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Pharmacokinetics of 5-aminolevulinic acid-induced protoporphyrin IX in skin and blood

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Abstract

The fluorescence and photosensitivity of endogenously synthesized protoporphyrin IX (PPIX) is increasingly used for the diagnosis and treatment of malignant and certain non-malignant diseases. A selective accumulation of PPIX can be induced by application of 5-aminolevulinic acid (5-ALA), which is a precursor of PPIX in the cellular biosynthetic pathway of heme.

The purpose of this study was to monitor the in vivo accumulation of PPIX in different locations of the skin after oral ingestion and to determine the pharmacokinetics of 5-ALA and PPIX in human blood plasma for various routes of application. At the same time we wanted to achieve an optimal treatment scheme but also study possible side-effects of 5-ALA administration.

After oral application of 5-ALA in a concentration of 40 mg kg^{-1} body weight, the fluorescence intensities of PPIX in the skin showed maxima between 6.5 and 9.8 h depending on the location and decreased to values lower than 5% related to the maximum after a mean time of about 40 h. The measured absolute intensities of PPIX fluorescence varied strongly between different patients and different locations on one patient. In the plasma of blood samples, PPIX could be detected via its fluorescence for all studied routes of application with the exception of the ointment, where PPIX levels were below the detection limit of 1 $\mu g\, l^{-1}$. The highest mean concentration of 742 $\mu g\, l^{-1}$ PPIX in the plasma was measured 6.7 h after oral application. For inhalation of 5-ALA, a mean maximum concentration of 12 $\mu g\, l^{-1}$ could be detected 4.1 h after application, for intravesical instillation, the mean maximum concentration was found to be 1 $\mu g\, l^{-1}$ 2.9 h after application. The kinetics of 5-ALA in the plasma peaked much earlier with a maximum concentration of 32 mg l^{-1} about 30 min. after oral administration. The 5-ALA levels did not exceed normal reference values after topical application.

The results of our experiments suggest that for a systemic application of 5-ALA side-effects in sensitive patients cannot be excluded.

Keywords: Porphyrins; Fluorescence; Photodynamic diagnosis; Photodynamic therapy; Side-effects

1. Introduction

Usually, photodynamic diagnosis and therapy require the administration of exogenous photosensitizers. Besides synthetic photosensitizers like Photofrin®, there are several potential endogenous sensitizers synthesized during the biosynthesis of heme. Under physiological conditions, a feedback loop controls the metabolic steps of heme biosynthesis and excludes an accumulation of any photosensitizing concentrations of the involved intermediates [1]. The intracellular concentration of free heme in the liver inhibits the synthesis of 5-aminolevulinic acid (5-ALA) and thus all following metabolites as a rate-limiting factor [2]. Enzymatic defects associated with the metabolic steps result in abnormal quantities of the corresponding porphyrins in the biosynthetic pathway. The clinical manifestation of the resulting cutaneous porphyrias include acute or chronic photosensitization of the skin [1]. Investigations of Berlin et al. in 1956 [3] showed that the administration of an excess of exogenous 5-ALA bypasses the cellular feedback control mechanism even in normal organisms. The resulting accumulation of protoporphyrin IX (PPIX), an immediate precursor of heme in the biosynthesis, leads to comparable

Abbreviations: PPIX, protoporphyrin IX; 5-ALA, 5-aminolevulinic acid

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symptomatic photosensitization of the skin as seen in the cutaneous porphyrias [1,4].

The quantitative expression and regulation of heme production differs from tissue to tissue. Changes in heme biosynthesis of certain malignant cells, in particular, seem to correlate with a preferential accumulation of 5-ALA induced PPIX [4]. At present, the tumorselective potential of PPIX and its clinical value for photodynamic diagnosis and treatment of neoplastic lesions are part of extensive investigations [5–12]. Clinical experience has been reported for systemic and different topical routes of 5-ALA application [13–18].

Considering both the many different application schemes as well as the possible systemic side-effects of the 5-ALA administration, a thorough knowledge of the time course and the distribution of 5-ALA and the induced PPIX are of significant importance. Moreover, the pharmacokinetics of 5-ALA and its metabolites in vitro and in vivo provide the basis for a better understanding of the involved mechanisms. The systemic load of patients with 5-ALA and PPIX at different times after application can be evaluated analytically by means of a biochemical assay of the blood samples from the patients. In addition, the skin photosensitization due to PPIX after oral ingestion of 5-ALA can be estimated by measuring the fluorescence intensity of PPIX in the skin. The purpose of this study was to monitor PPIX on different locations of the skin after oral ingestion and to determine kinetics of 5-ALA and PPIX in human blood plasma for various routes of application.

2. Materials and methods

2.1. PPIX fluorescence kinetics in the skin

11 patients (mean age 70 a, SD 6.2 a, range 62 to 79 a) proposed for photodynamic diagnosis or treatment were included in this investigation. After informed consent had been obtained, all patients received 40 mg kg^{-1} body weight 5-ALA dissolved in mineral water per os in a single bolus. All patients were kept in a darkened room for 24 h after 5-ALA ingestion.

For a reproducible recording of PPIX fluorescence kinetics at a constant distance to the skin, a handheld detection device was designed to allow for a homogenous illumination of the probing location by the light of a modified Xenon short arc lamp (D-Light, Storz, Tuttlingen, Germany) (Fig. 1).

To ensure an efficient excitation of PPIX within its absorption band centered at $\lambda = 408$ nm, the light source was equipped with a bandpass filter system transmitting blue-violet light in the spectral range of $\lambda = 375$ to 440 nm. The total irradiation of the tissue was limited to 4 mJ cm^{-2} for each measurement and location in order to prevent photobleaching effects [19].

As shown in Fig. 1, the remitted fluorescence light was collected by a fused silica fiber (HCN600, core diameter 600 μm) and analyzed spectroscopically by means of an intensified optical multichannel analyzer (O-SMA 3, SI Spectroscopy Instruments, Gilching, Germany). A sufficient blocking of the excitation light was obtained by a combination of two longpass filters (KV 450, GG 455, Schott, Mainz, Germany) mounted behind the entrance slit of the monochromator. Emission spectra were recorded in the spectral range between $\lambda = 450$ and 750 nm and corrected according to the background and the wavelength sensitivity of the system.

To separate the specific fluorescence of PPIX from the autofluorescence of the skin, the ratio of the integral autofluorescence intensities at $\lambda = 635 \pm 5$ nm and $\lambda = 590 \pm 5$ nm was calculated for all spectra recorded from the skin prior to the application of 5-ALA and free of any porphyrin peaks. If this ratio is applied, the contribution of the autofluorescence to the integral fluorescence intensity at $\lambda = 635 \pm 5$ nm can

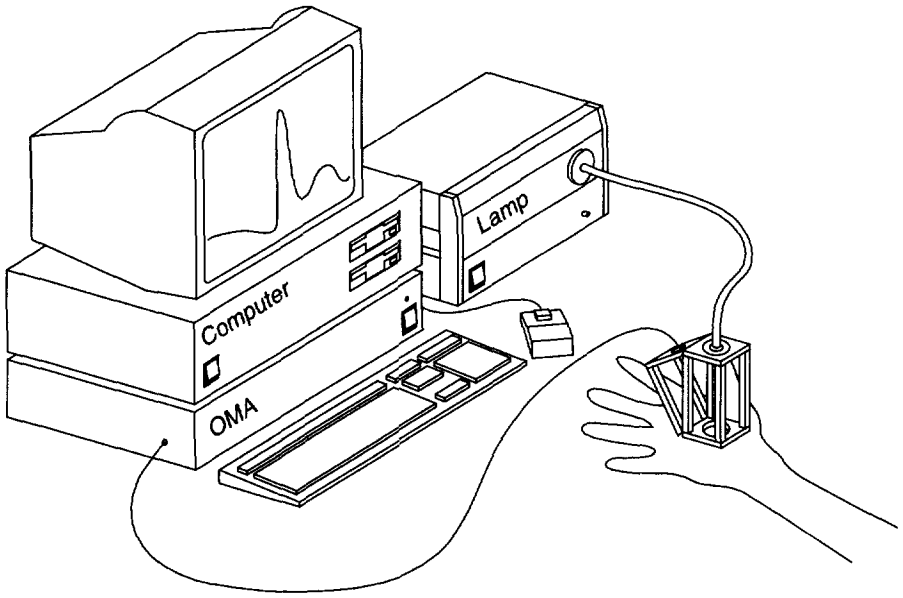

Fig. 1. Experimental setup used for the measurement of PPIX fluorescence kinetics in the skin.

Table 1
Doses and number of patients for different routes of application

Delivery	Dose	n
Oral	40 mg/kg body weight	12
Inhalation	500 mg (10%)	5
Intravesical instillation	1 g (2%)	5
Ointment	200 mg (10%)	1

be determined and then subtracted for each set of spectra. Subsequently, the resulting specific fluorescence intensities for PPIX at $\lambda = 635 \pm 5$ nm were plotted as a function of time.

2.2. Pharmacokinetics of 5-ALA and PPIX in blood plasma

Pharmacokinetic measurements in human blood plasma were performed for different routes of 5-ALA application. The applied doses for clinical 5-ALA assisted photodynamic diagnosis or therapy are listed in Table 1.

The application procedure for oral ingestion of 5-ALA is similar to the one described in the context of the skin measurements. For inhalation of 5-ALA, a concentration of 10% (500 mg 5-ALA in 5 ml isotonic saline) was applied 30 to 40 min. by means of a medical nebulizer (Inhalierboy, Pari, Starnberg, Germany). Intravesical instillation of the 5-ALA solution (1 g 5-ALA in 50 ml 1 M $NaHCO_3$) was performed via a catheter and incubated for a period of 2 to 4 h. For topical application as an ointment, a water-in-oil based cream containing 10% of 5-ALA (200 mg 5-ALA in 2 g cream) was applied and covered for 6 h with an occlusion pad.

Venous blood samples of all patients were collected immediately before and at various times between one and 48 hours after 5-ALA application. Blood was collected into EDTA tubes and protected from light during the whole preparation procedure. The separated plasma was stored at a temperature below 20 °C until analyzed.

The amount of 5-ALA in the plasma of two patients was determined by an HPLC method using fluorescence detection as described by Tomokuni et al. [20].

PPIX was extracted from the plasma using a mixture of acetonitrile and ethanol (1:8) as a solvent. Plasma and solvent in the ratio of 1:9 were mixed for one minute by a sonifier (Sonifier 250, Branson, Danbury, CT, USA). After one hour the mixture was centrifuged in an ultra centrifuge (Cryofuge 800, Heraeus, Hanau, Germany) for ten minutes at 5700*g*. Fluorescence spectra of the supernatant were measured with a fluorescence spectrometer (Luminescence Spectrometer LS 50, Perkin Elmer, Überlingen, Germany) at a spectral resolution of $\Delta\lambda = \pm 2$ nm. According to the Soret band of PPIX dissolved in ethanol, emission spectra of the supernatant were measured at the excitation wavelength of 405 nm. Subsequently, excitation spectra were recorded for all peak wavelengths appearing in the emission spectra. All emission spectra were corrected as to the background of the solvents used and the recovery rate. PPIX concentrations were calafter the calibration of fluorescence intensities obtained from standard solutions of PPIX (Porphyrin Products, Logan, UT, USA). The PPIX concentrations of these standard solutions were determined via the absorbance measured at 405 nm (Diode Array Spectrophotometer 8452A, Hewlett Packard) using Beer's law, the extinction coefficient $\epsilon = 1.71 \times 10^5$ l $mol^{-1}cm^{-1}$ for PPIX in ethanol [21] and the molecular weight of PPIX = 562.7 g mol^{-1}. Fluorescence intensities were measured in the spectral range of $\lambda = 635 \pm 5$ nm for a series of 10 diluted solutions prepared in the range between 10 μg l^{-1} and 10 mg l^{-1}, thus showing a linear relationship between absorption and fluorescence. By extrapolation to lower values, PPIX-concentrations of plasma samples could be determined down to 1 μg l^{-1}.

Kinetics based on the achieved values are fitted by using least squares fitting procedures [22] and rate constants according to a one-compartment model for 5-ALA and a three-compartment model for PPIX [5,6].

Spectral identification of the contributing fluorophores was achieved by comparing the resulting spectra with emission and excitation spectra recorded from standard solutions of uroporphyrin I/III, coproporphyrin I/III and protoporphyrin IX (Porphyrin Products, Logan, UT, USA) using the above mentioned combination of acetonitrile and ethanol (1:8) as a solvent. Mixtures containing uro-, copro- and protoporphyrin IX in varying ratios of concentration showed the contribution of the different porphyrins to the peak positions of the measured emission and excitation spectra.

By dissolving and extracting a known amount of PPIX in fresh and untreated human plasma the recovery rate of the procedure was found to be 72%.

3. Results

3.1. PPIX fluorescence kinetics in the skin

Fig. 2 shows fluorescence spectra (before, 7 h and 50 h after application) taken from a series of spectra recorded on

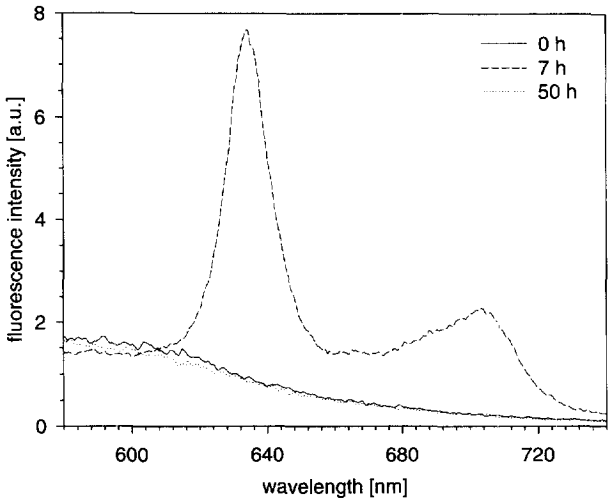

Fig. 2. Fluorescence spectra recorded on the forearm of one patient before,

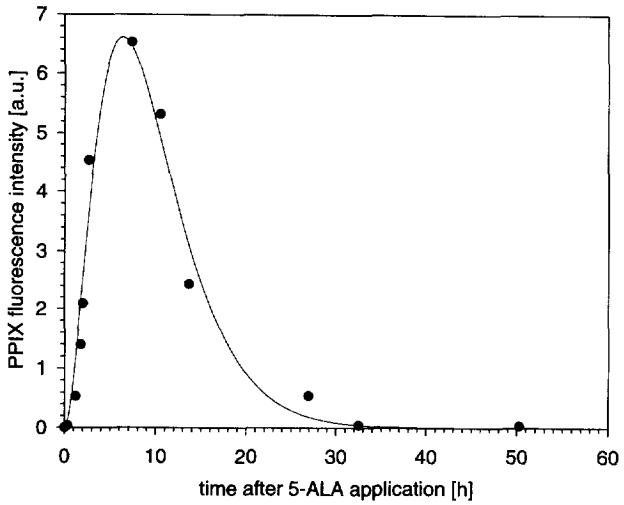

Fig. 3. PPIX fluorescence kinetics of forearm skin (same patient as in Fig. 2). Peak values at $\lambda = 635 \pm 5$ nm are corrected for autofluorescence and background.

Table 2
Mean parameters calculated from the fits of the PPIX fluorescence kinetics after oral application of 5-ALA. The left column shows the absolute mean peak times for different locations as averaged for all patients. For the second and third columns (relative), the ratios of the peak times and intensities related to the first location "back of the hand" are determined individually and averaged for the patients subsequently. Since the standard deviation (SD) was comparable for the different locations, the mean SD is calculated independently of the locations

Location	t_{max} (absolute) [h]	t_{max} (relative)	I_{max} (relative)
Back of the hand	6.5	1.0	1.0
Lip	7.9	1.3	9.4
Cheek	8.7	1.5	7.3
Forearm	9.8	1.7	1.4
SD [%]	15	10	25

a patient's forearm before and at various times between 30 min and 50 h after oral 5-ALA application.

In none of the spectra recorded from human skin, any indication for the presence of porphyrins other than PPIX could be found. The fluorescence kinetics for PPIX measured on the forearm is shown in Fig. 3 showing a peak after 7 h and a fast decrease to baseline levels within 40 h. The highest fluorescence intensities were measured for the areas of head and neck, especially the lips. It was observed that PPIX fluorescence intensities decreased towards the extremities. The lowest signals, however, were detected on the trunk. Table 2 summarizes the results of the PPIX fluorescence kinetics measured on some characteristic locations of the skin.

As seen in the right column of Table 2, the lowest mean intensity was observed at the back of the hand with the maximum intensity already 6.5 h after application. The kinetics of the lip with the highest intensities measured peaked after 7.9 h, followed by the cheek with the maximum intensity after 8.7 h and the forearm after 9.8 h. After a mean time of PPIX-fluorescence intensities decreased to values lower than 5% of the maximum for all locations.

3.2. Pharmacokinetics of 5-ALA and PPIX in blood plasma

Both emission and excitation spectra of the plasma recorded after application of 5-ALA showed the typical shape and peak positions of PPIX. There was no spectral indication for the presence of other porphyrins. Fig. 4 compares some characteristic examples of the kinetics of PPIX in blood plasma for various routes of application.

Table 3 summarizes the results of the determination of PPIX in the plasma according to the fitted kinetics and averaged for all patients: For oral application, we found an average peak concentration of 742 $\mu g\,l^{-1}$ PPIX in the blood plasma 6.7 h after application. For inhalation, an average peak concentration of only 12 $\mu g\,l^{-1}$ was reached already 4.1 h after application. The average peak concentration for intravesical instillation was 1 $\mu g\,l^{-1}$ and close to the detection limit. For topical application of 5-ALA using an ointment, no PPIX could be detected in the plasma. The average time $t_{0.05}$ at which the PPIX concentration decreased to values lower than 5% of the maximum was less than 35 hours for all routes of delivery.

In summary, the ratio of the maximum concentrations was found to be 742 (oral):12 (inhalation):1 (instillation).

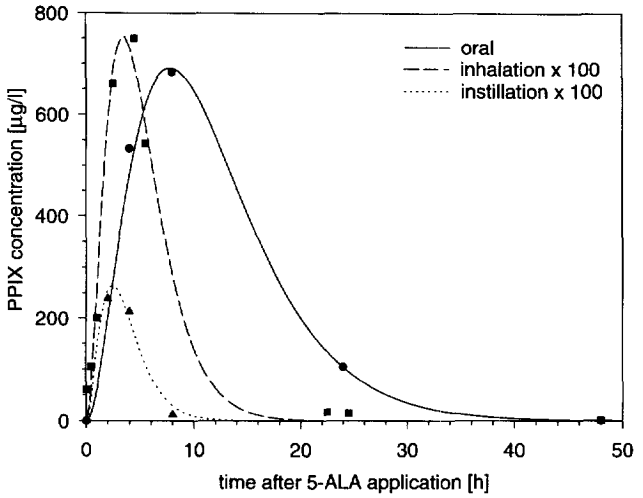

Fig. 4. Comparison of the PPIX concentrations in the plasma after systemic and topical application.

Table 3
Averaged results of the fitted PPIX plasma kinetics (corresponding standard deviations in brackets). A mean maximum concentration c_{max} was reached at the mean peak time t_{max} and decreased to values lower than 5% of the maximum at $t_{0.05}$ (n.d. = not detected)

Application	t_{max} [h] (SD)	$t_{0.05}$ [h] (SD)	c_{max} [µg/l] (SD)
Oral	6.7 (0.8)	35 (4.6)	742 (87)
Inhalation	4.1 (0.8)	21 (7.8)	12 (2.9)
Instillation	2.9 (0.5)	n.d.	1 (0.8)
Ointment	n.d.	n.d.	n.d.

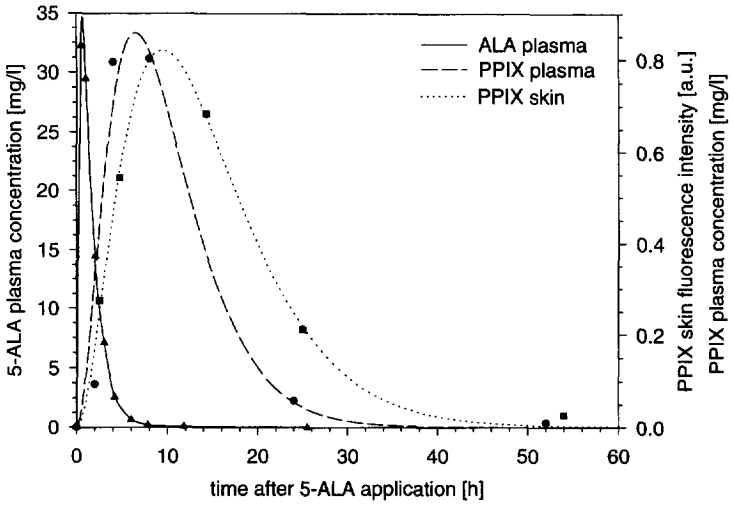

Fig. 5. Comparison of the PPIX skin kinetics (a.u.), PPIX (right axis) and 5-ALA (left axis) plasma kinetics after oral application.

The time course of the 5-ALA kinetics (Fig. 5) was much faster than all PPIX kinetics. A 5-ALA peak concentration of 32 mg l^{-1} was reached already 30 min. after systemic application with a return to baseline levels in less than 8 h. In contrast to these observations, the measured maximum concentrations for topical application did not exceed the detection limit of 0.1 mg l^{-1} set by the HPLC technique used in the experiments [20].

4. Discussion

4.1. PPIX fluorescence kinetics in the skin

The present study describes the in vivo kinetics of 5-ALA induced PPIX for various routes of application. For systemic administration (oral), the PPIX kinetics in normal skin was assessed by in vivo fluorometric measurements. Apart from that, we investigated time course and peak levels of both 5-ALA and PPIX in human blood plasma for systemic and various topical routes of delivery (inhalation, instillation, ointment).

All kinetics recorded from different locations of the skin showed similar time courses, although peak times and intensities seemed to depend on both location and individual variations between the patients. After oral administration of 5-ALA, the fluorescence kinetics of PPIX in normal skin reached maximum values after about 8 h followed by a decline to baseline levels after 40 h.

The patient-to-patient variability can be studied in Table 2. The lower standard deviation for relative t_{max} (second column) versus absolute t_{max} (first column) indicates that, once normalized individually, the variance in peak times decreases. The clearance of PPIX from the sensitized tissue appears to be even more sensitive to individual differences between the patients, resulting in a higher relative standard deviation of the clearance times (22%) compared to the abso-

In order to be able to interpret the relative peak intensities I_{max} listed in the last column of Table 2, additional variations in the optical properties of the tissue have to be taken into account. Individual variations in the optical tissue parameters of each location can explain the high standard deviation of up to 25% of even the relative values.

For the routine clinical use of orally administered 5-ALA, a detailed knowledge of the induced transient photosensitization of the skin is quite important. Considering the good correlation between PPIX fluorescence and photosensitization of human skin [13], we observed that the highest risk for phototoxic reactions was about 8 h after 5-ALA application. In fact, the exposure to artificial light within this time range led to mild erythema in two of the patients. On the other hand, our investigations also indicate that with a slight individual uncertainty a significant photosensitization of normal skin does not persist longer than 40 h after administration.

In contrast to reports of Edwards [23] and Mustajoki [24], our own yet unpublished observations support the results of recently published studies [18,25] indicating that, besides the transient skin photosensitivity, an exogenous systemic 5-ALA loading might provoke other porphyric or even non-porphyric side-effects in morbid patients. Most of our patients tolerated an oral dosage of 40 mg kg^{-1} body weight quite well. Only a small group of partly high-risk patients showed mild complications from nausea to occasional vomiting and neurological symptoms, but also severe side-effects like hypotension in combination with a remarkable vasodilatation. The last complication in particular, which occurred between 2 and 8 h after application, seems to be inconsistent with the known hypertension in the case of acute hepatic porphyrias. Thus, a systemic administration of 5-ALA in excess might result in a clinical manifestation partly different to that observed with various kinds of porphyrias.

4.2. Pharmacokinetics of 5-ALA and PPIX in blood plasma

To provide a deeper insight into the distribution mecha-

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