Evaluation of fluorescence measurement techniques for tumour detection in vivo
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CHAPTER 4

Fluorescence measurements of mTHPC in basal cell carcinoma and two malignant melanomas using the Double Ratio and the Normalised Fluorescence Ratio techniques

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Abstract

Two fluorescence detection techniques, the Double Ratio and the Normalised Fluorescence Ratio, were used for measurements of pharmacokinetics and tumour to normal ratios of mTHPC in basal cell carcinoma in four patients. Independent on differences in colour, these two techniques measured difference between BCC and normal skin as well as between two metastatic malignant melanomas and normal skin. The NFR was more suitable than the DR for pharmacokinetics measurements due to high mTHPC concentrations.

Conclusions

In this study the DR increased in the four patients who received ALA but remained at baseline in the controls. However, the NFR showed a difference with a patient in photon image analysis.

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References

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Introduction

Most photosensitisers (PS) used in photodynamic therapy (PDT) of malignant or non-malignant lesions show fluorescence after excitation with blue or green light. Fluorescence measurements are used as a non-invasive technique to determine pharmacokinetics of the PS in vivo. Because of the shallow penetration depth of the excitation light, fluorescence measurements are suited for superficial lesions, located on skin or in a hollow organ which can be reached with an endoscope (Sterenborg et al. 1996, Baumgartner et al. 1987). Factors that influence the fluorescence measured on the tissue surface are the depth at which the tumour is located and the preferential uptake of PS in the tumour (Saarnak et al. 1998, Chapter 6).

Fluorescence measurements on red or dark lesions, such as basal cell carcinoma (BCC) or malignant melanoma, need to be corrected for the high absorption in the lesions. Sterenborg et al. (1996, Chapter 2) used the Double Ratio fluorescence detection technique for detection of ALA-induced protoporphyrin IX fluorescence on normal moles to correct for the high absorption in the moles. This technique requires low PS concentrations for optimal sensitivity, which is not always the case when measuring fluorescence in connection with photodynamic therapy. Another technique is the normalised fluorescence ratio (NFR) which has been used to compare fluorescence in tissues with different optical properties (Chapter 8). The normalised fluorescence ratio (NFR) has a linear dependency with the PS concentration with a proportionality factor dependent on the type and concentration of autofluorophores and the PS fluorescence quantum yield. If the proportionality factors for tumour and normal tissue can be determined a tumour to normal PS concentration ratio can be calculated.

The fluorescence measurements in this study were performed in connection to a dose finding study of PDT of BCC using meta-tetra(hydroxyphenyl)chlorin (mTHPC) (Chapter 5). mTHPC is a PS suitable for fluorescence measurements since it emits fluorescence at 652 nm after excitation around 420 nm. Pre-clinical and clinical studies have shown that mTHPC is an efficient photosensitiser with relatively good tumour-selectivity (Bonnett et al. 1989, Braichotte et al. 1995).

The purpose of this study was to compare the suitability of the DR and NFR techniques to measure mTHPC pharmacokinetics in basal cell carcinoma, and tumour to normal concentration ratios in vivo.

Materials and methods

Measurement procedure

Meta-tetra(hydroxyphenyl)chlorin (mTHPC) was intravenously injected at a dose of 0.05 (patient 1), 0.075 (patients 1 and 2) and 0.1 (patients 3 and 4) mg/kg bodyweight. Patient 1 received mTHPC twice, with two months between the different doses. The patients were kept under subdued light for one week after injection. At approximately 4 h and thereafter every day up to 4 days after injection, fluorescence on 3-5 BCC and adjacent normal skin spots was measured. Patient 1 was measured up to 10 days after administration of 0.05 mg/kg mTHPC. On each site five measurements were made with the fibre slightly repositioned between the measurements. Fluorescence kinetic behaviour in BCC and normal skin was determined by calculating the normalised fluorescence ratio (NFR) (see Chapter 9). Fluorescence at 660-750 nm was divided by
autofluorescence at 550-600 nm. This ratio was normalised by the ratio of the autofluorescence measured at time \( t=0 \) h, before administration of mTHPC.

One patient with metastatic malignant melanoma received 0.1 mg/kg mTHPC and fluorescence was measured at 16, 40 and 64 h after administration. Two lesions were measured as well as the normal surrounding tissue. The lesions were heavily pigmented and rose several millimetres above the skin. To avoid total absorption of excitation and fluorescence light in the dark lesions, the measurement fibres were kept at a distance of approximately 1 mm above the lesions.

**Fluorescence set-up**

The fluorescence measurement set-up was similar to a set-up previously described by Sterenborg et al. (1996) (Chapter 2). A Mercury-lamp was used as light source and 405 nm and 435 nm were chosen for excitation with interference filters (Oriel 56541 and Oriel, respectively). Excitation light was delivered to the tissue through a 200 \( \mu \)m fibre and fluorescence was collected with two 600 \( \mu \)m fibres. The fibres were in contact with the tissue. Two detection paths measured the fluorescence, one with a Schott KV 500 and a Schott RG 645 filter and a Hamamatsu R 636-10 red-sensitive photomultiplier tube (PMT), the other with a Schott KV 550 filter and a green-sensitive Hamamatsu IP 128 PMT. Lock-in amplifiers were used and data were collected on a PC by a Pascal-program that monitored the measurements. The ratio between the fluorescence collected at wavelengths longer than 660 nm and fluorescence between 550 and 600 nm was calculated by the program.

**The Normalised Fluorescence Ratio (NFR)**

Fluorescence measurements have been described with a mathematical model (Sinaasappel and Sterenborg 1993). Excitation wavelength is \( \lambda_{exc} \) (405 nm) fluorescence \( F \) is measured at wavelength \( \lambda_{em1} (>645 \text{ nm}) \) and \( \lambda_{em2} (550-600 \text{ nm}) \). The normalised fluorescence ratio can be written as:

\[
NFR_{\lambda_{exc,\lambda_{em1,\lambda_{em2}}} = \frac{F_{\lambda_{exc,\lambda_{em1}}} \cdot F_{(Cp=0)\lambda_{exc,\lambda_{em2}}}}{F_{\lambda_{exc,\lambda_{em2}}} \cdot F_{(Cp=0)\lambda_{exc,\lambda_{em1}}}}}
\]

The concentration PS in the tissue is denoted \( C_p \) and \( F_{Cp=0} \) refers to a measurement where no PS is present. In the present study this was the measurement at time \( t=0 \) h, before PS was administered. The NFR has been shown to be equal to (see Chapter 9)

\[
NFR_{\lambda_{exc,\lambda_{em1,\lambda_{em2}}} = 1 + \alpha_{\lambda_{exc,\lambda_{em1}}} C_p}
\]

where \( \alpha \) is a proportionality constant depending on the macroscopic fluorescence yield \( Y_{p\lambda_{exc,\lambda_{em1}}} \) and \( Y_{a\lambda_{exc,\lambda_{em1}}} \) of the PS and the autofluorophores, respectively, and the concentration of autofluorophores \( C_a \):

\[
\alpha_{\lambda_{exc,\lambda_{em1}}} = \frac{Y_{p\lambda_{exc,\lambda_{em1}}}}{C_a \cdot Y_{a\lambda_{exc,\lambda_{em1}}}}
\]
The NFR has a simple linear relation to the PS concentration. If the proportionality constant \( \alpha \) is assumed to be equal in tumour and normal tissue, \( \alpha(\text{tumour}) = \alpha(\text{normal}) \), a tumour to normal concentration ratio (T/N-ratio) can be calculated from the measured NFR in the following way:

\[
\frac{\text{NFR(tumour)} - 1}{\text{NFR(normal)} - 1} = \frac{\alpha(\text{tumour}) \cdot C_p(\text{tumour})}{\alpha(\text{normal}) \cdot C_p(\text{normal})} = \frac{C_p(\text{tumour})}{C_p(\text{normal})}
\]  

(4)

### The Double Ratio (DR)

The DR is calculated by measuring the fluorescence \( F \) at \( \lambda_{\text{em}1} \) and \( \lambda_{\text{em}2} \) using two different excitation wavelengths \( \lambda_{\text{exc}1} \) (405 nm) and \( \lambda_{\text{exc}2} \) (435 nm). The fluorescence ratio between the PS fluorescence and autofluorescence is determined for each excitation wavelength, and thereafter a ratio between these two ratios is calculated.

\[
\text{DR}_{\lambda_{\text{exc}1}, \lambda_{\text{exc}2}, \lambda_{\text{em}1}, \lambda_{\text{em}2}} = \frac{F_{\lambda_{\text{exc}1}, \lambda_{\text{em}1}}}{F_{\lambda_{\text{exc}2}, \lambda_{\text{em}1}}} \cdot \frac{F_{\lambda_{\text{exc}2}, \lambda_{\text{em}2}}}{F_{\lambda_{\text{exc}1}, \lambda_{\text{em}2}}}
\]  

(5)

The DR has the following dependency on the \( C_p \) (Sinaasappel et al. 1993):

\[
\text{DR}_{\lambda_{\text{exc}1}, \lambda_{\text{exc}2}, \lambda_{\text{em}1}, \lambda_{\text{em}2}} = \frac{1 + \alpha_{\lambda_{\text{exc}1}, \lambda_{\text{em}1}} C_p}{1 + \alpha_{\lambda_{\text{exc}2}, \lambda_{\text{em}1}} C_p}
\]  

(6)

In Eq. 6 the DR has been normalised by the DR of the autofluorescence before PS was administered.

At high concentrations of PS, \( C_p \gg 1 \), the DR approaches the limit \( \alpha_{\lambda_{\text{exc}1}, \lambda_{\text{em}1}} / \alpha_{\lambda_{\text{exc}2}, \lambda_{\text{em}1}} \).

### Results

**Fluorescence and mTHPC kinetics**

Fluorescence kinetics in the patients is shown as the DR and NFR as a function of time after mTHPC administration. In Fig. 1a-c the mean and standard error of the mean (s.e.m.) of the DR of BCC and normal skin for 0.05 (a), 0.075 (b) and 0.1 (c) mg/kg mTHPC are shown. In the BCC a maximum DR around 1.7 is measured, though there is variation between the patients. Normal skin reaches a DR of 1.2-1.4. In patient 2 (Fig. 1b) the DR in the BCC is at some points at the same level as normal skin.
Figure 1a shows the results for the NFR. In order to find parameters to compare the mTHPC kinetics in different patients the data were fitted with one or two exponentials using the standard pharmacokinetics expressions

\[ c = 1 + c_0 (1 - \exp(-r_1 t)) \]  

(7)  

or

\[ c = 1 + c_0 (1 - \exp(-r_1 t)) \exp(-r_2 t) \]  

(8)

where \( c \) denotes the mTHPC concentration and \( r_1 \) and \( r_2 \) are rate constants for different pharmacokinetic phases. These expressions could be used since the NFR is proportional to the PS concentration (Eq. 2). The DR has a non-linear relation to the PS concentration (Eq. 6) and therefore we were not able to fit the DR data with these expressions (Eqs. 7-8). Since the NFR equals 1 when \( C_p = 0 \) (at \( t=0 \) h) the fit was forced to begin in \( c=1 \). The fit used a least-squares algorithm and a \( \chi^2 \) goodness of fit test was performed giving a p-value. A p-value >0.05 meant no significant

Figure 1. The DR as a function of time in patients receiving 0.05 (a), 0.075 (b) and 0.1 (c) mg/kg mTHPC. Each point is the mean and s.e.m. of measurements on BCC (n=15-25) or adjacent normal skin spots (n=5-20).
difference between the measured data and expected values found by the fit. All measurements performed on tumour (n=15-25) and normal skin (n=5-20) on each patient were used for the fit.

**Figure 2a**

![Graph](image)

**Figure 2b**

![Graph](image)

**Figure 2c**

![Graph](image)

Figure 2. The NFR as a function of time in patients receiving 0.05 (a), 0.075 (b) and 0.1 (c) mg/kg mTHPC. Each point is the mean and s.e.m. of measurements on BCC (n=15-25) or adjacent normal skin spots (n=5-20).

The results of the fits with Eqs. 7 and 8 are shown as solid and dashed lines in Fig. 2a-c and rate constants \( r_1 \) and \( r_2 \) are shown in Table 1 with the p-values. In Fig. 2a the data at 167 h and 1 data point at 95 h had to be excluded from the data of the normal skin to be able to make the fit.

**Table 1. Rate constants \( r_1 \) and \( r_2 \) found by fitting the NFR with Eqs. 7 or 8. The p-value was obtained from a \( \chi^2 \) goodness of fit test and p>0.05 denotes no significant difference between measured data and values found by the fit.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>mTHPC (mg/kg)</th>
<th>( r_1 ) (h(^{-1}))</th>
<th>( r_2 ) (h(^{-1}))</th>
<th>p-value</th>
<th>( r_1 ) (h(^{-1}))</th>
<th>( r_2 ) (h(^{-1}))</th>
<th>p-value</th>
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<tr>
<td>1(*)</td>
<td>0.05</td>
<td>0.039</td>
<td>0.0052</td>
<td>&lt;0.001</td>
<td>0.015</td>
<td>0.010</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>1(**)</td>
<td>0.075</td>
<td>0.145</td>
<td>0.0039</td>
<td>0.005</td>
<td>0.15</td>
<td>0.0023</td>
<td>&gt;0.975</td>
</tr>
<tr>
<td>2(*)</td>
<td>0.075</td>
<td>0.027</td>
<td></td>
<td>&gt;0.995</td>
<td>0.099</td>
<td></td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>3(*)</td>
<td>0.1</td>
<td>0.051</td>
<td></td>
<td>&lt;0.001</td>
<td>0.033</td>
<td></td>
<td>&gt;0.995</td>
</tr>
<tr>
<td>4(*)</td>
<td>0.1</td>
<td>0.060</td>
<td></td>
<td>&lt;0.005</td>
<td>0.017</td>
<td></td>
<td>&gt;0.995</td>
</tr>
</tbody>
</table>

\(*\) fitted with Eq. 7, \(**\) fitted with Eq. 8
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The NFR in BCC reaches a maximum of around 4, while the normal skin has a maximum value of 2. The kinetics behaviour between the patients varies, as is also seen in the rate constants. The measurements in patient 1 show maximum NFR before 100 h after administration. In patients 2-4 a maximum is not found within the measured time.

Figure 3 shows the red/green fluorescence ratio (Fig. 3a) and the DR (Fig. 3b) of two metastatic malignant melanomas and surrounding normal tissue at 16, 40 and 64 h after administration. Since the melanomas were black, the fluorescence signal of the melanomas before administration was too low to use these measurements for normalisation. After at least 16 h a difference is seen in the fluorescence ratio between malignant and healthy tissue. In the DR the difference is seen after 40 h.

Figure 3a

![Figure 3a](image)

Figure 3. A red/green fluorescence ratio (a) and DR (b) of mTHPC in two malignant melanomas and adjacent normal skin. Mean and std. (n=5) is shown.

The T/N-ratios calculated from the measurements on BCC using Eq. 4 are shown in Table 2. The first column shows the centre of the time range chosen, the second column the limits of the time range. The number of measurements, n, used for each T/N-ratio is shown in the last column.

Table 2. The T/N-ratio calculated using the kinetics data from patients 1-4. The time ranges after administration of mTHPC chosen are shown, as well as the number of measurement points (n) used for the calculations in each time range. The mean and std. of all patients measured within the given time range are shown.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>T/N ratio</th>
<th>mean</th>
<th>std.</th>
<th>n</th>
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<td>0.22</td>
<td>0.75</td>
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</tr>
<tr>
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<td>0.75</td>
<td>3</td>
</tr>
<tr>
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<td>20</td>
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<td>2.2</td>
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<td>5</td>
</tr>
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<td>0.9</td>
<td>5</td>
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<tr>
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<td>2.3</td>
<td>4</td>
</tr>
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<td>3.1</td>
<td>2.7</td>
<td>2</td>
</tr>
<tr>
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<td>4.0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>240</td>
<td>240</td>
<td>3.1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
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Discussion

Animal studies on different organs have shown a single- or biexponential pharmacokinetic behaviour of mTHPC in vivo (Whelpton et al. 1995, Whelpton et al. 1996), representing the uptake of mTHPC into tissue and subsequent metabolism and clearance from the body (Eqs. 7 and 8). Braichotte et al. (1995) measured mTHPC fluorescence in tumour and normal tissue in the oral cavity and normal skin of 4 patients and found maximum fluorescence at around 30-60 h, 50-90 and 100 h after administration in tumour, normal tissue inside the oral cavity and normal skin, respectively. In our study, measurements in most patients were not performed longer than 100 h. In one case (Fig. 2a) a NFR maximum in normal skin is seen before 100 h but in other patients no maximum was seen on this time scale. This was also the case for the BCC where in one patient a NFR maximum was found before 100 h (after 0.05 mg/kg mTHPC, Fig. 2a) and before 50 h (after 0.075 mg/kg mTHPC, Fig. 2b). In the other patients no maximum was found during the time measurements were performed.

Variations in fluorescence kinetics between different patients were observed. The half-lives ($t_{1/2}=\ln2/r_1$) found in our study for the uptake of mTHPC in BCC varied between 4.8 and 25.8 h and in skin between 7 and 46.2 h. There is no correlation between the mTHPC dose and the length of the half-lives. In patient 1 the rate constants after administration of two different doses of mTHPC could be compared. The data in Fig. 2a and b shows similar kinetics behaviour comparing the two different doses, though the rate constants found by the fits differed. In the case of 0.05 mg/kg mTHPC the first half-life was 17.8 h in BCC and 46.2 h in normal skin, while the half-life for the second phase ($t_{1/2}=\ln2/r_2$) was 133.3 and 69.3 h for BCC and normal skin respectively. After injection of 0.075 mg/kg the half-lives of the first phase were 4.8 and 4.6 h and the second phase half-lives were 178 and 301 h for BCC and normal skin, respectively. The p-values for the \(\chi^2\) goodness of fit test (Table 1) shows that the exponential expressions fitted normal skin better than BCC ($p>0.05$).

The NFR corrects the measured fluorescence for differences in optical properties in the different tissues. Using the NFR, the T/N-ratio between two differently coloured tissues, such as BCC and normal tissue, should be more reliable than using the red fluorescence only. The NFR depends on the tissue parameter \(\alpha\) (Eq. 4) which in this study was assumed to be equal for BCC and normal skin. This implies that the fluorescence yield \(Y_p\) of mTHPC is the same in BCC as in normal skin (Eq. 3). Aggregation of mTHPC probably influences the fluorescence yield (Ma et al. 1994, Vernon et al. 1995) but it was assumed here that the rate of aggregation would not differ between normal skin and BCC. The autofluorescence properties \(C_a\), \(Y_a\) are also assumed to be equal in BCC and normal skin (Eq. 3). Autofluorescence of skin tumours and normal skin have been studied, and no spectral differences could be found which would indicate different types of fluorophores between the two tissues (Sterenborg et al. 1994).

The Double Ratio can be used to compare fluorophore concentrations in tissue with different optical properties. In this study we believed that the mTHPC concentration in vivo exceeded the limit for the sensitive concentration range where the DR can be used. In most patients the DR showed a constant value after 20 h and onwards, while the NFR was increasing or decreasing. This study was performed in combination with PDT and therefore the mTHPC concentration was not kept lower than 0.05 mg/kg. For pharmacokinetics measurements the DR was not as suitable as the NFR.
Conclusions

The NFR and DR can be used for fluorescence detection of dark lesions such as basal cell carcinoma and metastatic malignant melanoma. The NFR is suitable for measurements of pharmacokinetics and tumour to normal ratios.

References


