Evaluation of fluorescence measurement techniques for tumour detection in vivo
Saarnak, A.E.

Citation for published version (APA):
CHAPTER 3

Individual variations in fluorescence of piglet skin in vivo after oral administration of ALA

A.E. Saarnak, H.J. van Staveren, J.F. Beek, M.J.C. van Gemert, H.J.C.M. Sterenborg
Chapter 3

Abstract
Four piglets received 60 mg/kg δ-aminolevulinic acid (ALA) orally. Protoporphyrin IX (PpIX) fluorescence in the skin was measured up to 30 hours after administration. One piglet was used as a control and did not receive any ALA. The set-up included optical fibres for excitation and detection. Two excitation wavelengths of 405 and 435 nm were used and fluorescence was detected with a 550 high pass filter and a green-sensitive photomultiplier, and with a 660 high pass filter and a red-sensitive photomultiplier. The double ratio (DR) of the four detected fluorescence intensities was calculated as a parameter to monitor variations in the PpIX concentration. The control piglet showed no increase in the DR. The DR increased in all four sensitised piglets and was still high 30 hours after ALA administration. A consistent fluorescence kinetics behaviour between the piglets was not found, indicating that the distribution in the body of the photosensitiser after oral administration is determined by properties which can differ between the animals.
Introduction

As a precursor to haem, δ-aminolevulinic acid (ALA) is naturally present in cells. Administration of additional ALA to tissue may cause increased levels of protoporphyrin IX (PpIX), an intermediate in the haem cycle (Kennedy et al. 1990). At present, much work is being performed investigating the possibility of photodynamic therapy (PDT) following oral administration of ALA, but only a few pharmacokinetics studies on patients have been reported (Grant et al. 1993, Loh et al. 1993, Mlkvy et al. 1995). In these studies patients received 30 or 60 mg/kg ALA orally. A fluorescence maximum in oral cavity squamous cell carcinoma was found 4-6 hours after ALA administration (Grant et al. 1993). In the gastrointestinal tract fluorescence maximum in colonic tumours at 4-6 hours after administration of 30 mg/kg ALA has been reported. After administration of 60 mg/kg ALA fluorescence intensity increased during the 7 hours of measurements (Loh et al. 1993). Another study reports that after 30 mg/kg ALA high PpIX levels were found in oesophagus and duodenum, and less in the colon (Mlkvy et al. 1995). The tumour selectivity increased in colon after 60 mg/kg ALA.

For fluorescence diagnostics or photodynamic therapy (PDT) with ALA it is important to know the distribution and kinetics of the ALA and PpIX in the body. The purpose of the present study was to measure fluorescence of PpIX in normal piglet skin in vivo after oral administration of ALA, and to determine whether the fluorescence kinetics is consistent among the different animals. In the measurements fluorescence was induced by different excitation wavelengths. We used the Double Ratio (DR), as it has been shown to depend on the photosensitiser concentration, while it corrects for spatial variations in optical properties of the tissue (Sinaasappel and Sterenborg 1993, Sterenborg et al. 1996 (Chapter 2)).

Materials and methods

Four piglets (i-iv), weighing 40-65 kg, received ALA orally. One piglet (v) did not receive any ALA and was used as a control. The piglets had not been fed for 24 hours. Prior to ALA administration, a stomach tube was inserted after an intramuscular injection of 15 mg/kg Ketamine in a 100 mg/ml solution. ALA (Finetech, Haifa, Israel) was dissolved in phosphate buffered saline (pH=3-3.5) and administered in a dose of 60 mg/kg through the stomach tube. After ALA administration the piglets were conscious for about 4 hours, whereafter the piglets were fully anaesthetised. A premedication of 0.1 ml/kg Stresnil (Azaperon 40 mg/ml) was given by intramuscular injection, followed by intubation and artificial respiration with 1 % Halothane, ~60 % N₂O and ~40 % O₂. The control piglet that did not receive any ALA was put under general anaesthesia during the whole experiment. Skin fluorescence was measured before ALA administration and every hour for 5-11 hours thereafter. In one piglet additional measurements were performed at 25 and 30 hours after administration. Three skin areas were chosen: behind the ear, on the side and on the buttock. These sites were shaved to avoid influence from hair fluorescence. On each site 4-6 measurements were made and the mean and standard deviation were determined.

The set-up for fluorescence measurements has been described in detail before (Sterenborg et al. 1996 (Chapter 2)). Excitation light of 405 and 435 nm was obtained from a Hg-lamp using interference filters. The excitation light was delivered to the tissue through a 300 μm quartz fibre
and the fluorescence light was transmitted through two 600 \( \mu \text{m} \) glass fibres. Two fluorescence band intensities were simultaneously measured. One band with a 660 nm high pass filter and a red-sensitive photomultiplier, the other with a 550 nm high pass filter and a green-sensitive photomultiplier. Standard lock-in techniques were used, and the detection system was connected with a computer which calculated the DR of the four detected fluorescence intensities. The DR was calculated in the following way (Sinaasappel and Sterenborg 1993):

\[
\frac{\left( \frac{F_{405,660}}{F_{405,550}} \right)}{\left( \frac{F_{435,660}}{F_{435,550}} \right)}
\]

where \( F_{405,660} \) represents the fluorescence intensity induced with 405 nm and detected at 660 nm. According to theory the DR is equal to the following expression:

\[
DR = \frac{1 + aC_p}{1 + bC_p}
\]

where \( C_p \) is the photosensitiser concentration. The constants \( a \) and \( b \) depend on the concentration of autofluorophores and the fluorescence yields of autofluorophores and photosensitiser in tissue. The wavelengths for detection were chosen so that \( a \gg b \).

**Results**

Figures 1 and 2 show the DR as a function of time after ALA administration. In both figures the measurement performed before ALA is plotted at \( t=0 \). Figure 1 shows the DR of the three skin sites (neck, side, buttock) on piglet \( i \). The error bars denote \( \pm \) one standard deviation of the mean (\( n=4-6 \)). The DR at the different sites show good agreement, as was the case for the other piglets (not shown here).

Figure 2 shows the mean of the DR measured on all three sites of the different piglets, each curve representing one piglet. In the left upper corner a typical error bar (\( \pm \) one standard deviation, \( n=15 \)) is shown. Fluorescence maximum of the DR in piglet \( i \) is about 2.3, in piglets \( ii-iv \) about 1.8. In
piglets i-iii a decrease in the DR is seen after 4 hours, followed by an increase up to 7 hours after administration. In piglet iii the DR after 25 and 30 hours is still about 1.8.

\[
\begin{align*}
\text{DR} &= \frac{1}{2} \\
\text{DR} &= \frac{1}{2}\left(\frac{a}{b}\right)
\end{align*}
\]

Figure 2. Double ratio (Eq. 2) versus time of piglets i-v after oral administration of 60 mg/kg ALA at t=0. Piglet v did not receive any ALA. Each series shows measurements on one piglet, and one point represents the mean of the measurements on the three sites. In the left upper corner a typical error bar (±1 standard deviation) is shown.

Discussion

In Eq. 2 the constants a and b depend on the macroscopic fluorescence yield of the different fluorophores. These constants have not yet been determined in vivo and might be different for each human or animal. However, in Eq. 2 is seen that for large \( C_p \) the DR will approach \( \frac{a}{b} \). This limit for PpIX has been estimated in humans by applying ALA topically on skin. In that case the ratio \( \frac{a}{b} \) was determined to be between 2 and 3 (Sterenborg et al. 1996 and unpublished data). We can rewrite Eq. 2 as:

\[
bC_p = \frac{1-\text{DR}}{\text{DR}-(a/b)}.
\]

Assuming the ratio \( \frac{a}{b} \) to be equal to 3 and a and b to be equal for all piglets, we can insert the values 2.3 and 1.8 for DR, the measured maxima found for piglet i and ii-iv respectively, in Eq. 3. For these values of the DR, \( bC_p \) equals 1.9 and 0.7 respectively. This suggests that at 6 hours after administration the photosensitiser concentration in the skin is 2.7 times higher in piglet i than in piglets ii-iv.

The advantage of the DR method is that the fluorescence signal is corrected for spatial variations in absorption and scattering of tissue as well as for changes in excitation and detection geometry. If a and b are assumed to be the same for all piglets, variations in the DR are only caused by variations in the photosensitiser concentration. The different time course of the DR seen between the different piglets (Fig. 2) might be caused by individual variations in the distribution of ALA and PpIX between different organs in the body. After 4 hours a decrease is seen in piglets i-iii. At that time the piglets were anaesthetised after having been conscious since the ALA administration. In measurements on a piglet that was not included in this study, we had seen that the anaesthesia influenced the digestive uptake of ALA. By delaying the total anaesthesia with 4 hours we expected
Chapter 3

the ALA uptake not to be influenced by the total anaesthesia. However, we cannot exclude that the
anaesthesia influenced the uptake of ALA and PpIX in the skin after 4 hours. Other kinds of
anaesthesia have been shown to influence ALA production in liver and kidneys (Buzaleh et al.
1994), an effect we have not investigated in this study.

According to Eq. 2 the DR is 1 when the photosensitiser concentration is 0, which is at t=0. Piglets i
and iii had a DR higher than 1 and piglets iv and v lower than 1 at t=0. This might be due to a low
signal to noise ratio since autofluorescence at the longer wavelengths of the spectrum is weak
compared to 550 nm fluorescence.

Conclusions

In this study the DR increased in the four piglets which received ALA but remained at baseline in
the control piglet. Using the DR to estimate the relative photosensitiser concentration in the
different piglets, a difference with a factor 2.7 in photosensitiser concentration was found at DR
maximum between piglet i and the other piglets. This indicates that the distribution of ALA and
PpIX after oral administration of ALA could be different in each animal. Anaesthesia might also
influence the uptake of ALA.

Acknowledgements

The authors would like to thank C.W.J. Verlaan and M.G. Klein at the Department of Experimental
Surgery of the Academic Medical Centre for their assistance in the piglet experiments. This study
was partially supported by the John L. Emmett Foundation.

References

Buzaleh AM, Enriquez de Salamanca R, del C. Batlle AM, Effect of multiple doses of volatile anesthetics on heme
enzymes, Gen Pharmac, 25:1179-1183, 1994

Grant WE, Hopper C, MacRobert AJ, Speight PM, Bown SG, Photodynamic therapy of oral cancer: photosensitisation
with systemic aminolaevulinic acid, Lancet, 342:147-148, 1993

Kennedy JC, Pottier RH, Pross DC, Photodynamic therapy with endogenous protoporphyrin IX: Basic principles and

Loh CS, MacRobert AJ, Bedwell J, Regula J, Krasner N, Bown SG, Oral versus intravenous administration of 5-
aminolaevulinic acid for photodynamic therapy, Br J Cancer, 68:41-51, 1993

photodynamic therapy (PDT) of gastrointestinal tumors with 5-aminolaevulinic acid (ALA) induced protoporphyrin IX

Sinaasappel M, Sterenborg HJCM, Quantification of the hematoporphyrin derivative by fluorescence measurement
using dual-wavelength excitation and dual-wavelength detection, Applied Optics, 32:541-548, 1993

Sterenborg HJCM, Saarnak AE, Frank R, Motamedi M, Evaluation of spectral correction techniques for fluorescence