showed a different behaviour compared to mTHPC with a slower build-up in liver and a faster one in tumour. In liver, SC102 measured by extraction did not agree with the fluorescence measurements. SC102 kinetics measured in plasma follows a behaviour similar to our fluorescence measurements in liver (Rovers, personal communication). It is possible that the liver measurement is dominated by the plasma SC102 content, and that extracted tissue does not contain as much plasma due to the excision of tissue. This could explain why some measurements in the liver had to be fitted by Eq. 9, where an extra term $A_0 \exp(-r_1 t)$ was added to the standard pharmacokinetics expression $A_0 (1-\exp(-r_2 t)) \exp(-r_3 t)$. The extra term could be interpreted as the PS pharmacokinetics in blood which was present in the liver.

The general pharmacokinetics behaviour between F, NFR and mTHPC concentration agreed well. The T/N-ratio determined from NFR was in good agreement with the mTHPC and SC102 concentration since the influence of optical properties was cancelled. The measurements of F show that using PS fluorescence at one wavelength underestimates the PS content in liver compared to tumour due to the much stronger absorption. This results in a highly overestimated T/N-ratio. The T/N-ratio corrected for $\alpha_T$ and $\alpha_N$, $C_{\text{PT}}/C_{\text{PN}}$ (Eq. 6), had the best agreement with the T/N-ratio found with mTHPC concentrations. This indicates that despite the large scatter of the data, and the fact that the least-squares fit neglects the possible error in the extracted PS concentration, the $\alpha_T$ and $\alpha_N$ found in Fig. 7b might be reasonable values showing the difference between tumour and liver.
The differences in $\alpha$ seen in tumour and liver were not due to differences in optical properties. The parameter $\alpha$ depends on the fluorescence yield of the PS, $Y_p$, and the autofluorescence parameters $Y_a$ and $C_a$ (Eq. 5). The fluorescence yield $Y_p$ of porphyrins has been shown to be higher in monomers than in aggregates (Vernon et al. 1995). This might also be the case for mTHPC which in vivo is found in aggregated as well as in monomer form (Ma et al. 1994). Temporal as well as local differences in PS fluorescence yield might be an effect of structural changes and binding to the surrounding environment.

The spectral shape of autofluorescence of the liver and tumour showed differences between 510-580 nm where the fluorescence in liver was relatively lower than in tumour due to blood absorption. Fluorescence at 620-650 and 670-720 nm was relatively higher in tumour due to porphyrin production. This could cause differences in the measured $\alpha_T$ and $\alpha_N$. However, Fig. 7b indicates that the autofluorescence intensity in liver is higher than in tumour since $\alpha_T > \alpha_N$ (see Eq. 5).

The spread in the data of the fluorescence measurements as well as the extracted mTHPC and SC102 concentration makes it difficult to state whether the mathematical model is correct or not, as is seen in Fig. 7b. Possible causes of the spread of the data might be inhomogeneity in the tissue structure and distribution of mTHPC and SC102. One experimental problem is necrosis formation which occurs in these fast-growing tumours. The tumours have been seen to consist of 50-60% necrosis. Fluorescence originates from the surface layer and the excitation light probably does not reach the necrotic part in the centre. Extraction on the other hand is performed on the whole tumour, including the necrotic inner part where no PS will be present. Since concentration obtained by extraction assumes a homogeneous distribution in the whole tumour, the PS concentration determined by extraction will be underestimated.

From the results of our measurements it is clear that it is difficult to compare fluorescence in vivo with PS concentration determined in vitro. The inaccuracy of PS extraction and the difficulty to know the exact PS structure during fluorescence measurement are problems which should be solved for further investigations.

**Conclusions**

The two fluorescence techniques showed approximately the same mTHPC kinetics behaviour as the mTHPC concentration. Liver and tumour showed different NFR values independent of differences in optical properties. With the NFR tumour to normal ratios could be determined which agreed well with ratios found with photosensitiser concentrations.

**References**


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The difference in Fmax to liver and other organs not due to differences in optical properties. This parameter is influenced by the spectral overlap of the excitation and emission profile within the sample. The difference can be explained by the fluorescence in the liver being lower than in other organs due to blood and temperature effects. The differences may be due to differences in the excitation and emission profiles, which in turn may be due to differences in the tissue structure and biochemical composition. The spread in the data of the fluorescence measurements is consistent with the expected noise level.

Conclusions

The two fluorescence techniques are essentially the same in terms of THPC concentration and the results show that the liver concentration is lower than in other organs not due to differences in optical properties. The differences in Fmax between the liver and other organs may be due to differences in tissue structure and biochemical composition.

References

