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Chapter

Protoporphyrin IX Analysis from Blood and Serum in the Context of Neurosurgery of Glioblastoma

Anna Walke, Eric Suero Molina, Walter Stummer and Simone König

Abstract

Protoporphyrin IX (PPIX) is formed from δ-aminolevulinic acid (ALA) during heme biosynthesis. Due to its cyclic tetrapyrrole core structure, it absorbs in the visible region of the electromagnetic spectrum and is thus colored. Both ALA and PPIX have become of great interest to neurosurgery, because in high-grade glioma, ALA diffuses into the tumor and is converted to PPIX. Fluorescence-guided resection (FGR) takes advantage of both the enrichment of PPIX in the tumor and its fluorescent properties, which enable visualization of tumor tissue. ALA-mediated FGR thus maximizes the extent of resection with better prognosis for patients. Tumor cells are able to produce porphyrins naturally or after administration of ALA, which is also reflected in elevated plasma fluorescence of cancer patients. PPIX might thus serve as a biomarker for monitoring of the tumor burden. A liquid chromatography-mass spectrometry (LC-MS)-based method is presented to quantify PPIX in blood and serum in the context of current fluorescence-based diagnostics. The method is able to distinguish between zinc PPIX, a component of red blood cells of importance in the detection of lead poisoning and iron deficiency anemia, and metal-free PPIX. In a proof-of-principle study, it was used to follow a time course of a glioblastoma patient undergoing surgery and confirmed elevated PPIX blood levels before ALA administration. During surgery, these blood levels increased about four-fold. The here developed 10 min reversed-phase LC-target MS method now allows patient screening with high specificity and throughput.

Keywords: protoporphyrin IX, zinc protoporphyrin IX, iron deficiency, lead poisoning, porphyria, biomarker, cancer, ion trap mass spectrometry, brain tumor, glioblastoma, blood

1. Introduction

Cyclic tetrapyrroles are biologically important molecules as they form the core structure of prosthetic groups such as porphyrins (e.g. heme), natural pigments like chlorophyll, and of vitamers (cobalamins, e.g. vitamin B12). Due to their large conjugated systems, they absorb in the visible region of the electromagnetic spectrum and are thus colored [1, 2].

Porphyrins occur naturally as metal complexes with the red pigment in blood cells, heme, being the best-known example. Hemes (**Figure 1**), the cofactors of

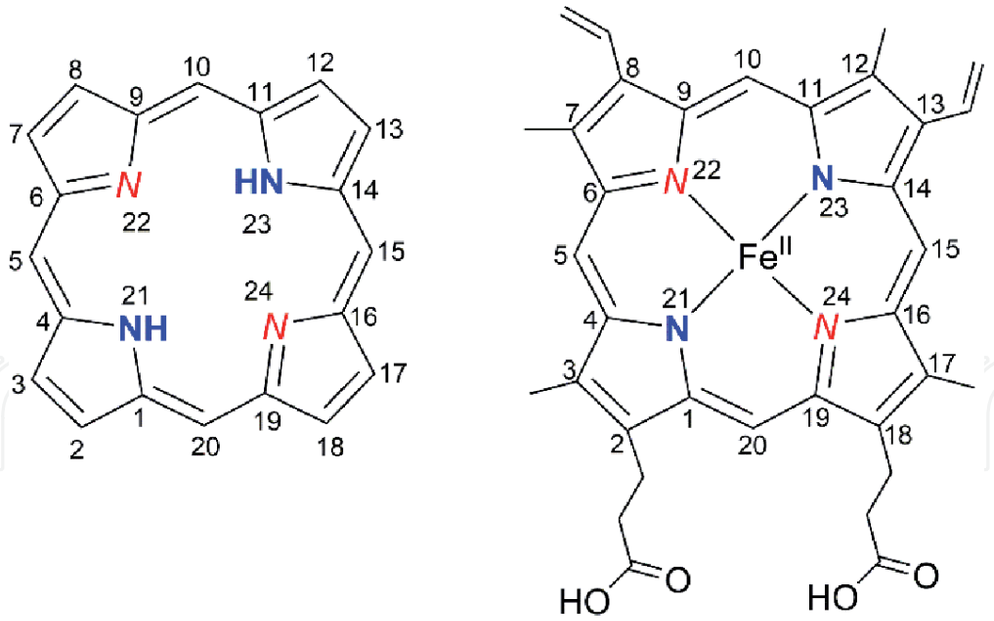

Figure 1.
Porphyrin structures. Porphin (left), the simplest representative, and heme b (right), the prosthetic group of, e.g., hemoglobin, myoglobin, catalase, and cytochromeP450. Other members of the heme group (e.g. heme c in cytochrome C) differ slightly in the side chains. Porphyrins chelate divalent ions such as iron in heme. Due to their delocalized system of π-electrons they fluoresce after excitation. Different nitrogen forms in the pyrrole ring are labelled (red/italic, blue/bold).

hemoproteins (e.g. hemoglobin, myoglobin) [3, 4], are complexed to iron and occur ubiquitously. They are critical to life, because hemoproteins are involved in the transport of diatomic gases (respiration), chemical catalysis and electron transfer [5].

During heme synthesis from glycine and succinyl-CoA (**Figure 2**) a number of intermediates including δ-aminolevulinic acid (ALA) are produced until, ultimately, protoporphyrin IX (PPIX) is converted to heme by insertion of a divalent iron (Fe (II), catalyzed by ferrochelatase) [1].

Both ALA and PPIX have become of great interest to neurosurgery, because in gliomas, ALA diffuses into the tumor and induces PPIX-synthesis [6]. A surgical method has been developed taking advantage of both the enrichment of PPIX in the tumor

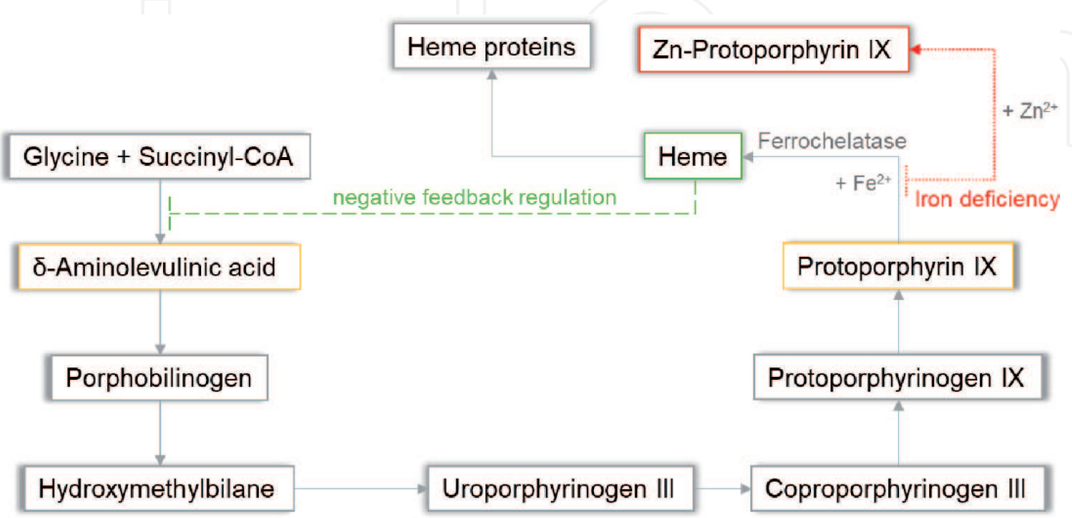

Figure 2.
Heme synthesis from succinyl-CoA to PPIX and ultimately heme. Synthesis of ALA is the rate-limiting step and under negative feedback control of heme (green/ dashed). A deficiency of iron supply limits heme synthesis and leads to PPIX accumulation. Zinc can then substitute for iron and ferrochelatase catalyzes the formation of zinc PPIX (red/dotted) [1].

and its fluorescent properties, which enable visualization of the tumor area (fluorescence-guided resection (FGR)) [7]. Alternating between normal white light illumination and violet-blue excitation light, the vital tumor tissue can be identified by its red/pink color during surgery; the distinction of infiltration zone and healthy brain tissue is improved. ALA-FGR thus maximizes the extent of tumor resection with improved prognosis for patients. In this context, the question arose whether or not PPIX blood levels could be used to monitor tumor regrowth, because the spectral characteristics of blood from cancer patients differ from those of control subjects [8–11].

The use of a simple analytic procedure would be cost-effective at lower strain for the patient. We have thus used liquid chromatography coupled to mass spectrometry (LC-MS) to quantify PPIX in blood and serum and describe it in the context of current practice in PPIX diagnostics below.

2. Protoporphyrin IX detection

2.1 Erythrocyte protoporphyrin analysis

Erythrocyte protoporphyrin (EP) served as a diagnostic marker for lead poisoning and environmental lead pollution as well as for iron deficiency anemia at the end of the 20th century [12]. From 1972 to 1991, it was officially recommended as the primary screening test for childhood lead poisoning by the Center for Disease Control and Prevention in the United States [13–15]. For the clinical diagnosis of porphyrias [16], rare disorders resulting from enzyme variability in heme biosynthesis, the porphyrin pattern is determined in blood, urine and faeces based on fluorescence techniques.

Taking advantage of the strong absorption of porphyrins in the Soret band (380– 430 nm) and their fluorescence, spectrophotometric and -fluorometric methods have been preferred for EP determination so far. The free erythrocyte porphyrin (FEP) test [17, 18], was, however, based on liquid-liquid extraction (LLE) at acidic pH, which dissociated zinc protoporphyrin (ZnPPIX) to metal-free PPIX during the extraction process. Thus, a sum parameter with different - and unknown - contributions of free PPIX and ZnPPIX was measured leading to false conclusions. The ratio of ZnPPIX to metal-free PPIX in erythrocytes varies, because in lead poisoning and iron deficiency anemia, ZnPPIX is accumulated in the blood, whereas in protoporphyria, the metal-free PPIX is elevated [12, 19, 20].

Hence, neutral ZnPPIX-specific LLE methods were developed, but they suffered from poor extraction efficiency [20, 21]. The widely applied ethyl acetate-acetic acid LLE method had three problems [17, 18]: First, the low extraction efficiency of PPIX from whole blood in comparison to the extraction from pre-diluted blood, which provided better precision of analysis; second, impurities in ethyl acetate influencing fluorescence and requiring pre-tests of reagent batches; third, the instability of PPIX standards prepared with deionised water. Thus, EP analysis required great attention to detail, because method modification, sample contamination or aging of standards and reagents had a great impact on analysis [22]. As a result, inter-laboratory comparison of EP results was generally poor while intra-laboratory precision was good [23].

2.2 Spectrophotometry and –fluorometry

In 1977, a hematofluorometer (HF) was designed for the detection of ZnPPIX in a drop of whole blood without sample pretreatment, which allowed immediate, simple and inexpensive detection [24]. Spectrophotometric and -fluorometric analysis became conventional analytical practice, but the inter-laboratory

agreement for EP levels was still poor and standardization has not been achieved [25]. PPIX levels measured with the FEP-test and HF-values of ZnPPIX did not match [13, 23]. Problems arose as a result of the limited PPIX stability towards light exposure, its tendency to form molecular aggregates in aqueous solution and buffer/solvent-dependent variation in absorbance [13]. Consequently, different values have been published for the molar absorptivity of PPIX, leading to discrepancies in the calculation of PPIX and ZnPPIX concentrations from measured absorbance using Beer's Law or fluorescence emission intensity [13]. In addition, interferences such as bilirubin, increased hemoglobin, riboflavin, quinine as well as several drugs including doxorubicin or amoxycillin disturbed HF measurement; results improved with extended washing of erythrocytes [13, 26].

The application of dual-wavelength excitation in the HF technique [24] allowed the recording of background fluorescence, which then could be removed from the analyte spectra [27]. In 2019, it was shown that even the non-invasive measurement of erythrocyte ZnPPIX in children and women after childbirth was possible on the wet vermillion of the lower lip using this principle [28, 29].

The fluorescence method is rapid and easy to use, but it is limited by high background fluorescence. Complex algorithms for the processing of the obtained spectra are required. As fluorescence is measured as a sum parameter of all contributing analytes, results can easily be overinterpreted, especially when measuring at the lower limit of detection. This has to be taken into account when free PPIX is the target instead of ZnPPIX or the sum parameter of both. ZnPPIX is the predominant species of EP in circulating erythrocytes while free PPIX is only present in trace amounts [20, 30, 31]; the specific quantification of metal-free PPIX next to ZnPPIX is thus challenging.

2.3 Liquid chromatography

The absorption in the Soret band is broad and the resolution of absorption spectra is low resulting in spectral overlap. Chromatographic separation of porphyrins is thus recommended [25]. It isolates the analyte from the sample matrix and concentrates it. Conclusively, the application of high-performance liquid chromatography (HPLC) coupled with fluorescence detection (FLD, UV-visible detection was not sensitive enough) [30, 32–37] and MS became highly popular. Initially performed thin layer chromatography assays were gradually replaced in the 1980s [36].

HPLC-FLD was demonstrated in the differentiation of porphyrin species in porphyria [33, 37] or lead poisoning [36]. It is now the technique of choice for porphyrin analysis in routine and research laboratories [28, 36]. Methods are time-consuming with typical elution times above 20 min [34, 35]. Moreover, the analysis of lipophilic PPIX remains challenging, because of its comparatively low recovery. Porphyrins respond quite differently in HPLC and attempts to measure them all in a single run have been abandoned. Porphyrins also have different excretion patterns in the body so that the majority of laboratories determines free PPIX and ZnPPIX in whole blood or plasma, and other porphyrins (uroporphyrin I and III, coproporphyrin I and III, **Figure 3**) in urine, using individual HPLC-FLD methods optimized for each purpose [28, 33–35, 37].

Advanced column media and higher pressure LC instrumentation (ultra high-performance (UHP) LC) further improved resolution and analysis time at lower solvent consumption although conventional HPLC was still widely applied. UHPLC from biological matrices like blood or tissue is not yet a routine technique in porphyrin analysis, because advanced sample preparation is required to avoid column contamination and clogging. Still, the use of UHPLC in conjunction with MS has great potential regarding sensitivity and speed of analysis [38].

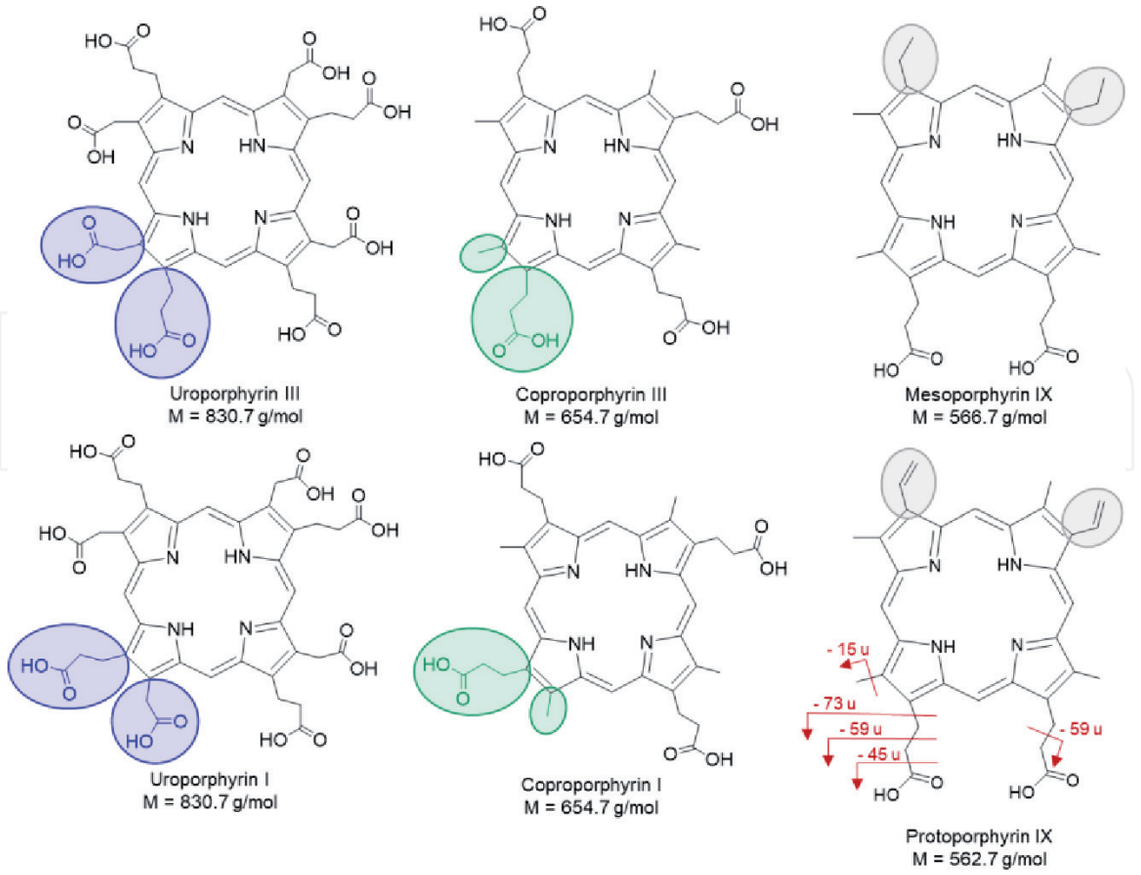

Figure 3.
Structures of type I and III isomers of uro- and coproporphyrin in comparison to PPIX and mesoporphyrin (MPIX). Differences of type I and III isomers are highlighted in blue (uroporphyrin) and green (coproporphyrin). Differences of MPIX and PPIX structures at positions 8 and 13 are shown in grey. Fragmentation of PPIX in MS analysis is given in red (in the ion trap, cleavage of ethylic side chains at positions 8 and 13 was not observed, for MS spectra see ***Figure 4****).*

2.4 Mass spectrometry

MS detection has a number of advantages over FLD [26, 27, 39]. Both high mass accuracy and the possibility of breaking the analyte molecules in the gas phase and measuring their fragments (MS/MS) contribute to unequivocal species identification, because complex samples can often not be fully chromatographically resolved. HPLC-MS was applied for the determination of porphyrin profiles in biological matrices [11, 39–43] using predominantly electrospray ionization (ESI) interfaces. Additionally, there were efforts to explore atmospheric pressure chemical ionization [42] for the purpose.

In urine, uroporphyrin, coproporphyrin and other porphyrins were measured [41, 42]. As urine is a matrix with comparatively low complexity compared to blood and tissue, minimal sample pretreatment (acidification) was sufficient allowing high-throughput analysis. Providing excellent sensitivity and specificity by operating in multiple reaction monitoring mode, the presented method was superior to FLD [41]. Another approach in urine used porphyrin esterification followed by extraction and single ion monitoring MS [42].

Regarding porphyrin detection in more complex matrices, Sullivan and coworkers [39] quantified twelve porphyrins including PPIX in liver extracts based on their mass, because most porphyrins co-eluted. MS/MS was not carried out. The method suffered from problems with peak-splitting and matrix suppression [39]. Furthermore, highly acidic extraction was performed so that this approach was not suitable for the individual determination of metal-free PPIX and ZnPPIX.

For the investigation of porphyrins in blood, predominantly plasma or red blood cells were used [11, 40, 43]. In plasma, MS was applied for the quantification of coproporphyrin isomers for monitoring of drug interactions [43], the elucidation of fluorescing compounds after detection of elevated fluorescence [11], and the qualitative analysis of porphyrin patterns facilitating the differential diagnosis of human porphyrias [40]. Despite all these efforts, no short and sensitive HPLC-MS/MS method for specific PPIX quantification from whole blood or serum was yet available although great data have been shown for less complex cell culture extracts [44, 45].

3. Protoporphyrin IX: a potential biomarker for cancer screening

More recently, besides the analysis of porphyrin metabolites [39] and profiles for toxicological and pharmacological applications [40–42], PPIX has been investigated as tumor marker for bladder, colorectal and kidney cancer [10, 11, 32]. Tumor cells are able to produce porphyrins naturally or after administration of ALA, which is also reflected in elevated plasma fluorescence of cancer patients. The spectral characteristics of blood from normal control subjects differ significantly from those of cancer patients in renal cell carcinoma, prostate cancer and colorectal adenocarcinoma [8–11].

PPIX analysis is, however, not straightforward in a clinical setting. Factors such as unrelated diseases and medication may influence the measured porphyrin concentration [8]. Lualdi and co-workers [11], e.g., confirmed their findings of enhanced plasma fluorescence in colorectal adenocarcinoma patients by HPLC coupled to high-resolution MS and detected mainly PPIX and coproporphyrin I. Ota et al. [32] applied HPLC-FLD for the determination of PPIX in plasma of bladder cancer patients after ALA administration. The patients showed significantly higher plasma PPIX concentrations compared to healthy adults. It was extrapolated that the accumulation of PPIX in cancer cells is common to almost all types of cancer [8–10] and that the specific measurement of PPIX is advantageous for cancer screening [32].

A further application of PPIX is above-mentioned photodynamic diagnosis, where PPIX is applied as an intraoperative marker especially for brain tumors. Using ALA-induced PPIX-fluorescence in tissue during surgery of high-grade glioma, the resection is more complete and the patients have a higher 6-month progression-free survival compared to those without FGR [7]. Unfortunately, due to the infiltrative growth of these tumors, complete tumor resection is still impossible and tumors can recur. Clinically, diagnosis of high-grade glioma and glioblastoma multiforme (GBM) as well as their recurrence requires multidisciplinary strategies such as contrast enhancement magnetic resonance imaging, computer tomography and biopsy [6, 7, 46, 47]. Therefore, a sensitive and cost-effective method for tumor monitoring is highly desirable supporting early diagnosis and treatment of GBM as well as better prognosis for patients. So far, the survival prognosis for GBM patients is one of the lowest in modern day oncology [47]. As PPIX is an approved marker for GBM tissue in ALA-FGR, here, the hypothesis was tested if it could also be a blood biomarker for GBM screening and diagnosis.

4. PPIX quantification

For the detection of PPIX in whole blood or serum, we developed an HPLC-MS method using an HP1100 HPLC (Agilent, Waldbronn, Germany) coupled to an Esquire 3000 ion trap mass spectrometer (Bruker Corp., Bremen, Germany).

Mesoporphyrin (MPIX) (**Figure 3**) was chosen as internal standard (IS), because it provided high structural similarity to PPIX and isotope labeled standards for PPIX were not available. Distinction of PPIX from ZnPPIX was possible during sample preparation. The method described below allowed the quantification of metal-free PPIX in whole blood, the determination of endogenous PPIX in serum and the measurement of endogenous ZnPPIX in whole blood (200 μl, respectively).

4.1 Sample preparation

PPIX LLE extraction from serum and whole blood was achieved with only water and acetonitrile (ACN). Hemolysis with water was crucial for good recovery as observed by others working with pre-dilution [22]. It was followed by protein precipitation with ACN; concomitant porphyrins were extracted into the supernatant [48], which was further purified using anionic-exchange solid phase extraction (SPE) cartridges. The extracts of whole blood and serum had a pH 8–9 so that PPIX, ZnPPIX and MPIX had deprotonated propionic acid side chains and were negatively charged. No conversion of ZnPPIX into metal-free PPIX was observed before loading the extracts onto the SPE cartridge. All three porphyrins were retained on the cartridge presenting quaternary ammonium groups. MPIX and PPIX were eluted using ACN containing 2% formic acid (FA), ZnPPIX with increased FA content (20%). No elution or hydrolysis of ZnPPIX was detected at 2% FA; only metal-free PPIX was seen in the first eluate. The higher percentage of FA in the second step caused the acidic release of the Zn^{2+} ion. ZnPPIX was thus detected as metal-free PPIX.

4.2 Gas phase fragmentation

As already demonstrated in the literature for other porphyrins [40], PPIX ionizes as singly charged $[M+H]^+$ species (*m*/z 563.3) in ESI-MS. MS/MS fragmentation preferentially occurs on the side chains (**Figure 3**). The spectra in **Figure 4** illustrate the stepwise fragmentation of PPIX from preselected precursor ions in subsequent MS/MS and MS/MS/MS experiments in the ion trap. The abundant loss of an ethanoic acid substituent (-CH_2COOH; 563-59 u) results in a fragment ion at *m*/*z* 504.3 that is used as precursor for MS^3 fragmentation which then generates further side chain losses (-CH_3, 15/30 u; -COOH, 45 u; -CH_2COOH, 59 u; -CH_2CH_2COOH, 73 u).

For selected compounds, it was discussed that even-electron ions generated in the ESI source can produce radical cations with odd-electrons by hemolytic cleavage. The most common process in radical fragmentation is the elimination of a methyl group as proposed for flavonoids, antraquinones and terpenoids [49]. It was shown that the radical elimination of the methyl group is a low energy process in flavonoids. The loss of 59 u by radical cleavage was also already described for FePPIX in previous studies on metalloporphyrins and other compounds with extended π-electron systems [50–52].

The fragmentation of MPIX is similar to PPIX. MPIX has two saturated ethyl side chains at positions 8 and 13 (**Figure 3**) so that precursor and fragment ions differ by four mass units in comparison to PPIX.

ZnPPIX was measured in MS/MS mode as it showed lower ionization efficiency than MPIX and PPIX. The time-segmented method switched from MS/MS mode for ZnPPIX detection to MS^3 mode for MPIX and PPIX measurement. This approach proved advantageous in comparison to continuous MS/MS and MS^3 switching for porphyrins and significantly increased sensitivity. In comparison to the ion traces of matrix-free porphyrin standard solution, the background signal was about five times higher in whole blood extracts, but that did not hamper detection with the specific MS^3 method.

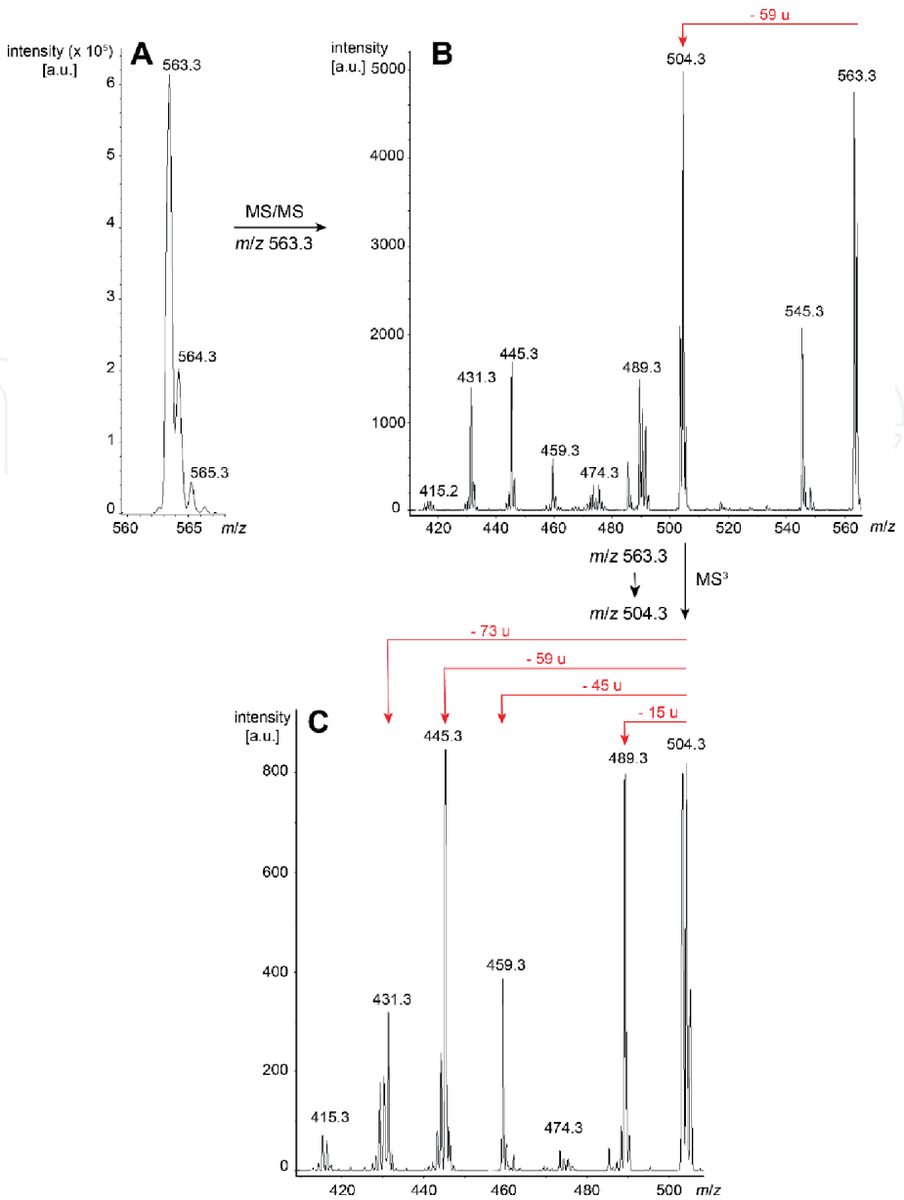

Figure 4.
*Fragmentation of PPIX in the ion trap. A: $[M+H]^+$ ion of PPIX at m/z 563.3 in full scan mode. B: MS/MS spectrum of precursor ion at m/z 563.3. The loss of the ethanoic acid side chain (59 u) is dominant. C: MS^3 spectrum for two-step fragmentation (m/z 563.3 -> m/z 504.3). The major ions result from side chain cleavage (red arrows, for structure see **Figure 3**).*

4.3 Chromatography

HPLC separation of PPIX was not straightforward, because of its high lipophilic nature. Problems included low resolution on capillary C_8 LC and high carry-over on endcapped C_{18} phase. The low flow rate of capillary LC (5 µl/min) was also disadvantageous for porphyrin separation. Analytical LC with its higher flow rate (300 µl/min) in conjunction with a semi-porous C_{18} column media and an almost isocratic gradient allowed much more efficient operation at great resolution. **Figure 5** shows the separation of a standard solution of ZnPPIX (10 pmol on column), MPIX and PPIX (5 pmol on column each).

4.4 Procedure

All experiments were performed in accordance with the declaration of Helsinki and by approval of the Ethics Committee of the Ärztekammer Westfalen-Lippe (2017-169-f-S).

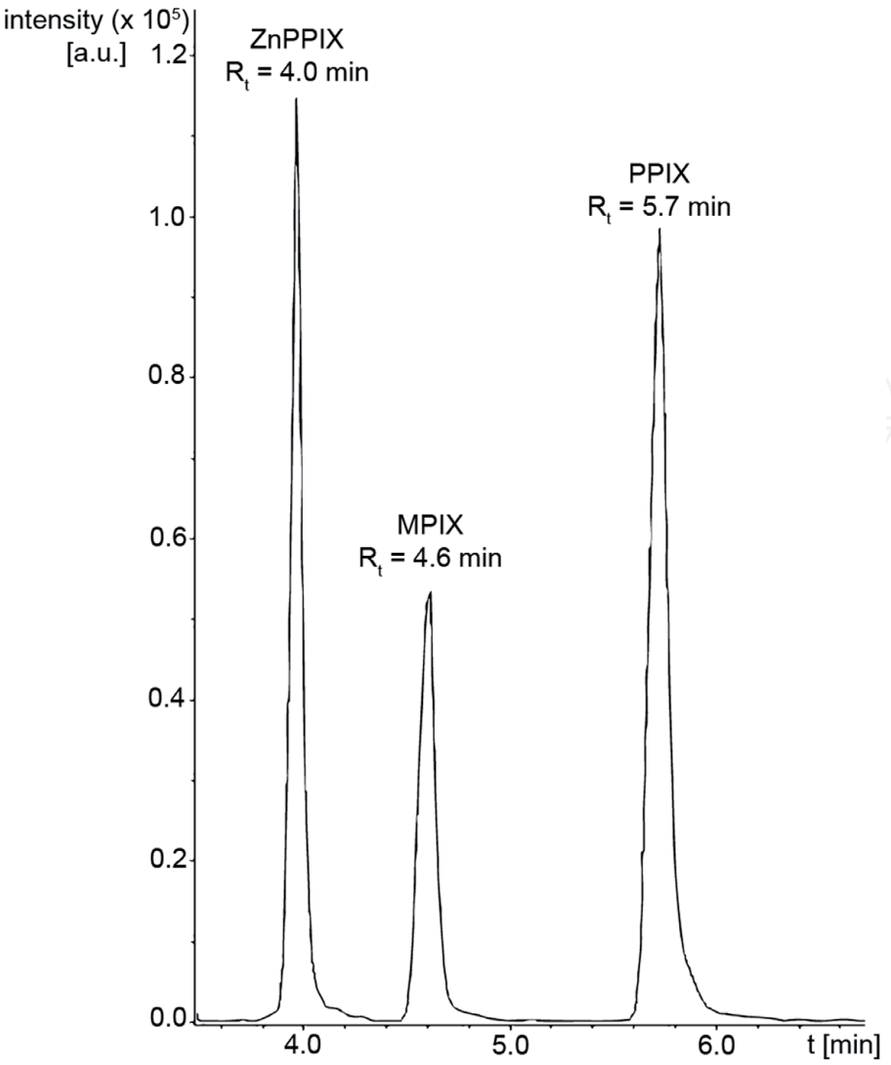

Figure 5.
Extracted ion chromatogram (EIC) of porphyrin standard (ZnPPIX m/z 565.2; MPIX m/z 449.3; PPIX m/z 445.3) as detected with a time-segmented method on the Esquire 3000 ion trap (for parameters see 4.4).

PPIX was obtained from Enzo life sciences GmbH (Lörrach, Germany), MPIX and ZnPPIX from Merck KGaA (Darmstadt, Germany). ACN, water, FA and methanol (MeOH) were all LC-MS grade and purchased from Merck KGaA as were ammonium hydroxide solution and dimethyl sulfoxide (DMSO). SPE cartridges came from Restek GmbH (Bad Homburg, Germany).

For drying of samples, a SpeedVac system (Savant SPD 111V SpeedVac concentrator with vapor trap Savant RVT 5105) was used (Thermo Fisher Scientific, Schwerte, Germany). For centrifugation, a Universal 320R Hettich centrifuge (Tuttlingen, Germany) was utilized.

Hemolysis of 200 µl whole blood or serum was performed by adding 800 µl of water and shaking at room temperature for 1 h. Protein precipitation and porphyrin extraction was achieved by adding 2 ml of ACN, shaking at room temperature for 1 h and centrifuging at 14.000 rcf at 20 °C for 30 min. The clear supernatant was then transferred to an SPE cartridge, which was treated as described in **Table 1**. The 1st and 2nd eluates were dried and finally reconstituted in 15 µl DMSO for HPLC-MS (for parameters see **Tables 2** and **3**).

Raw data were converted using the msConvert toolkit from ProteoWizard software (version 3; [53]). MPIX and PPIX were quantified using the total areas for the three most abundant fragment ions in MS^3 mode (MPIX: extracted ion chromatogram (EIC) *m/z* 449.3, 479.3, 493.3; PPIX: *m/z* 445.3, 459.3, 489.3) and Skyline software (version 20.1; [54]).

Step	Solvent
Cartridge conditioning	2 ml ACN
Equilibration	2 ml water
Sample load	porphyrin extract (~3 ml)
1st Wash	2 ml 5% ammonium hydroxide solution
2nd Wash	2 ml MeOH
1st Elution	2 ml ACN with 2% FA
2nd Elution	2 ml ACN with 20% FA

Table 1.
Protocol for the purification of neutral porphyrin extract with SPE cartridges.

LC-Parameter		
Column	Poroshell C_{18} (2.7 µm, 2.1 mm i.d.) with guard column	
Flow	0.3 ml/min	
Solvent A	95% water/4.9% ACN/0.1% FA	
Solvent B	95% ACN/4.9% water/0.1% FA	
Gradient	t [min]	solvent B [%]
	0.0	70
	1.0	100
	5.0	100
	5.5	70
	10.0	70
Injection volume	10 µl	
MS-Parameter	ESI (+)	
Capillary	−4.5 kV	
End plate	−500 V	
Nebulizer	30.0 psi	
Dry gas	9 l/min	
Dry temperature	320°C	
Scan range	400–700 *m/z*	

Table 2.
HPLC-MS parameter for porphyrin detection.

Analyte	Time [min]	Mode	Isolation *m/z*	Fragmentation amplitude
ZnPPIX	3.4–4.3	MS/MS	625.2 ± 5.0	0.43
MPIX	4.3–5.3	MS/MS	567.3 ± 2.0	0.90
		MS^3	508.3 ± 2.0	0.70
PPIX	5.3–7.0	MS/MS	563.3 ± 2.0	0.85
		MS^3	504.3 ± 2.0	0.70

Table 3.
Parameters for MS/MS detection of ZnPPIX and MS^3 detection of PPIX and MPIX.

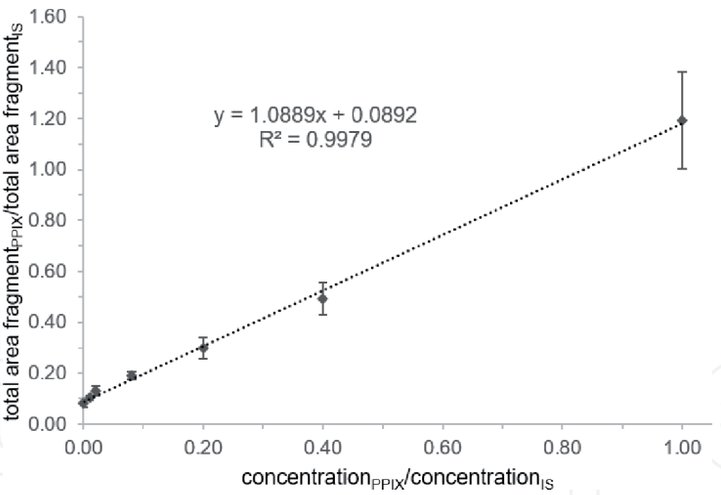

Figure 6.
Calibration curve for PPIX spiked into the whole blood of a healthy volunteer. MPIX (IS) was spiked at 500 fmol/μl. Five replicate injections (10 μl each) were run.

Calibration was performed in the relevant matrix (whole blood of a healthy volunteer) using 500 fmol to 50 pmol PPIX spikes and 500 fmol/μl MPIX (**Figure 6**). The contribution of native PPIX was determined in an aliquot spiked with MPIX only.

5. Measurement of clinical samples

Samples from a patient harboring a GBM and undergoing surgery were collected at different time points before, during and after surgery. The first sample was obtained 1 h prior to ALA administration for the determination of the basic free PPIX level, the following samples were collected 5 and 7 h after ALA administration. Whole blood samples were refrigerated and stored in the dark. Porphyrins were extracted in triplicate using 200 μl of whole blood for each experiment.

Figure 7 shows the results. The level prior to ALA administration was measured twice (two different extractions from the same blood sample) and was 33.9 ±0.4 and 34.9 ± 1.9 pmol PPIX/ml whole blood. After ALA administration, the PPIX level rose about four-fold to 100.9 ± 2.7 and 114.4 ± 11.4 pmol PPIX/ml, respectively, at

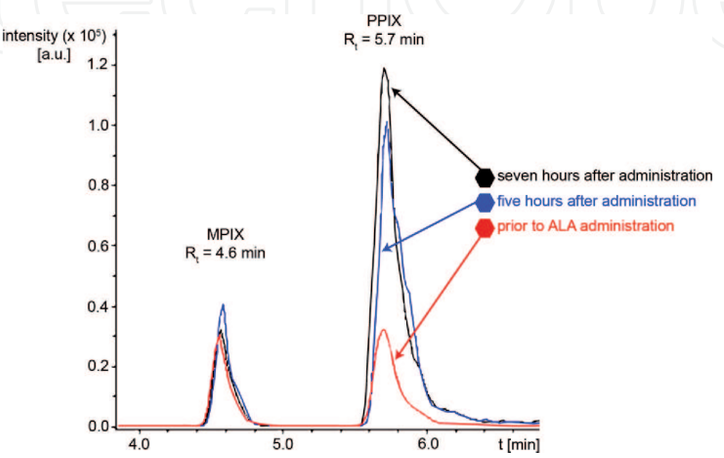

Figure 7.
Overlay of three EIC traces (MPIX m/z 449.3; PPIX m/z 445.3) from measurement of whole blood from a GBM patient taken at different time points. The red line shows a sample prior to ALA administration, whereas the blue and black lines mark the samples 5 and 7 h after ALA administration.

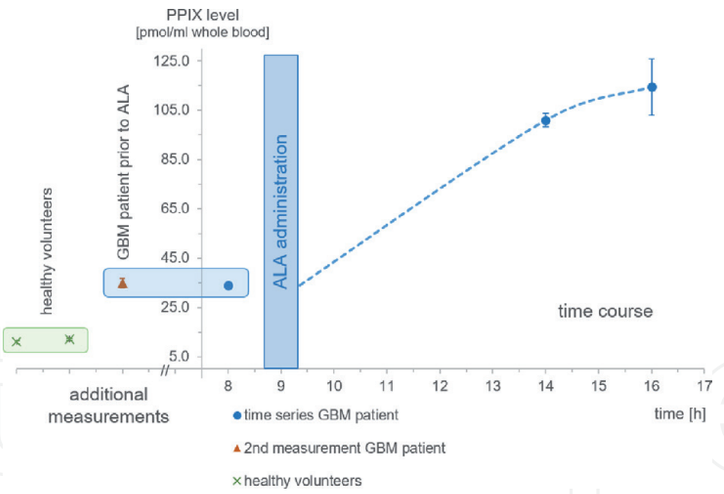

Figure 8.
Time series of a GBM patient with ALA administration at 9 am, illustrated by blue bar and blue points. The pre-ALA PPIX level was confirmed in the same blood sample (orange triangle). PPIX levels in blood of healthy volunteers were determined for comparison (green crosses).

the later time points. For comparison, PPIX levels were determined in two healthy volunteers without ALA administration. They reached only about a third of the pre-surgery level of the patient (11.0 ± 0.7 and 12.1 ± 0.4 pmol PPIX/ml). These observations are summarized in **Figure 8**. So far, having tested one patient only, the results support the hypotheses of elevated PPIX in the circulation as discussed in the literature for other types of cancer [8–11, 32] and the timely and dramatic increase after ALA administration. An extended study involving more probands is planned.

6. Conclusion

MS has the huge advantage over fluorescence-based porphyrin detection that it can pinpoint the individual molecules by their mass and, adding to the specificity, by their fragmentation pattern, which can be generated in the mass spectrometer. Ambiguities as known from fluorescence spectroscopy due to varying or overlapping absorbance maxima and extinction coefficients or fluorescing matrix interferences do not occur; background corrections with complex spectral fitting algorithms are not necessary.

A LC-MS method for the quantification of metal-free PPIX in whole blood was developed. It is short (10 min) and robust (analytical LC, ion trap MS) and provides the necessary specificity and sensitivity. ZnPPIX, MPIX and PPIX can be baseline resolved without the carryover problems observed earlier. The LLE sample preparation provides high extract purity with good recovery. Importantly, ZnPPIX and PPIX can be properly distinguished during SPE clean-up. Matrix effects which would negatively affect HPLC-MS analysis were not observed.

The method is applicable to serum in the same manner, however, serum and plasma PPIX levels are much lower than those of whole blood. Unfortunately, the values reported in the literature lack confidence (**Table 4**). There is no reference range for PPIX in serum or whole blood for healthy individuals. PPIX plasma concentrations in healthy subjects after ALA administration were low and erratic, ranging from below the limit of quantification to hundreds of nmol/l [32, 55, 56]. Often, PPIX was not detected in plasma samples at all [55]. In serum, PPIX levels are lower still, even after ALA administration.

Our results indicate the same extract purity for spiked PPIX extracted from serum as from whole blood. The recovery of PPIX was even slightly better.

	PPIX in plasma (HPLC-FLD) [nmol/l]	Reference
Protoporphyria	2-15	[56]
Healthy adults after ALA administration	0.2-2.8	[32]
	17.8–444.3	[55]

Table 4.
Reported PPIX levels in plasma, measured with HPLC-FLD.

Nevertheless, quantification requires more effort, because only trace amounts of endogenous PPIX were detected in sera of healthy adults so far.

In proof-of-principle experiments the LC-MS method was applied to blood samples from a GBM patient, which confirmed both the elevated PPIX levels in the blood of GBM patients and the increase following ALA administration. The method is now ready for patient screening. Additional resolution and sensitivity for high throughput analysis could be achieved with instrumentation such as a triple quadrupole mass spectrometer, if desired.

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Conflict of interest

The authors declare no conflict of interest.

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