

MEASUREMENTS OF ALA METHYLESTER DIFFUSIVITY IN NORMAL SKIN *IN VIVO*: A PILOT STUDY

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Abstract: In photodynamic therapy the patient is given a tumor-selective drug, e.g. Methyl-Aminolevulinic Acid, which will convert to the fluorescent compound Protoporphyrin IX in the cells. By illuminating Protoporphyrin IX with 635 nm, the excitation energy will be transferred to oxygen molecules, which forms very reactive singlet oxygen, leading to tumor destruction. It is important to know the different processes of the treatment in detail in order to obtain the best treatment result. In our work we have identified the diffusivity of the drug as an important factor, because it determines the distribution and concentration of the drug in tissue, and is important for the efficacy of photodynamic therapy. These measurements are the first step towards our next goal, being the construction of a dosimetry model for the treatment. As the topically applied substance (an ALA derivative) is non-fluorescent, no such measurements have been successful so far. We have used microdialysis on normal skin, and traced the drug with both High Performance Liquid Chromatography-Fluorimetry, and Liquid Chromatography Mass Spectrometry.

Introduction

5-aminolevulinic acid (5-ALA) is a precursor compound in biosynthesis of haem which as an intermediate product has a photosensitizer called protoporphyrin IX (PpIX). In Photodynamic Therapy (PDT), PpIX is excited with 635 nm light, frequently resulting in that a nearby oxygen molecule is transferred to its excited singlet state by exchanging energy with the excited PpIX molecule. Singlet oxygen is a cytotoxic compound that can destruct and kill tumor cells. 5-ALA can be administrated topically as a cream in PDT of basal cell carcinoma. The distribution of the drug (5-ALA) depends on molecular diffusion properties and tissue vascularity [1]. Because of the poor penetration of drugs into the

skin [2], drug transport properties can limit the treatment of thick lesions [1]. In PDT the concentration and distribution of the drug is an important dosimetric parameter. Since ALA is a hydrophilic molecule, its penetration through cellular membrane and into interstitial space of tissue is low. Ester derivatives of ALA, such as ALA methylester (M-ALA), are more lipophilic than the free acid, can also be used as drugs for topically administration in PDT [3]. As ALA and its derivatives are non-fluorescent, and also because the concentration of these molecules in the skin after topically administration is very low, no diffusivity measurements of 5-ALA or its esters have been reported yet. In our project we administered M-ALA cream (METVIX[®] 20 %, OSLO, Norway) to normal human skin, and used microdialysis for sampling its concentration at a depth of 0.5 mm inside the skin. Microdialysis is a technique to monitor the chemistry of the extracellular space in living tissue. The principle of microdialysis is based on the passive diffusion of a compound along its concentration gradient. The samples, collected by microdialysis, were analyzed using two different methods, High Performance Liquid Chromatography-Fluorimetry (HPLC-Fluorimetry), and Liquid Chromatography Mass Spectrometry (LC-MS). In this paper we describe how these methods were employed for analyzing our samples.

Materials and methods

Metvix was applied on the skin of the forearm skin of a volunteer. A microdialysis device was used to sample the subcutaneous fluid at certain time intervals. A flow rate of 0.3 μ l/min and a permeable membrane with 100 KD cut-off were used for microdialysis sampling. The permeable membrane was placed at a depth of 0.5 mm in the skin. Microdialysis fluid was pumped through the system, allowing M-ALA and other compounds with molecular weight less than 100 KD to be transferred to the liquid by diffusing through the

membrane. Samples were collected in 500- μ l Ependorf tubes and were kept in -80 °C until analyzing. For measurement by HPLC-Fluorimetry, M-ALA in the sample was coupled with a fluorescent molecule 4-(2-cyanoisondonyl) phenylisothiocyanate (CIPIC) [4] and the derivative was injected into the HPLC system, using a reverse phase column Synergi Fusion-RP (C18 with polar embedded groups and low MS bleed), mobile phase containing 0.1 % formic acid in water. The sensitivity for detection of M-ALA using MALDI-TOF MS was assayed using a preparation of pure M-ALA in buffer. All MALDI-TOF experiments were performed on a 4700 Proteomics Analyzer (Applied Biosystems, USA). Di-hydroxy benzoic acid (DHB) was used as the MALDI matrix. The signal at 147 D, corresponding to M-ALA, overlapped with a signal from DHB and could thus not be used by itself for detection of M-ALA. Instead, the signal at 147 D was selected for fragment ion analysis with the instrument operated in MS/MS-mode. In the M-ALA samples, a characteristic fragment ion signal at 114 D could be detected and was used for identification of M-ALA. Samples were directly injected to LC without any derivitization [5]. Fractions were collected around the retention time where M-ALA had shown to elute was collected and subjected to MALDI-TOF MS analysis. However, M-ALA could not be detected in these samples, likely due to coelution of some unknown substances, interfering with the MALDI sample preparation.

Results and discussion

HPLC with fluorimetric detection was not sensitive enough to measure all different concentrations of M-ALA in the samples. The detection limit for M-ALA was in the region of 100 ng/ml and 300 ng/ml in buffer and sample, respectively (Figure 1).

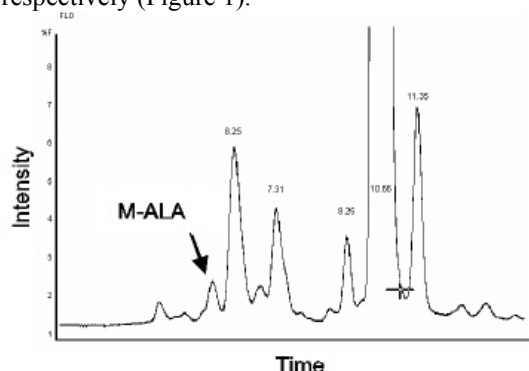


Figure 1: HPLC-Fluorimetric for M-ALA in a fortified sample.

Using MALDI-TOF MS, the detection limit for M-ALA was lowered to approximately 5 ng/ml

(Figure 2). Adjusting the mobile phase and LC/MS procedure to obtain the desired retention and separation are under way.

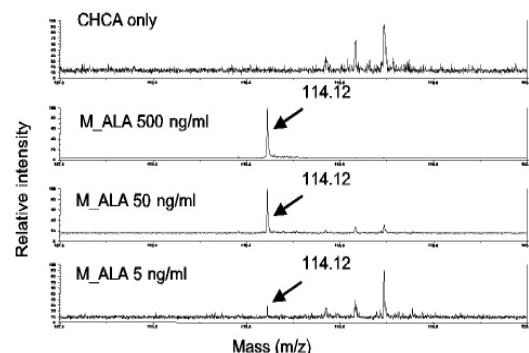


Figure 2: MS/MS spectra of M-ALA signal at m/z 146.2

Conclusions

This paper presents a study aiming at measuring M-ALA concentration as a function of time after topical application at a specific depth (0.5 mm) of normal skin *in vivo* using microdialysis. One of the aims of this study is to understand the diffusivity of the M-ALA, which would be necessary for developing an improved dosimetry model for PDT. For further studies the presented project will be performed for tape stripped normal skin.

References

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