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Published in:
ADVANCES IN LASER AND LIGHT SPECTROSCOPY TO DIAGNOSE CANCER AND OTHER DISEASES II, PROCEEDINGS OF

DOI:
10.1117/12.206834

1995

Pharmacokinetic studies of δ-amino levulinic acid-induced protoporphyrin IX build-up in some malignant tumours

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ABSTRACT

Laser-induced fluorescence was used for the monitoring of the δ-amino levulinic acid (ALA)-induced protoporphyrin IX (PpIX) build-up in non-melanoma malignant tumours of the skin and some cancers in the head and neck region. An optical-fibre based point monitoring system was utilised in the recording of fluorescence spectra at different time intervals after the administration of ALA. In the cases of skin tumours ALA was normally applied topically to the area. Only in one patient with an aggressive skin tumour ALA was administered intravenously. For the PpIX induction in the head and neck tumours the ALA was given orally. An example of a tumour fluorescence image is also presented.

INTRODUCTION

Photodynamic therapy (PDT) in an investigational local tumour treatment modality based on the light-induced activation of various photosensitising agents, administered to the body at different time intervals before the laser irradiation. As the photosensitising tumour-localising agents also exhibit characteristic fluorescence they have been utilised in the development of a new diagnostic method for the early photodetection (PD) of tumours based on the specific drug-related fluorescence combined with the autofluorescence from tissue chromophores [1-4]. The most commonly used drug is a haematoporphyrin derivative (Photofrin®) which is administered intravenously normally 24-48 hours before the procedures. After this time interval the concentration of the drug is higher in the tumour than in normal surrounding tissue, depending on various factors, such as the molecular properties of the dye and the tumour vasculature [5]. An unwanted side-effect with Photofrin is the transient skin sensitisation, which has played a major role in the slow clinical acceptance of the agent and the treatment modality. The skin response is dose dependent and in PD procedures the drug dose can be reduced drastically with the avoidance of the skin sensitisation, provided sensitive fluorescence equipment is used [3,4]. Second-generation sensitises have been developed and some of them exhibit much shorter periods concerning the skin affection [6]. Recently, newer and easier ways of drug administration for PDT and PD have been introduced. Especially, the introduction of ALA has opened up the possibility of topical and oral administration. The local application is of special interest for tumours in the skin or in the bladder [7-9]. The oral administration of the substance is applicable for other tumours, such as carcinomas in the head and neck region. ALA has been administered intravenously in experimental tumour systems [10] and in some case to a patient [11].

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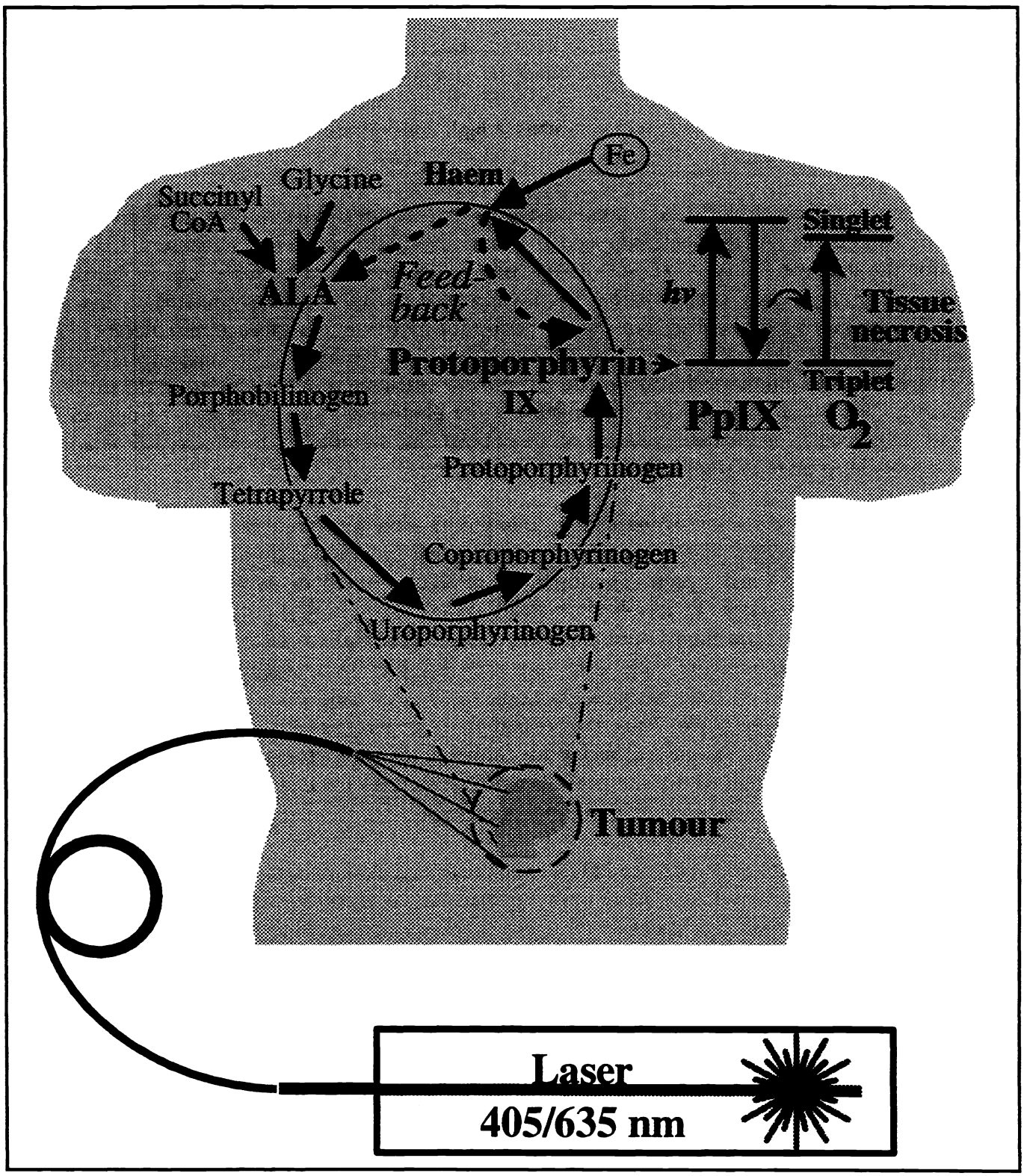

Figure 1. *A schematic diagram of the haem cycle. The feed-back system at the starting point of the cycle is disrupted if ALA is given excessively to the organism and a fast build-up of protoporphyrin IX occurs. The conversion of PpIX to haem is very slow leading to an accumulation of PpIX. PpIX is activated by utilising laser light at 635 nm for the induction of photodynamic tumour treatment. Laser light at 405 nm is used for fluorescence diagnostics* [8].

The time interval between the drug delivery and the laser irradiation for obtaining optimal tumour contrast varies for different substances. Also the way of administration certainly influences the tissue build-up of the drug. Therefore, it is of importance to develop clinically adopted reliable methods for drug detection in the tissue. The therapeutic as well as the diagnostic procedures should be scheduled within a time window when tumour tissue exhibits a high concentration and the normal tissue a low content of the sensitising agent.

Laser-induced fluorescence (LIF) is a non-invasive method for real-time *in-vivo* monitoring of the chromophores in tissue. As the sensitising agents exhibit specific fluorescence signatures, LIF can be utilised for the drug detection. Although the intensity of the fluorescence signal cannot be directly interpreted in terms of drug concentration, the fluorescence intensity is clearly related to the tissue content. The monitoring of the drug can also be performed by tissue fluorescence microscopy [12] or chemical extraction of the drug content. The disadvantage with these methods, in comparison to LIF, is that they are invasive with tissue destruction do not provide real-time results. Besides, in the procedure of preparing the tissue for the *in vitro* investigations, the photosensitising agent may be degraded or destroyed. In particular, in the determination of the optimal time window for treatment, LIF is a useful tool, as it provides prompt *in situ* results.

LIF can be used in the detection of the endogenous sensitisation with the transformation of ALA to highly fluorescent substances. ALA is the first step in the haem cycle and is formed in the condensation procedure of coenzyme A and glycine. The formation of ALA in the body as well as the synthesis of haem from PpIX is regulated by feed-back systems. When ALA is administered in excess to the organism the cell regulation of the ALA formation is disrupted and PpIX is formed during several steps within the cycle as shown in Figure 1 [8]. Each step in the haem cycle is regulated by enzymes. Due to differences in the content of these enzymes in normal and malignant tissue some of the steps are facilitated and others slowed down. Thus, the content of the enzymes regulating the first two steps in the cycle, is high in malignant tissue, enhancing the formation of tetrapyrole in malignant tissue [13] On the other hand the content of ferrochelatase is low in malignant tissue and therefore the step from PpIX to haem is slow and an accumulation of PpIX occurs [14].

Beside the topical application of ALA for PDT in non-melanoma skin malignancies [7,8] a few patients with oral cavity and colonic cancers [15,16] were treated after oral administration of ALA. The orally administered ALA was given in doses of 30-60 mg/kg b.w. For tumour demarcation by means of laser-induced fluorescence, lower ALA doses are desirable. When the system is overloaded with ALA, the bioavailability in normal mucous membrane is above a threshold for PpIX synthesis also in normal tissue and the demarcation towards malignant tissue is less pronounced [16,17]. With lower ALA doses (5-7.5 mg/kg b.w.), the availability of ALA in normal tissue is too low for the enzymatic capability. On the other hand PpIX can still be synthesised in tumour tissue as the enzymatic pattern is organized to meet the higher metabolic demand from proliferating tissue. Most probably, there is a threshold for the ALA dose, which varies from organ to organ, for optimal tumour demarcation. The time interval for maximal tumour build-up of PpIX in tumour tissue may also vary with the grade and stage of the tumour as well as with the tumour type. For the evaluation of the optimal time window for treatment and diagnostics, farmacokinetics studies are important. *In vitro* fluorescence microscopy investigations have been performed for farmacokinetic studies of the distribution of PpIX in different tissue types in a few patients [15,16]. The microscopy fluorescence studies showed a maximum PpIX build-up 4 to 6 h

after the oral administration of ALA. The fluorescence intensity in the specimens showed about double the intensity in the tumour as compared to normal surrounding connective tissue.

We have utilised LIF for real-time *in vivo* investigations of the PpIX build-up in different human malignant tumours after topical, oral or intravenous ALA administration. In cases of topical administration in non-melanoma skin malignancies we have used a 20%-ALA containing cream and the fluorescence was monitored before the application and at about 1.5 h and 6 h after the ALA application. For PD investigations in the bladder 1 or 3 % ALA solutions were instilled in the bladder [18,19]. Low-dose (5-15 mg/kg b.w.) oral ALA administration was utilised in the detection of tumour in the head and neck region [17,20]. Intravenous ALA injection was applied in one single patient with an aggressive basal cell carcinoma on the chest wall [11]. Some examples of the farmacokinetic studies we have performed are presented in this paper. Normally, a point monitoring clinically adopted fluorosensor was used for the collection of the fluorescence spectra [2]. Also a multi-colour fluorescence imaging system was used and some example is given [19].

MATERIAL AND METHODS

Patients with different kinds of malignancies were investigated by means of LIF at different time intervals after the ALA administration. In cases of non-melanoma skin malignancies, mainly superficial or solid basal cell carcinomas, the patients were investigated in conjunction with the sensitisation for PDT procedures. Oral administration of low-dose (5-15 mg/kg b.w.) ALA was administered in patients with malignant tumours in the head and neck region. Beside one non-Hodgkin lymphoma, all patients had squamous cell carcinomas of various stage and differentiation. The intravenous ALA injection was given to a patient with an aggressive solid basal cell carcinoma invading deep into the chest wall.

For the topical application ALA powder (Porphyrin Products, Logan, Utah) was dissolved in sterile water and mixed in a water-in-oil emulsion [8]. The ALA cream was prepared with 20% ALA and applied topically to the tumour and a border zone of about 5-10 mm surrounding the visible tumour. The fluorescence investigation was performed with a point monitoring system at about 1.5 h and 6 h and with an imaging system 6 h after the application. In the case of intravenous injection of ALA, the powder was dissolved in sterile saline and administered at a dose of 30 mg/kg b.w. through the intermediate cephalic vein [11]. For the oral administration, ALA powder was dissolved in orange juice (at a pH of about 3) [17]. The ALA was administered to the patients immediately after the preparation.

The fluorescence investigations were performed with a fibre based mobile clinical fluorosensor with the excitation light at 405 nm. In the measurements the fibre was placed perpendicularly to the tissue under investigation. At least 2 spectra were recorded from a given area. The fluorescence was evaluated as the background-free intensity at 635 nm and expressed in terms of an internal reference for minimal influence from uncontrolled variations in the measurements. The fluorosensor and the measurement procedures are described in detail in Ref. 2. For the imaging investigation a multi-colour fluorescence system was utilised and a tissue area of about 2-3 cm^2 can be investigated [19].

RESULTS AND DISCUSSION

In all patients a time dependent build-up of PpIX was observed in the different tumours, irrespective to the way of ALA administration. A fast build-up time for PpIX was monitored in the patient to whom

ALA was administered intravenously [11]. The patient had an aggressive basal cell carcinoma invading about 1-1.5 cm into the tissue of the thoracic wall from the skin down to the muscle fascia above the ribs. The tumour diameter was about 10 cm at the bottom with the "tissue walls" sloping upward towards the normal skin on the thoracic wall. Thus, the tumour formed a crater-like structure. The fluorescence spectra were recorded from the tumour edge sloping about 80° towards the tumour crater. Spectra were also recorded from the normal surrounding skin. The tumour edge exhibited more viable tumour tissue than the tumour crater but also some areas at the sloping edge exhibited small nests of necrotic tissue. Six fluorescence spectra are shown in Figure 2. Five of them were recorded from the tumour edge before ALA and 15, 30 and 45 min and 1 h after ALA administration and one spectrum from the skin. The tumour spectra were recorded from approximately the same spot.

As seen in the figure, already 15 min after the injection a small sign of PpIX peaking at about 635 nm is visible. Thirty minutes *post* injection the peak at 635 nm is sharper and also a second peak at about 700 nm is discernible. The next measurement at 45 min shows a clear dual-peaked PpIX fluorescence also seen in the spectrum recorded 60 min after the ALA injection. The measurement from the skin also shows PpIX-related fluorescence. It should be noted that the spectra are autoscaled when read out and the maximum fluorescence intensity counts on the y-axis vary. This is particularly evident when comparing the spectrum recorded 45 min after ALA with the spectrum 60 min after. In the latter spectrum the total number of fluorescence counts are lower. Most probably this is due to the fact, that the fibre tip was placed onto a spot with both necrotic and viable tumour tissue. In Ref. [11] all data from the measurements are presented.

As can be seen in the figure a fast build-up of PpIX is seen in the tumour after the intravenous injection. It is also clear that within the time interval from the injection until at least 45 min *post* injection, an increase in the fluorescence intensity occurs. The tumour fluorescence can also be expressed as a ratio formed by the background-free intensity at 635 nm and the autofluorescence peaking at about 500 nm. This evaluation gives a ratio of 0.10, 0.45, 0.75 and 0.95 at 15, 30, 45 and 60 min, respectively. If the tumour ratio at 60 min is compared with the ratio for the normal skin (0.01) the demarcation is about 95:1. The advantage of forming a ratio is evident, in particular in clinical investigations performed through an endoscope, with several uncontrolled variations, such as the distance to the probe due to the breathing of the patient and difficult tissue topography and irregularities in the tumour shape. Also technically related factors, such as variations in the excitation source and the detection equipment sensitivity, may influence non-ratio results.

Some examples of fluorescence spectra recorded 6h after oral administration of 15 mg/kg b.w. of ALA are given in Figure 3. The tumour, a T3 squamous cell carcinoma, was located in the oropharyngeal cavity involving the uvula and upper anterior part of the tonsil. The tumour spectrum is characterised by a clear PpIX fluorescence. A build-up of PpIX is also seen in the tongue and the normal palate to a certain degree. As in the patient with the intraveous ALA injection the skin fluorescence was also monitored and almost no PpIX related fluorescence was detected. With the autofluorescence included in the criterion, a dimensionless ratio is achieved. This calculated ratio of the background-free I(635nm) and I(500nm) from the spectra presented in Figure 3 gives the value of 1.4, 0.02 and 0.11 for tumour, normal palate and tongue, respectively. By dividing the ratio for the tumour with the ratio for the normal palate and the tongue, an enhanced tumour demarcation is achieved.

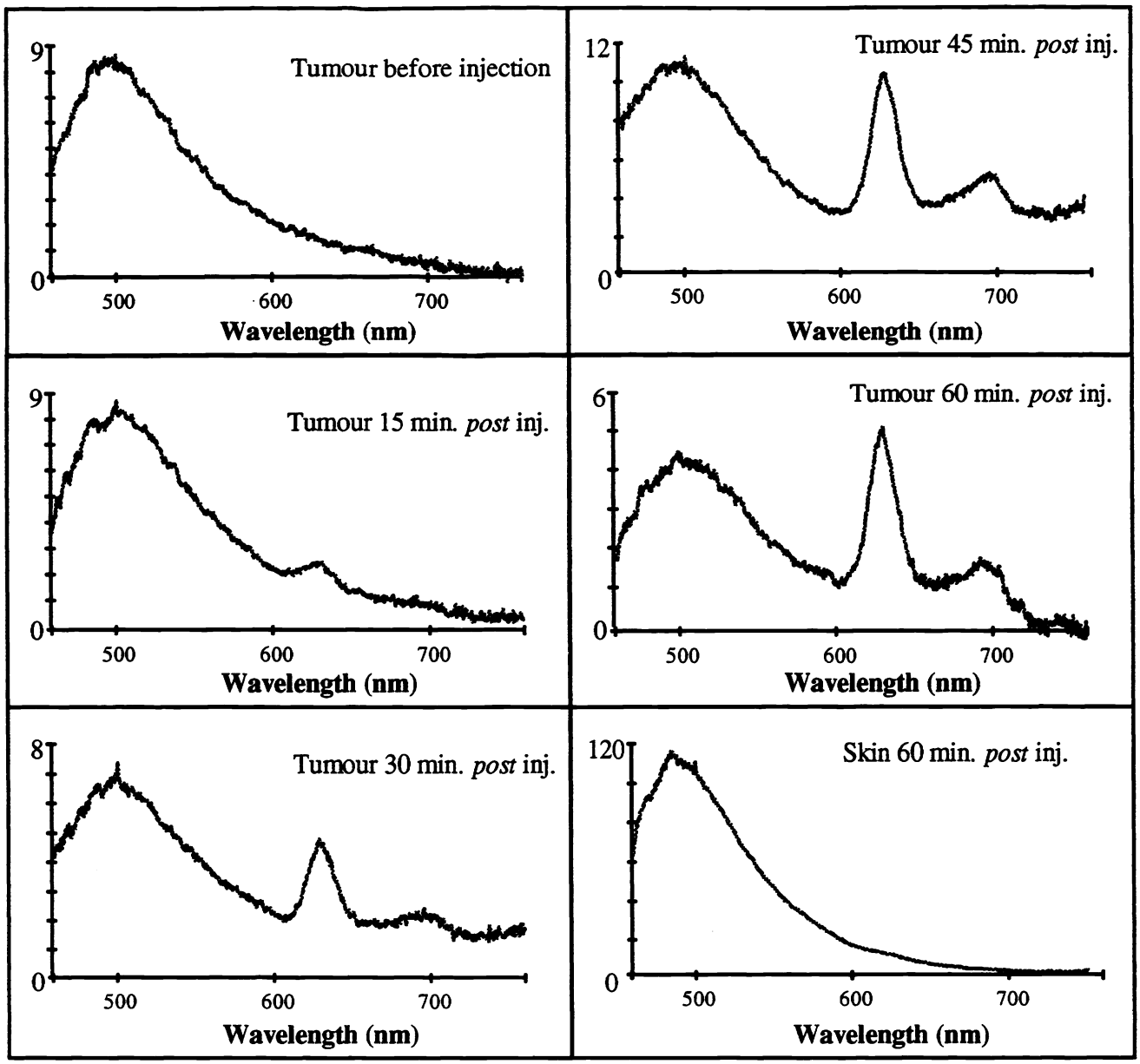

Figure 2. *Fluorescence spectra recorded in a patient with an aggressive basal cell carcinoma destructing the tissue on the thoracic wall. The ALA was administered intravenously at a dose of 30 mg/kg b.w. The fluorescence spectra were recorded before the injection and at different time intervals after the injection. Also the normal skin was investigated. The fluorescence intensity is expressed in normalised units with an autoscaled read-out. The excitation wavelength was 405 nm* [*11*].

Figure 4 shows the evaluated data of the fluorescence measurement from the same patient as presented in Figure 3. The fluorescence investigations were performed before the oral ALA administration and at 8 time intervals, 15 and 45 min, 1, 2, 3.3, 6, 8.5 and 24 h after ALA. The fluorescence was recorded from the tumour and also the tongue, normal palate and the skin. The tumour shows two intensity maxima. The first maximum appears already 15 min *post* ALA and represents the first-pass metabolism of ALA in the tumour. At this time the tumour demarcation towards normal palate in terms of

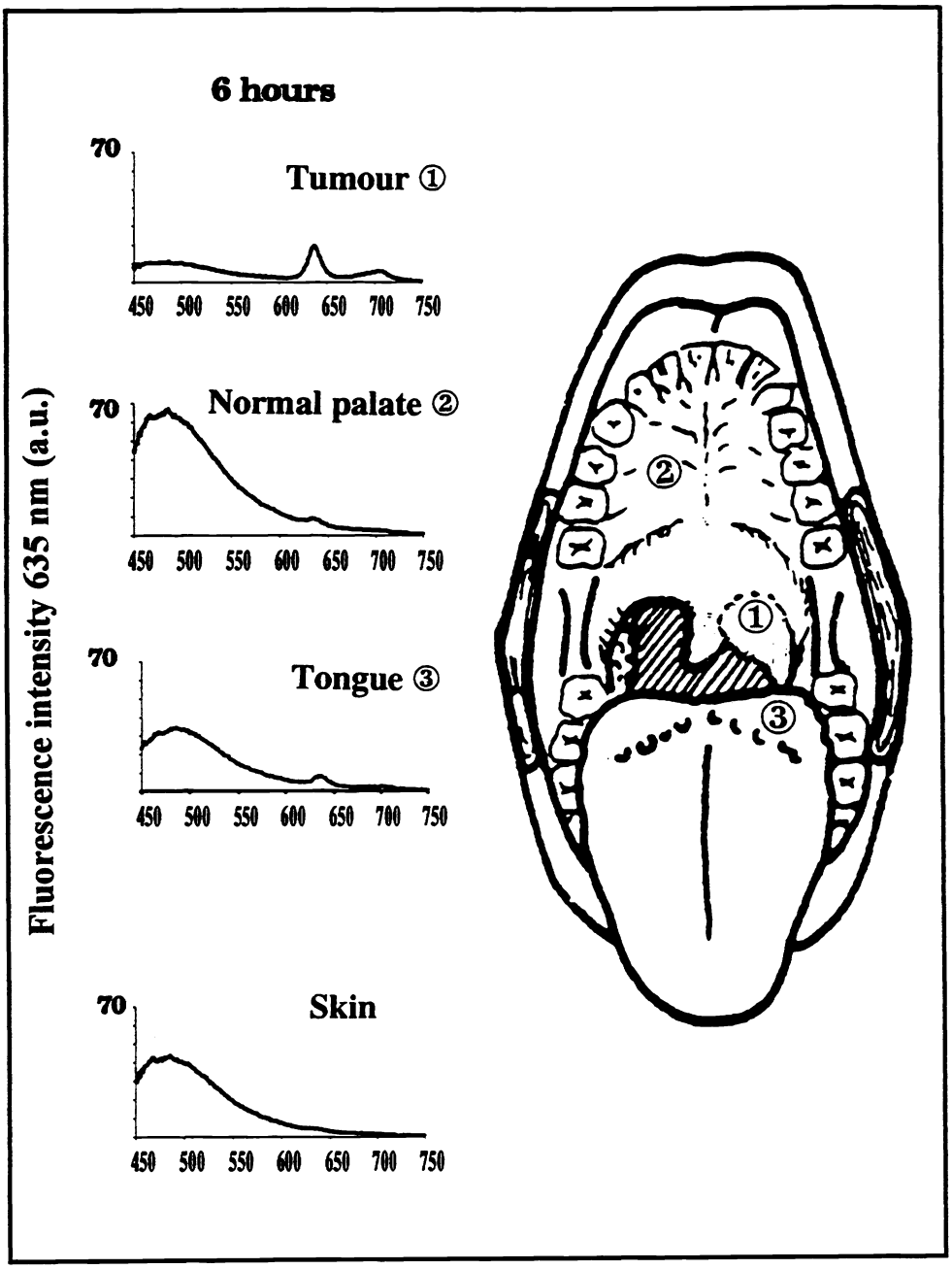

Figure 3. *LIF spectra recorded 6h after oral administration of 15 mg/kg b.w. to a patient with an oropharyngeal T3 squamous cell carcinoma. The tumour location ① is indicated in the figure as well as the investigation spot on the normal palate ② and the tongue ③. The spectrum from the skin was collected from the buccal area* [21].

background-free fluorescence intensity at 635 nm is about 10:1 and towards tongue 4:1. The PpIX build-up for non-malignant tissue shows a slower rate as compared to tumour and at 2 h the tongue exhibits about the same value as tumour and normal palate about double the value. A second maximum is seen for all three tissue types after about 8 hours. At that time the tumour demarcation is about 4:1 for normal palate and 2:1 for tongue. At 24 h the levels of the PpIX has come down to the background level for the normal palate and the levels of tongue and tumour have also come down drastically. The

skin shows very low PpIX-related fluorescence during the whole period. In Ref. [21] the ratio of the PpIX-related signal and the autofluorescence will be presented. Thus, the autofluorescence provides an internal tissue reference.

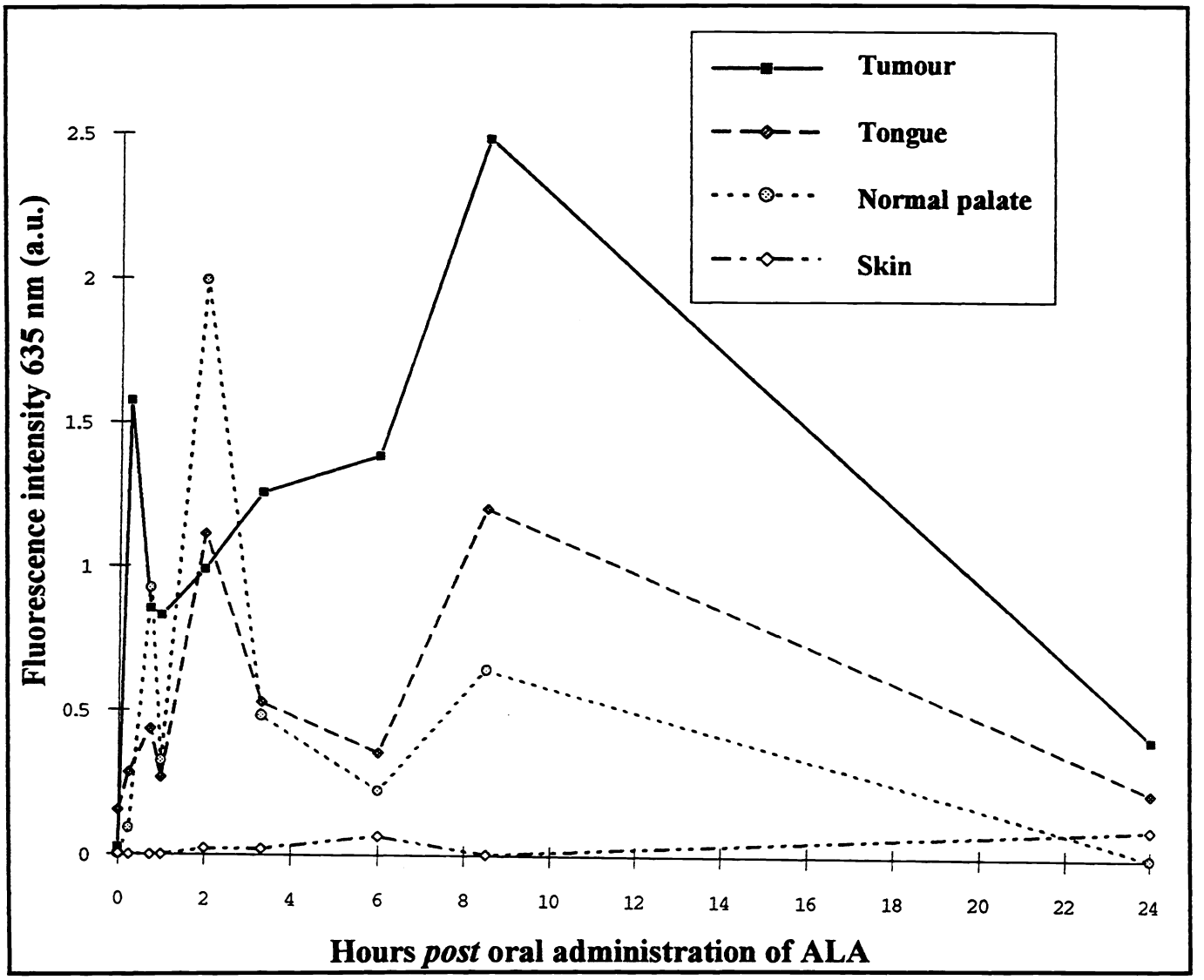

Figure 4. *Evaluated fluorescence data recorded at different time intervals in the same patient as presented in Figure 3. The fluorescence intensity at 635 nm was evaluated as the background-free peak lifted off the curve [21].*

The dose of 15 mg/kg b.w. used in the patient is probably too high for PD purposes as the bioavailability of ALA in normal tissue is too high for strong tumour demarcation. Especially, the normal palate exhibits a comparatively high PpIX-related fluorescence at 30 min and 2h after the ALA administration. The metabolic capacity of the mucous membrane in the palate seems to possess a large capacity of PpIX biosynthesis, which has also been shown for the gastrointestinal mucous cells [16]. The metabolic activity in secretory epithelial cells can be expected to be higher with the increased demand of haem. For an enhanced tumour demarcation a lower dose gives a better tumour selectivity [15].

An example from fluorescence measurements after topical application of ALA is shown in Figure 5. In this case a superficial basal cell carcinoma and the margins outside the visible tumour border was applied with 20% ALA 4 hours before the fluorescence investigation. The patient was then treated with PDT. As seen in the tumour spectra a prominent dual-peaked fluorescence occurs in the tumour area. The normal skin about 2-3 mm outside the visible tumour border shows only a small sign of PpIX related fluorescence. A fall-off in the autofluorescence is also seen in the tumour as compared to the normal skin as has been demonstrated also in Figure 2 and 3 for skin, normal palate and tongue.

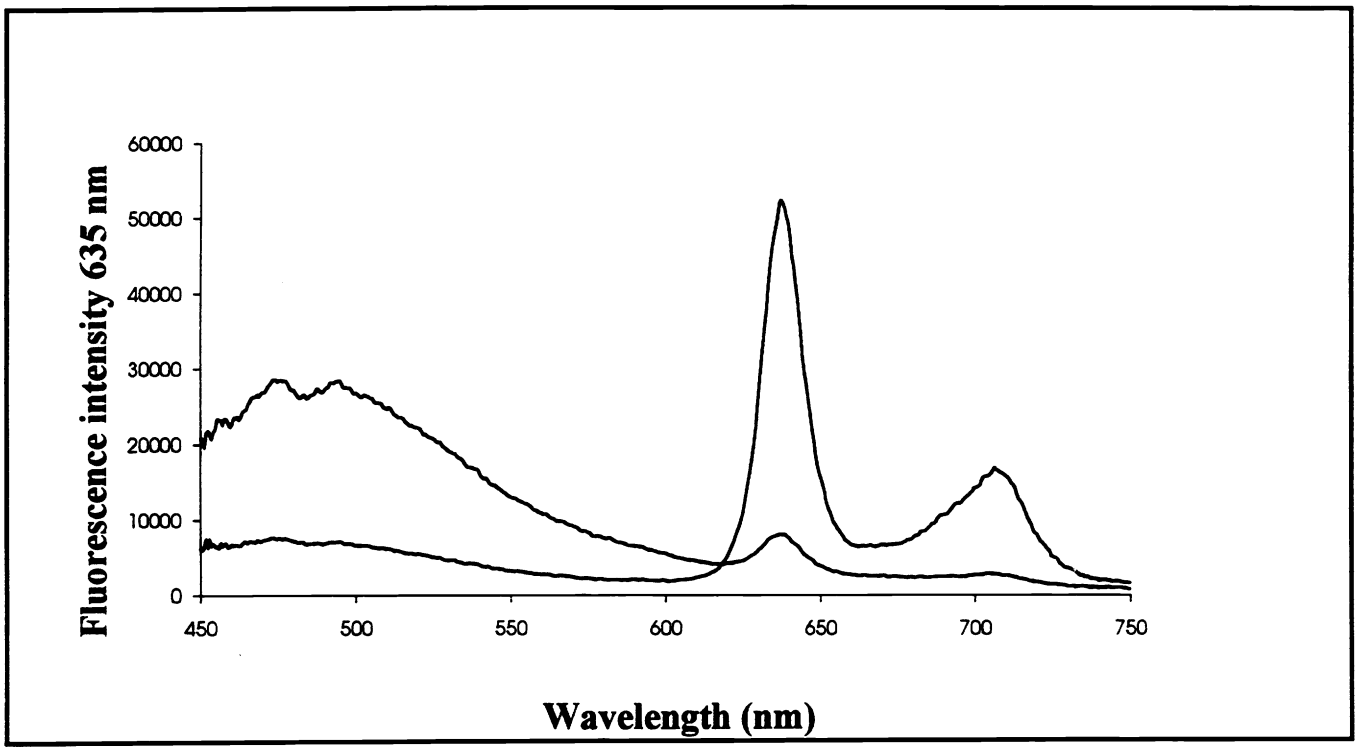

Figure 5. *Two LIF spectra recorded in a patient with a superficial basal cell carcinoma. The lesion with a margin of about 7 mm outside the visible tumour border was topically applied with 20% ALA cream 4 h before the investigation. The PpIX-related dual-peaked fluorescence signal in the red spectral region is clearly seen in the tumour area. The autofluorescence in the tumour tissue is very low as compared to normal surrounding skin* [22].

Figure 6 shows the evaluated fluorescence data recorded from two different superficial basal cell carcinomas and the surrounding normal skin. The recordings were performed at two time intervals after the application. In the figure the fluorescence intensity at 635 nm is shown for tumour and normal skin as well as the tumour demarcation. For the first case a demarcation of the tumour is only seen after 5h 30 min. In the second tumour a clear demarcation is seen 1h 20 min and 6 h after the ALA application. The reason for the low demarcation in the first case might be that the malignant tissue stretches out in the skin outside the visible border. This is of special interest for the planning of the PDT area. In such case the whole area with the inclusion of the margin outside the tumour should be included.

A tumour enhanced multi-colour fluorescence image of a basal cell carcinoma together with a photo of the same tumour and surrounding skin is shown in Figure 7. The false-colour coded image is superimposed on the tumour area with an update rate of about 3-5 frames/sec. The image is calculated pixel by pixel and the contrast enhanced ratio is processed as a colour-coded image. The brighter the

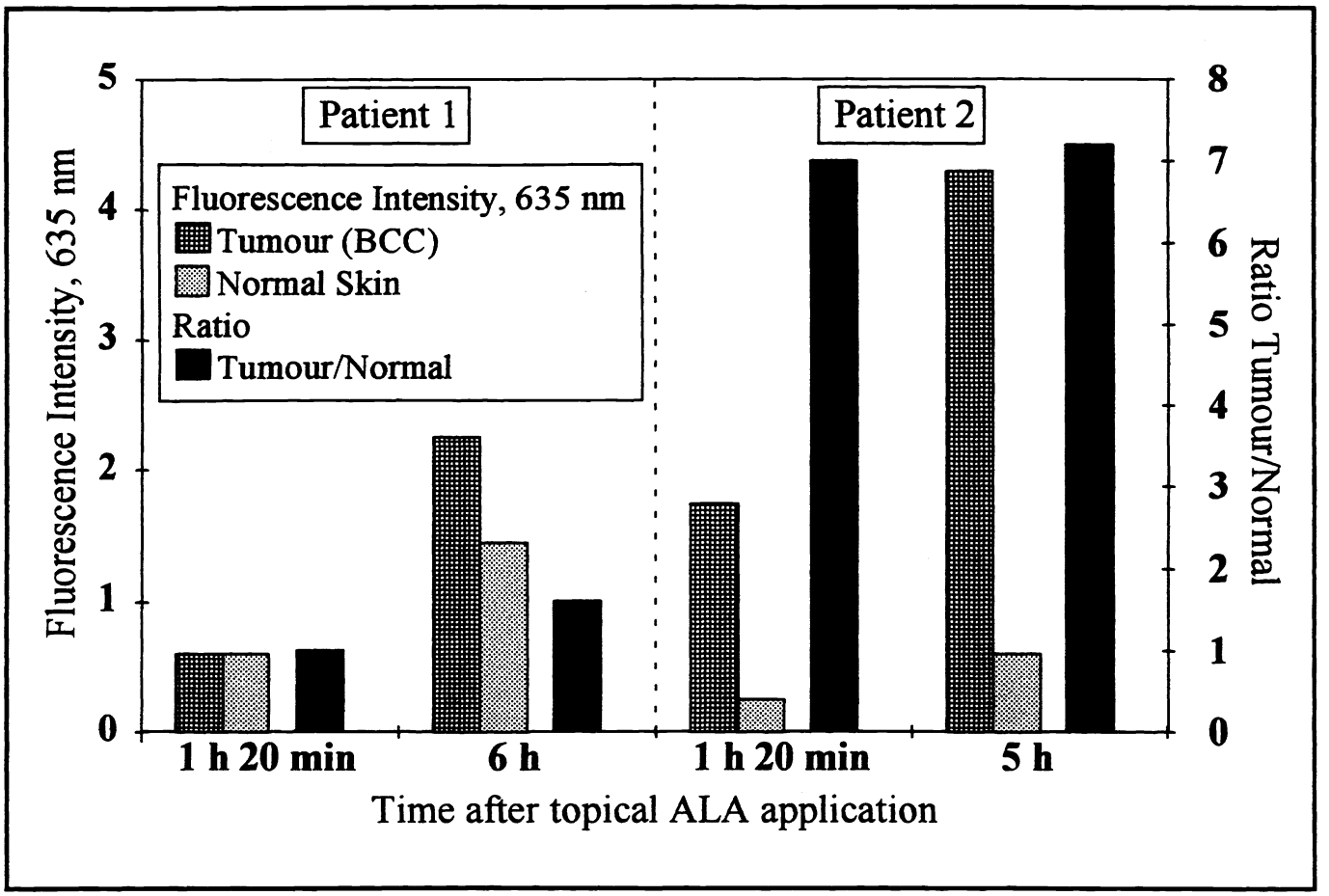

Figure 6. *Evaluated LIF data recorded from two different superficial basal cell carcinomas (BCC) and the normal surrounding skin about 10 mm outside the visible tumour border. ALA was topically applied to the whole area, including the border zone. The fluorescence intensity evaluated as the background-free fluorescence at 635 nm is shown for tumour and normal skin at two different time intervals. The tumour demarcation ratio is also shown for the two cases* [22].

image the higher is the tumour ratio. At the top in the figure just at the upper border of the tumour a dark spot is seen which is not included in the fluorescence image as it does not fulfil the tumour criterion being a benign pigmented nevus. The intensity of the processed image can be varied and thus mixed in to any desired degree during the investigation of the tumour. The initial results from some endoscopically based investigations in patients with different kinds of malignant tumours are described in Ref. [19].

CONCLUSION

A new way of tumour sensitisation for PDT and PD has been studied which involves the introduction of endogenous sensitisation utilising the haem precursor ALA. When administered excessively to the organism the regulating feed-back systems are disrupted and a build-up of the photodynamically very active and highly fluorescent substance PpIX occurs. Due to variations in the enzymatic pattern in different tissue types an enhanced accumulation takes place in malignant tissue. ALA is so far the only sensitising agent that can be administered orally which has advantages as compared to the intravenous

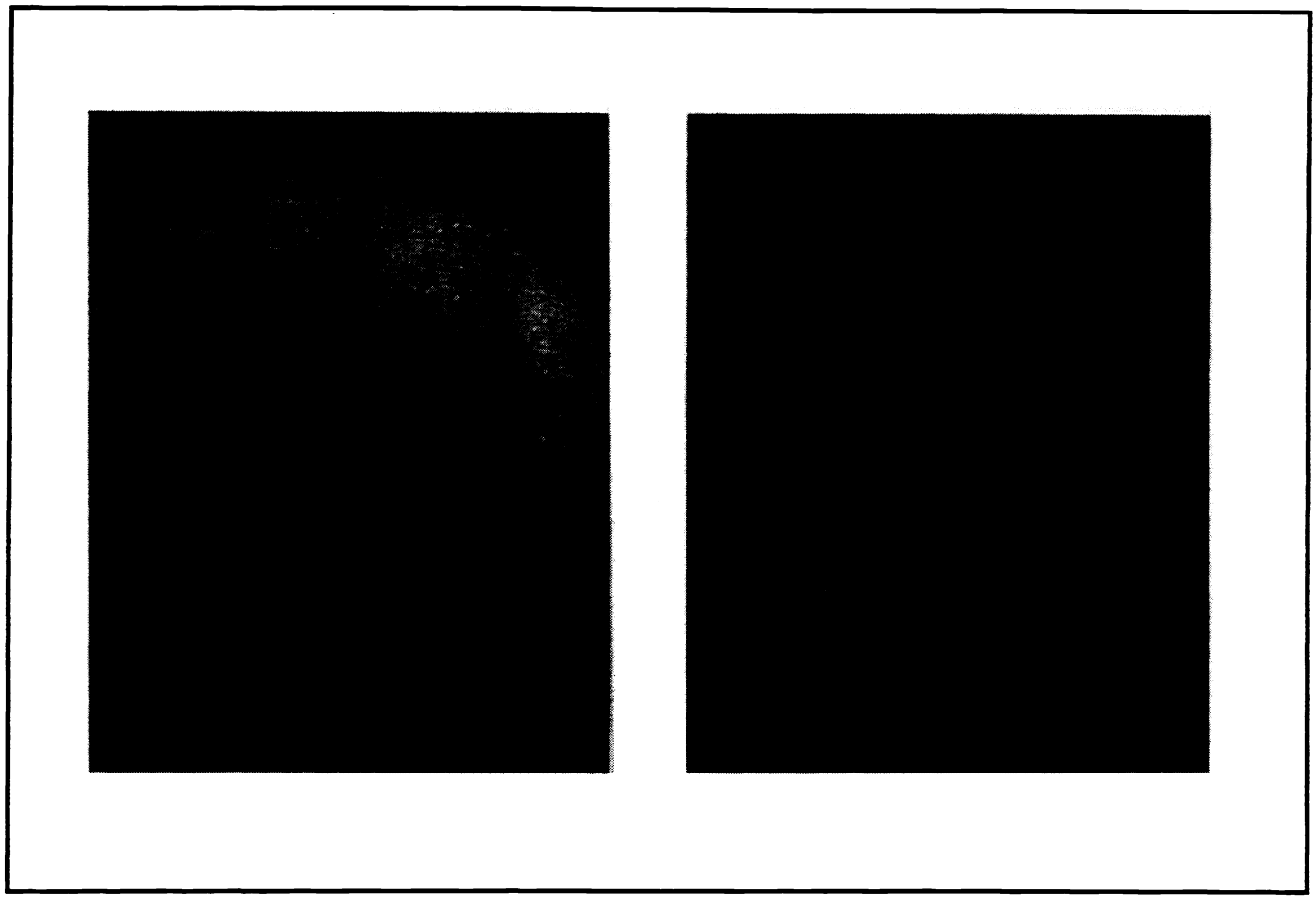

Figure 7. *A photo of a superficial basal cell carcinoma located on the back of a patient (to the left). The tumour diameter is about 2 cm. The tumour enhanced image is seen (to the right) superimposed on the normal image of the tumour. The brown spot at the upper border of the tumour is not covered with the processed colour-coded image as it does not fulfil the tumour criterion being a benign pigmented nevus* [*19*].

injection which normally has been utilised for different haematoporphyrin derivatives. Furthermore, local administration of ALA is also possible with the topical application on skin tumours and instillation in the bladder. Beside these newer ways of administration ALA can also be given intravenously.

Low-dose oral ALA is to be preferred for tumour diagnostic purposes in order not too exceed the threshold for the bioavailability of ALA especially in active secretion tissue, such as the gastrointestinal- or oral cavity mucous cells. Due to a very early first pass metabolism of PpIX in malignant tumour tissue the therapeutic and diagnostic procedures could take place very soon after the ALA administration, which from a practical point of view would be valuable. In order to find the optimal time window for treatment and diagnostics, pharmacokinetics studies are important. Of particular interest is the evaluation of the usefulness of LIF since the method has the advantage of being non-invasive and can be performed *in vivo* with immediate results. It would be of specific interest if LIF could be used in the interactive PDT planning for the determination of the optimal treatment time with the highest tumour selectivity. The use of LIF in early tumour diagnostics in conjunction with oral administration of ALA might also have a great potential.

ACKNOWLEDGEMENTS

The work was financially supported by the Swedish Cancer Society, the Swedish Research Council for Engineering Sciences, the Swedish National Board for Industrial and Technical Development, the Knut and Alice Wallenberg Foundation and the Kamprad Foundation. The support from Dick Killander and the collaborators within the Lund University Medical Laser Centre and Karl Erik Gierskssky at the Norwegian Radium Hospital is gratefully acknowledged.

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