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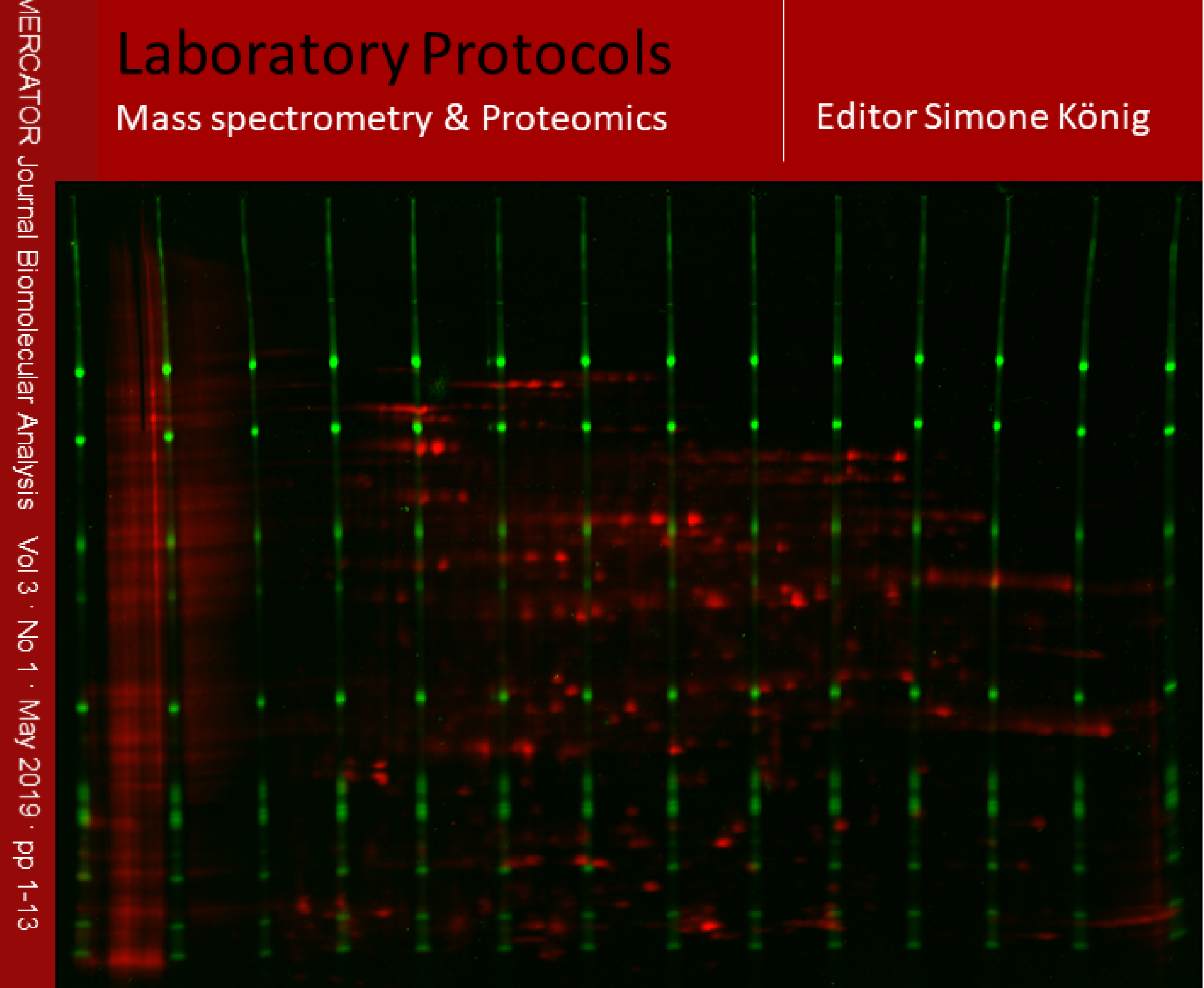

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Cover image
Mercator gel (run by D. Ackermann at CUP) representing the award-winning CoFGE technology for standardized gel electrophoresis

Protocol

Determination of δ-aminolevulinic acid-induced protoporphyrin IX in blood

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Abstract

In neurosurgery, δ-aminolevulinic acid-induced fluorescence-guided resection (ALA-FGR) of *high grade* gliomas (HGGs) improves tumor resection. ALA is a precursor of protoporphyrin IX (PPIX) and accumulates in tumor tissue assisting surgery by its fluorescence. In an effort to investigate whether ALA-induced PPIX can also be a serum biomarker for HGGs, a method for its detection in blood was developed. It involved analyte purification by liquid-liquid-extraction and its detection using target-MRM with an ion trap mass spectrometer coupled to capillary liquid chromatography. Analysis was possible from both serum and anticoagulated whole blood, but only ¼ of the starting sample volume was needed in case of the latter (4 ml serum *vs.* 1 ml whole blood). However, handling was also more challenging for whole blood so that, ultimately, an optimized method for the analysis of serum is aimed for. It will also resolve the problem that PPIX is measured as the sum of PPIX and endogenous Zn-bound PPIX in whole blood, while the detection of the latter can be avoided in serum. Preliminary results indicate higher PPIX levels in whole blood of patients suffering from HGG, who received ALA prior to venipuncture, in comparison to healthy controls. A time-series of PPIX levels in whole blood of a HGG patient undergoing surgery agreed with the observed fluorescence changes in tissue. These promising results encourage further method optimization and investigations both in whole blood and serum, which provides the purer analyte, but much less of it.

Introduction

Protoporphyrin IX (PPIX) is a hydrophobic, biogenic molecule and the direct precursor of heme. Although all living cells are able to synthesize heme, 85% are produced in bone marrow and most of the rest in the liver. PPIX occurs ubiquitious in all living cells [1]. Intracellular heme synthesis takes places in mitochondria and surrounding cytoplasm. Heme is formed via an eight-step enzyme cascade from glycine and succinyl-coenzyme A (succinyl-CoA) (Figure 1) [1; 2].

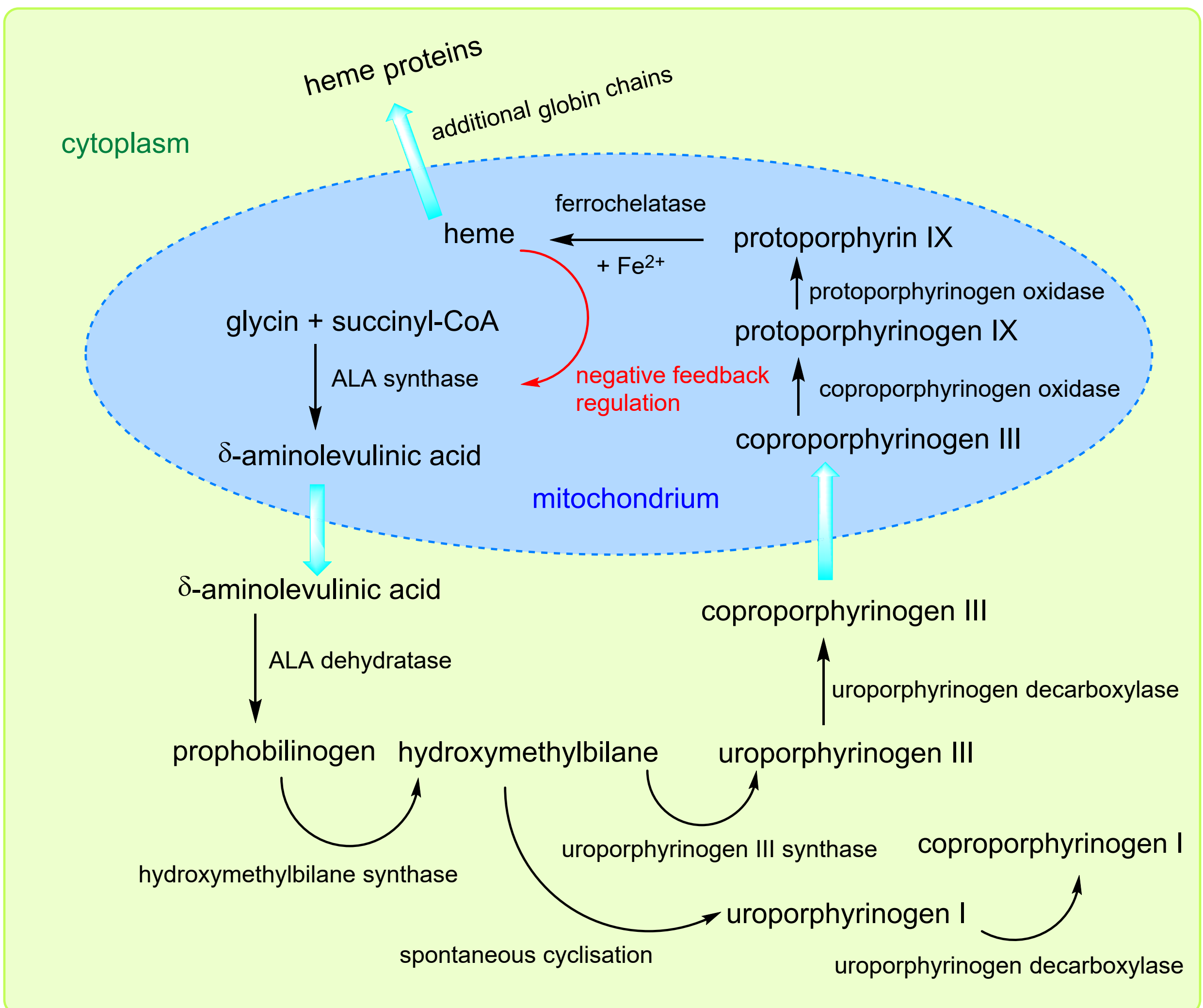

Figure 1: In heme biosynthesis multiple enzymes, cofactors and transporters are involved. Condensation of glycin and succinyl-CoA is the first and rate-limiting step to form PPIX and heme. It is restricted by negative feedback regulation through the end product heme [1; 2].

Besides the formation of δ-aminolevulinic acid (ALA), a second step is important to understand the formation and accumulation of PPIX within the cell. The transformation of PPIX to heme is catalyzed by ferrochelatase with Fe^{2+} as cofactor (Figure 2); this reaction represents a bottleneck in heme formation. Availability of Fe^{2+} and ferrochelatase activity are key factors for this reaction [1; 3].

Figure 2: Transformation of PPIX to heme, catalyzed by ferrochelatase. Fe^{2+} is inserted into a tetrapyrrole ring to form heme - a chelate [2].

Intracellular amounts of free porphyrins are low, because heme biosynthesis and degradation to bilirubin are well regulated. Otherwise, free porphyrins - as free heme - would damage the cell by the generation of reactive oxygen species [4; 5]. Due to its structure and conjugated π-system, PPIX absorbs light at ~400 nm - the specific absorption in the region of the Soret band is a common feature of all porphyrins. PPIX excitation results in two characteristic emission peaks at 635 and 704 nm [6]. In neurosurgery, these properties of PPIX are used in δ-aminolevulinic acid-induced fluorescence-guided resection (ALA-FGR) of high-grade glioma (HGG) providing improved tumor resection [3]. ALA is administered orally at a dose of 20 mg/kg body weight four hours prior anesthesia induction [7; 8]. From different in and ex vivo studies it is known that fluorescence in tissue is expected to peak 6 - 8 hours after administration [8–10].

ALA was first introduced in 1998 and since then has been extensively investigated. Nowadays it is approved for HGG resection in Europe, the USA and further countries [7; 8; 11]. After oral administration, ALA enters circulation. No active transport of ALA into normal brain tissue was registered and the diffusion from blood to normal brain is very low [3; 12]. In case of HGG, the blood-brain-barrier is disrupted allowing the diffusion of ALA into the tumor infiltration zone. The tumor selective mechanisms for cellular uptake of ALA and accumulation of PPIX are not fully understood yet. A dysregulation of different cellular transporters and enzymes of heme biosynthesis like GABA, pepT1 and pepT2 besides reduced activity of ferrochelatase and reduced availability of Fe^{2+} are suspected to contribute to ALA uptake and PPIX accumulation in malignant tissue via the heme synthesis pathway. The disruption of blood brain barrier was shown to be essential [3; 9].

During surgery, selective PPIX accumulation in HGG tissue is visualized by intraoperative fluorescence microscopy. Switching between normal white light illumination and violet-blue excitation light, the necrotic area of the main tumor is identified and the differentiation of infiltration zone and healthy brain tissue is enhanced. ALA-FGR enables more complete tumor resection with improved prognosis for patients [7]. However, malignant glioma grow in a diffuse and infiltrative manner and patients suffer from recurrent tumors [3]. It is therefore envisaged to diagnose tumor reoccurrence and growth based on PPIX levels in blood, which would be cost-effective at lower strain for the patient. Elevated PPIX levels in the plasma of bladder cancer patients in comparison to healthy adults after ALA administration have been detected by photodynamic screening before [13; 14]. We thus developed an analytical method to detect PPIX in blood.

In clinical laboratories, PPIX is not a standard parameter in blood analysis and the determination of porphyrins is limited to specialized laboratories [15]. PPIX is found in three different forms in whole blood (Figure 3) [16].

acidic dissociation

ZnPPIX

e.g. iron deficiency or lead poisioning

free PPIX

e.g. protoporphyria

heme

heme biosynthesis e.g. hemoglobin

Figure 3: Three different forms of PPIX in whole blood. In iron deficiency or lead poisioning Zn-bound PPIX (ZnPPIX) is elevated. Using acids while extracting porphyrins from blood, ZnPPIX dissociates into free PPIX and Zn^{2+}. Free PPIX is also found in traces in plasma. In whole blood PPIX is complexed with Fe^{2+} to form heme [16].

The determination of free erythrocyte porphyrin (FEP) gained importance in the 1970s when different groups monitored lead poisioning by elevated ZnPPIX levels in whole blood [17; 18]. The analytical method for the determination of the sum parameter consisting of ZnPPIX and free PPIX – the so called FEP test – is based on a two step liquid-liquid-extraction (LLE) with fluorophotometric detection (FLD) [17–19]. About 95% of extracted porphyrins from whole blood comprise PPIX, the rest is made up of plasma PPIX and coproporphyrin in plasma and erythrocytes [16]. Reported PPIX levels in whole blood, plasma and serum vary considerably (Table 1). The analytical determination of PPIX from blood has been developed since the 1970s. High performance liquid chromatography (HPLC) with FLD or coupling with mass spectrometry (MS) is used for chromatographic separation and detection of PPIX. When determining PPIX in complex matrices like blood, urine, faeces or tissue, MS is recommended for sensitive and specific detection. Absorption maxima and extinction coefficients of naturally occurring porphyrins overlap resulting in unresolved response when using FLD or UV/vis absorption for detection. MS offers more specific information like highly-resolved mass-to-charge ratios (*m*/*z*) and structural information through gas phase fragmentation [22]. In routine analysis, nevertheless, FLD is often used to overcome problems like increased matrix effects or signal suppression [23]. Chromatographic separation is typically performed on reversed phase C_{18} stationary phases in combination with gradient elution using methanol, acetonitrile, water and supplements like formic acid or ammonium acetate to adjust pH [22–24].

Table 1: Reported PPIX levels in different matrices.

Literature	δ-ALA administration	PPIX level/ pmol/mL	Matrix	Extraction
[14]	yes	0.2 - 2.8	plasma (healthy)	Acetic acid (HOAc, 50 %)/ dimethylformamide/ isopropanol
		41.8 - 69.1	plasma (bladder cancer)	
[15]	no	40 - 70[1]	erythrocytes	?[2]
[20]	yes	< LOQ up to 444[3]	plasma	HOAc (conc.)/ Ethyl acetate (EtOAc)
[21]	no	490	EDTA-whole blood (male)	HOAc (conc.)/EtOAc with reextraction in 1.5 M hydrochloric acid (HCl)
		563	EDTA-whole blood (female)	

Method Development

PPIX standard was obtained from Enzo life sciences. For multiple reaction monitoring (MRM) a method was devised on Synapt G2 Si ion mobility mass spectrometer with M-Class UPLC (Waters Corp., Manchester, UK). Reversed phase liquid chromatography (LC) was performed essentially isocratically (1-10 min 3-95% B, 10-30 min 95% B, 30-32 min 95-3% B, 32-55 min 3% B, flow rate 0.3 µl/min, aqueous solvent A (0.1% FA), organic solvent B (0.1% FA, ACN; 1.8 µm ACQ M-Class HSS T3 C18 75 µm x 200 mm column with a ACQ M-Class Sym 100 C18 5 µm, 100 Å, 20 mm trap column). Standard PPIX was used at 500 fmol/µl measuring an overview scan *m/z* 500-630 and an MS/MS function *(m/z* 563.3, 0.2 s, collision energy ramp 35-44 eV). The MS/MS spectrum of PPIX was dominated by the losses of the side chain moieties (Figure 4 and Table 2). The first 59 u loss was thus chosen for MRM.

Extraction from anticoagulated whole blood was performed according to a method published in 1984 for quantitative protein precipitation and the FEP test [18; 25]. Whole blood (1 mL), anticoagulated with ethylenediaminetetraacetic acid (EDTA), was supplemented with 3 µL of mesoporphyrin IX (MPIX, 15 pmol/µL in dimethylsulfoxide (DMSO)) as internal standard (ISTD). Following the addition of destilled water (3 mL), the tube was shaken for hemolysis on a horizontal shaker for 30 min. Then, a mixture of methanol / 1 M HCl (9:1, v/v, 20 mL) and chloroform (5 mL) was added. After short vortexing, a one-phase, to the eye homogenous, solution formed. With the addition of destilled water (15 mL) proteins were precipitated and three different phases could be separated by centrifugation (30 min, 4000 x g). The upper phase was discarded. The lower organic phase containing chloroform was collected. The protein interphase was extracted for a second time with the addition of water (4 mL). Finally, the chloroform phases were combined and

[1] Levels below LOQ of analytical method.

[2] No analytical method described.

[3] In most of the samples no PPIX was detectable, overall PPIX levels were low and erratic.

extracted twice by the addition of 1.5 M HCl (5 mL each) and centrifugation (30 min, 4000 x g). The upper aqueous phases were collected and evaporated to dryness using a speedvac system. The residue was reconstituted with DMSO (6 µL) and transferred into an amber vial with insert. Figure 5 illustrates the LLE procedure from anticoagulated whole blood.

PPIX analysis was performed by coupling capillary liquid chromatography to an ion trap mass spectrometer (Agilent HP1100, Esquire3000, Bruker; CapLC-IT; Zorbax XDB-C_{18} column (3.5 µm, 150 x 0,3 mm, endcapped); gradient elution (equilibration 50% B for 3 min, increase B up to 100% within 17 min, hold 100% B for 10 min, switch to 20% B within 1 min, hold 20% B until end of run)). The solvent system was build up of an organic phase (B) with high elution strength and an aqueous phase with low elution strength (A) (A: ACN/ water/ formic acid (5.0/ 94.9/ 0.1, v/v/v), B: ACN/ THF/ water/ formic acid (75.0/ 20.0/ 4.9/ 0.1, v/v/v/v); flow rate 0,125 mL/min splitted 1:25 resulting in 5 µL/min on column). The injection volume was 4 µL. The LC-system was controlled via ChemStation software (Agilent, LC 3D A.09.03). An MS^3 method was set up in the EsquireControl software (Bruker Daltonics, version 5.0; Table 3, Table 4). An external calibration curve with ISTD was recorded in the range of 2 - 13 pmol PPIX on column (Figure 6).

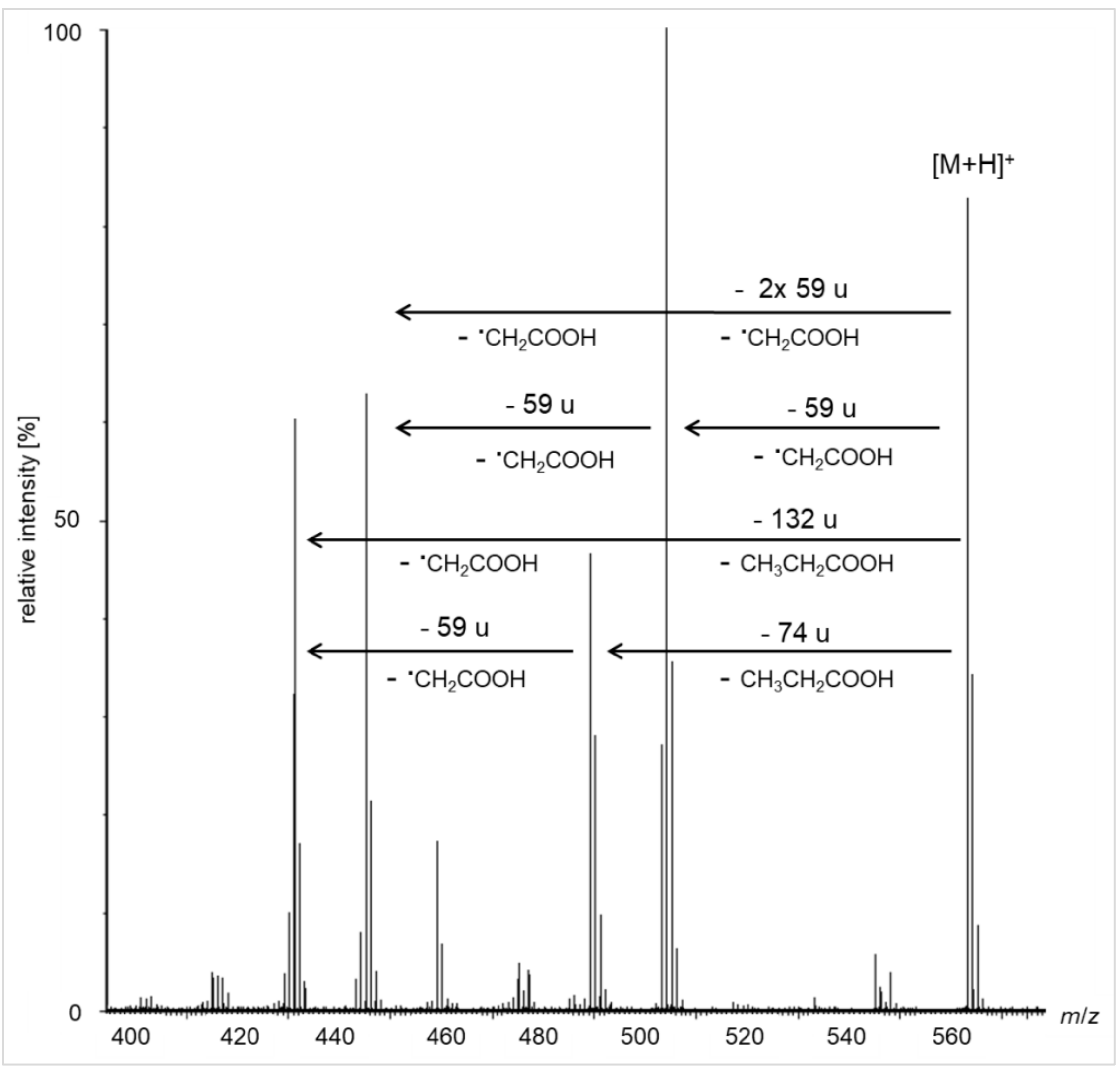

Figure 4: MS/MS spectrum of PPIX (Synapt G2Si).

Table 2: Overview of major PPIX fragments.

m/z	**Ion species**	**Proposed structure**
563.3	$[M + H]^+$	
504.3	$[M - {}^{\bullet}CH_2COOH + H]^+$	
489.3	$[M - CH_3CH_2COOH + H]^+$	
445.3	$[M - ({}^{\bullet}CH_2COOH)_2 + H]^+$	
431.3	$[M - {}^{\bullet}CH_2CH_2COOH - {}^{\bullet}CH_2COOH + H]^+$	

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Figure 5: Schematic survey of 2-fold LLE for extraction of PPIX from EDTA-whole blood. MPIX was deployed as ISTD and added to the sample before hemolysis.

Table 3: Ion trap parameters for positive electrospray PPIX analysis.

Capillary voltage/ kV	Nebulizer gas/ psi	Dry gas/ L/min	Temperature/ °C	Target mass
3.5	10.0	4.0	300	563

Table 4: Isolation and fragmentation ion trap parameters for MPIX (ISTD) and PPIX (analyte).

Experiment	Parameter	MPIX	PPIX
MS/MS	*m*/*z* target ion	567.3	563.3
	isolation range	± 2	± 2
	cut off *m/z*	156	155
	amplitude	0.9	0.85
MS^3	*m*/*z* target ion	508.3	504.3
	isolation range	± 2	± 2
	cut off *m/z*	139	138
	amplitude	0.7	0.7
	m/*z* scan	400 - 530	400 - 530

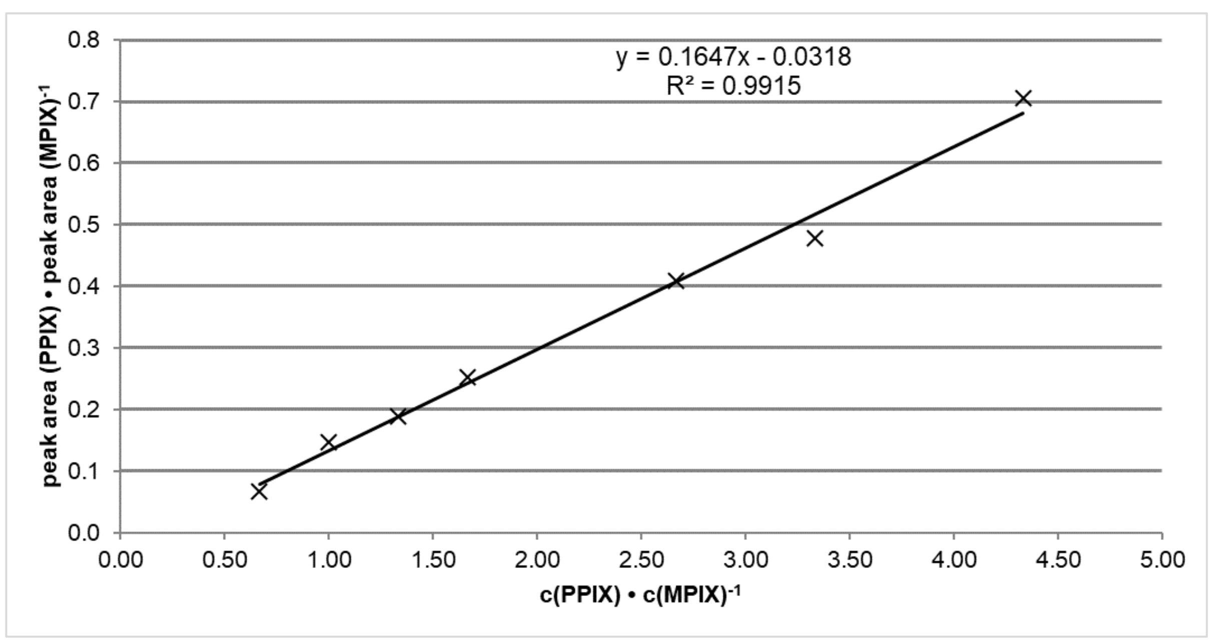

Figure 6: Calibration for extracted PPIX from blood via peak area with MPIX as ISTD (0.75 pmol/µL). Concentration range 0.50 - 3.25 pmol/µL.

Subsequently, blood samples from two different patients harboring glioblastoma multiforme (GBM) and one healthy adult were analyzed (ethic votum 2017-169-f-S). GBM patients received an oral dose of 20 mg ALA/kg body weight prior to surgery and venipuncture. The blood sample from the healthy volunteer was taken without ALA administration. The results indicated a higher PPIX level in blood of GBM patients after ALA administration in comparison to a healthy volunteer (Table 5). These values can hardly be related to literature data (normal values: 490 pmol/mL for males, 563 pmol/mL for females [21], Table 1) as both the analytical approaches and the results vary greatly. Our aim was rather the relative determination of PPIX under our predefined conditions. Although peak area from blood samples was partially outside the calibration range, the calculated PPIX levels suggested a 5- to 7-fold higher PPIX concentration in the blood of GBM patients.

Table 5: PPIX level in EDTA-whole blood (1 ml extract) from one healthy volunteer and two GBM patients, who received ALA prior to venipuncture.

	PPIX level / pmol/mL
Healthy volunteer	76
Patient 1	562
Patient 2	355

Furthermore, a time-series of the PPIX levels in EDTA-whole blood of a GBM patient was measured. To that end, 3 mL EDTA-whole blood per time point (4.5, 5.5, 6.3, 8.3, 12.3 h after oral ALA administration) were collected and 1 mL was used for extraction as described above. Representative data are shown in Figure 7. Both porphyrins were validated by their MS^3 spectra.

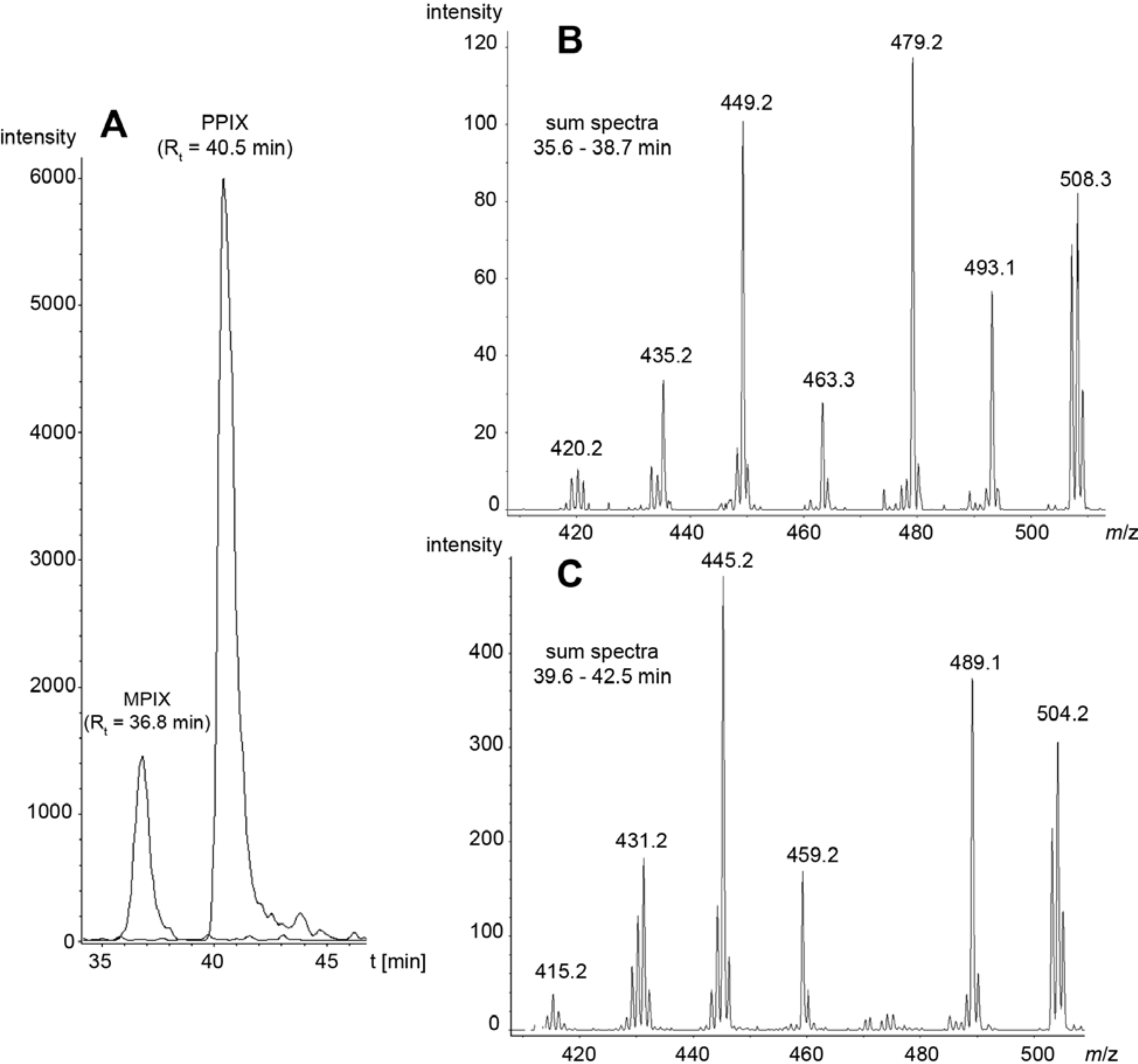

Figure 7: CapLC-IT analysis of PPIX extracted from EDTA-whole blood of a GBM patient 6.3 h after oral ALA administration. **A**: Extracted ion chromatogram of *m/z* 445.3 (PPIX, R_t 40.5 min) and 449.3 (ISTD MPIX, R_t 36.8 min). **B**: Summarized MS^3 spectra (*m/z* 567.3 –> 508.3) from 35.6 - 38.7 min for MPIX. **C**: Summarized MS^3 spectra (*m/z* 563.3 –> 504.3) from 39.6 - 42.5 min for PPIX.

PPIX levels from whole blood increased with time after ALA administration apparently levelling out at 8 h (Figure 8). More time points might describe the curve better, but also they mean more strain for the patient. Still, ambiguities such as the 5.5 h-outlier in the curve can only be clarified by more experiments. Nevertheless, our preliminary data demonstrate the potential of the approach. They agree with data obtained with an vivo rat model [9; 10] which showed maximum fluorescence in tumor tissue 6 h, and in plasma 8 h after oral ALA administration.

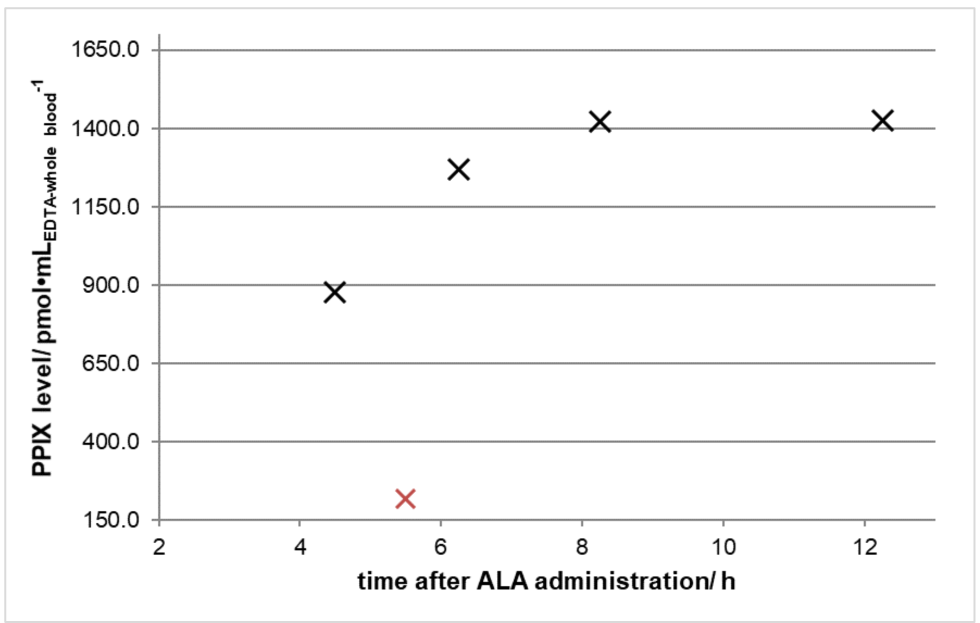

Figure 8: Time-dependent increase of PPIX in EDTA-whole blood of a GBM patient after ALA administration (1 mL whole blood).

Conclusion

An MS-based target method for the determination of PPIX in anticoagulated whole blood is presented. It detects native PPIX as a sum parameter of ZnPPIX and free PPIX after double LLE using MPIX as ISTD. The chromatographic separation is performed on endcapped C_{18} stationary phase with additional 20% THF in the mobile phase B – two essential parameters for improving peak shape and avoiding peak tailing. The method generated promising results for HGG patient samples. Still, further method development is necessary to thouroughly validate the method. LLE from whole blood is time- and solvent-consuming; phase separation is hard to perform reproducibly. Serum would be a better sample matrix to start with as it provides purer PPIX, but it contains much less of the analyte. Nevertheless, such optimization is underway with the aim of achieving a sample quality ready for nanoLC.

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