Protoporphyrin IX purification from blood and serum for mass analysis – Considerations with respect to neurosurgery

Protoporphyrin (PPIX) and its precursor 5-aminolevulinic acid (ALA) are routinely used in fluorescence-guided resection (FGR) in neurosurgery of glioblastoma. Orally administered prior to surgery, ALA diffuses preferentially into the tumour-bearing brain region and is there transformed into PPIX as part of the heme biosynthesis. During the resection of high-grade gliomas (HGG), the fluorescent properties of PPIX enable the differentiation of healthy and malignant brain tissue – resulting in a more complete tumour resection and, ultimately, a better prognosis for patients.1,2

Despite the use of ALA-FGR, complete tumour removal is impossible – due to the infiltrative growth of HGG, e.g., glioblastoma multiforme (GBM), tumours often reoccur. Clinical diagnosis of HGG/GBM is furthermore not straightforward and requires expensive imaging technology as well as tissue biopsies. So far, the prognosis for GBM patients remains one of the lowest in modern day oncology.1,3,4

We thus investigated PPIX as a potential blood biomarker for the diagnosis of primary and recurrent GBM using liquid chromatography/mass spectrometry (LC/MS).5 Some cancer research studies to that effect using plasma have already been published.6-8 It was also demonstrated that extracellular vesicles originating from glioma cells contained PPIX,9 but none of these investigations used mass spectrometry (MS) as detector.

Whole blood contains the most PPIX, but it is not the best matrix for such studies, because of unspecific contributions from erythrocyte ZnPPIX. For serum or plasma, though, the published PPIX levels, obtained with traditional methods, are low, often below the limit of detection, and not reliable.10,11 Taking advantage of the high sensitivity of MS, we have been aiming for a reproducible LC/MS workflow for the use of serum and tested our protocol in comparison to whole blood.5 Thereby, the reliable purification, respective recovery, of PPIX from the biological matrix proved particularly difficult and we have therefore evaluated different preparation methods. Initial liquid–liquid extraction (LLE) with water and acetonitrile (ACN) was crucial for best sensitivity.5,12 However, an additional purification step was required to minimize LC-column aging and clogging.

We first tried to separate the small molecular weight substances from macromolecules using syringe and centrifugal filters. It soon turned out, however, that some of the membrane material we tested – polytetrafluoroethylene, polyvinylidene fluoride, modified polyether sulfone, nylon and cellulose – in particular nylon (polyamide 6.6) and cellulose – quantitatively adsorbed PPIX. This observation led to experiments using punched-out membrane pieces for the specific extraction of PPIX directly from the biological matrix, because PPIX could subsequently be easily removed from the membrane using dimethyl sulfoxide (DMSO). The results from crude preliminary experiments were indeed promising considering a recovery of about 60–70% for PPIX from a reference extract, but handling was difficult, and reproducibility was hampered by the need to produce and process small to tiny pieces of nylon. The ratio between the amount of porphyrin and the nylon surface area was crucial as well as the pH during sample loading and the elution volume. Moreover, the extraction of mesoporphyrin (MPIX), our internal standard, from spiked serum was not as efficient as that of PPIX when working with serum instead of standard solution (Figure 1). At that point we switched to anionic-exchange solid-phase extraction (ae-SPE), but we did feel that the potential of nylon with respect to PPIX isolation should be kept in mind.

Ultimately, ae-SPE worked best for our purpose, but, interestingly, not all sorbent material performed with the same quality. We tested three products with different types of ae-sorbent.

All cartridge bed materials consisted of quaternary amine functionalized polymers with strong ae-properties; they differed only in the polymer and the particle size (Table 1). We evaluated a reference solution mimicking the LLE extract and spiked whole blood as well as native and spiked serum. The recovery on styrene (Sty)-based cartridges was the lowest for all matrices (Figure 2) when using a typical SPE workflow including bed preparation, ion exchange, analyte washing and elution (Figure 3) and thus the determination of native PPIX in serum was not attempted with this material. The other two cartridge types performed similarly with recoveries above 80% for spiked PPIX and MPIX (Figures 2A and 2B).

Fully water-wettable polymeric-based cartridges possess the advantage, compared with silica and partially water-wettable material, that an aqueous sample can be loaded directly without performing sorbent conditioning and equilibration steps (Figure 3)13 and for the hydrophilic–lipophili balanced (HLB) copolymer this has been suggested by the manufacturer. Furthermore, polymer-based cartridges can be allowed to run dry – as easily happens during vacuum-based handling – without any negative impact on...
performance. This simplifies ae-SPE and saves solvent and time. Comparing this shorter workflow for all cartridge types (Figures 2C and 2D), again the HLB copolymer and the mixed-mode (MM) ae-sorbent outperformed the Sty-based cartridge type, with the former fairing slightly better than the latter. No loss in recovery or reproducibility and no differences in extract purity were noticed for all of them. The determination of native PPIX from serum was possible with all three cartridge types. The averaged relative standard deviation (RSD) for triplicate LC/MS runs of MPIX and PPIX was 8 and 5%, respectively. The averaged RSD of ae-SPE (n = 2) of MPIX ranged from 3% on HLB to 6% on MMae cartridges and for PPIX it was 6% for all products. In an extension to the work of Zhang and co-workers, our results demonstrated that not only the HLB-based sorbent was amenable to the shortened SPE protocol.

Another decisive factor was the ease of use when working with a vacuum station. It was not recommended to exceed a flow rate of about one drop per second in order to allow sufficient interaction between sorbent and analyte, because, in comparison with normal- or reversed-phase interactions, the kinetic exchange process in the ion-exchange process is slower. While HLB and Sty-based cartridges were easily run with the recommended flow rate, adjusting the flow for MMae cartridges was laborious and time-consuming for no obvious reason.

Importantly, ZnPPIX and PPIX could be properly distinguished during ae-SPE, because MPIX and PPIX eluted at 2% formic acid (FA) and ZnPPIX did not up to a concentration of 20% FA. Considering all parameters, we have thus voted for the use of the HLB material for our study. For the extraction of low-abundance endogenous PPIX from serum (500 μL), containing about 2–3 pmol PPIX/mL in healthy volunteers, and whole blood this sorbent allowed the shortest possible workflow as well as easy handling and it outperformed – even if only marginally in one case – the competing products. We will extend the use of our procedure to tumour tissue. Although not demonstrated here, we expect it to perform equally well when purifying PPIX from LLE extracts of tissue homogenates.

Experimental: Adsorption experiments were performed using round disks made of nylon membrane (Roth, Karlsruhe, Germany) with a surface area of 20 mm² that were manually punched-out with a metal hollow punch. The disks were washed using ACN/water
(70:30, v/v) and subsequently transferred into either a standard solution (ACN/water, 70:30, v/v) or serum extract each spiked with MPIX and PPIX (90 pmol each). Loading of porphyrins was performed on a horizontal shaker for 1 h at room temperature. As the pH turned out to be crucial it was adjusted to pH 6 using FA (98–100%, Merck, Darmstadt, Germany) and sodium hydroxide solution (1 M, Merck). For elution of porphyrins the nylon disks were transferred into DMSO (Merck) and shaken for 1 h. A ratio of about 10 μL DMSO to 1 mm² nylon membrane worked best. The extracts were measured in triplicate using our previously developed multiple reaction monitoring (MRM) LC/MS method. SPE was performed using Resprep MAX (Restek, Bad Homburg, Germany), Oasis MAX (Waters, Eschborn, Germany) and Supel Select Sax (Merck) cartridges; their properties are listed in Table 1. For details on blood and serum extraction refer to our earlier publication. PPIX purification was carried out either from reference solution (PPIX and MPIX, 50 pmol each, in ACN/water 70:30, v/v; adjusted to pH 8 with aqueous 28–30% ammonia solution) or LLE extracts from whole blood and serum spiked with PPIX and MPIX (50 pmol each) for ae-SPE. Following equilibration of the cartridges with ACN and water the sample was loaded, washed with 5% ammonium hydroxide, methanol and 2% FA in ACN (Figure 3). The eluates were dried using a SpeedVac concentrator (Savant SPD 111 V; Thermo Fisher Scientific, Schwerte, Germany) and reconstituted in 100 μL DMSO for recovery tests and 35 μL DMSO for native serum investigation prior to triplicate LC/MS runs. Sample purification was performed twice per cartridge type. Subsequent experiments omitted sorbent conditioning and equilibration.

**Figure 2** Recovery rates for MPIX and PPIX purified using MMae, HLB and Sty-based cartridges with the protocol shown in Figure 3 (A,C). Bars show averaged recovery (blue/dotted for MPIX; red/dashed for PPIX), error bars indicate the deviation in two separate purifications and triplicate LC/MS measurements. For whole blood and serum there was a contribution of native PPIX beside spiked PPIX resulting in recovery rates greater than 100%. Peak area for native PPIX extracted from 500 μL serum (B,D).
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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the article and upon request from the authors.

REFERENCES

1. Stepp H, Stummer W. 5-ALA in the management of malignant glioma. Lasers Surg Med. 2018;50(5):399-419. https://doi.org/10.1002/lsm.22933

2. Stummer W, Pichlmeier U, Meinel T, Wiestler OD, Zanella F, Reulen H-J. Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: A randomised controlled multicentre phase III trial. Lancet Oncol. 2006;7(5):392-401. https://doi.org/10.1016/S1470-2045(06)70665-9

3. Batash R, Asna N, Schaffer P, Francis N, Schaffer M. Glioblastoma multiforme, diagnosis and treatment; recent literature review. Curr Med Chem. 2017;24(27):3002-3009. https://doi.org/10.2174/0929873324666170516123206

4. Stoyanov G, Dzhenkov D, Ghenev P, Illiev B, Enchev Y, Tonchev AB. Cell biology of glioblastoma multiforme: From basic science to diagnosis and treatment. Med Oncol. 2018;35(3):27-36. https://doi.org/10.1007/s12032-018-1083-x

5. Walke A, Suero Molina E, Stummer W, König S. Protoporphyrin IX analysis from blood and serum in the context of neurosurgery of glioblastoma. In: Mitulovič G, ed. Mass Spectrometry. London: IntechOpen; 2021. https://doi.org/10.5772/intechopen.95042

6. Ota U, Fukuhara H, Ishizuka M, et al. Plasma protoporphyrin IX following administration of 5-aminolevulinic acid as potential tumor marker. Mol Clin Oncol. 2015;3(4):797-801. https://doi.org/10.3892/mco.2015.549

7. Lualdi M, Cavalleri A, Battaglia L, et al. Early detection of colorectal adenocarcinoma: A clinical decision support tool based on plasma porphyrin accumulation and risk factors. BMC Cancer. 2018;18(1):841-849. https://doi.org/10.1186/s12885-018-4754-2

8. Kalaivani R, Masilamani V, Sivaji K, et al. Fluorescence spectra of blood components for breast cancer diagnosis. Photomed Lasers Surg. 2008;26(3):251-256. https://doi.org/10.1089/pho.2007.2162

9. Jones PS, Yekula A, Lansbury E, et al. Characterization of plasma-derived protoporphyrin-IX-positive extracellular vesicles following 5-ALA use in patients with malignant glioma. EBioMedicine. 2019;48:23-35. https://doi.org/10.1016/j.ebiom.2019.09.025

10. Hagemann O. Laborlexikon (ISSN 1860-966X): Porphyrine. http://www.laborlexikon.de/Lexikon/Infoframe/p/Porphyrine.htm. Accessed April 28, 2021.

11. Dalton JT. Clinical pharmacokinetics of 5-aminolevulinic acid in healthy volunteers and patients at high risk for recurrent bladder cancer. J Pharm Exp Ther. 2002;301(2):507-512. https://doi.org/10.1124/jpet.301.2.507

12. Doran D, Mitchell DG. Problems in the determination of erythrocyte protoporphyrin by ethyl acetate-acetic acid extraction. Ann Clin Biochem. 1994;21(2):141-145. https://doi.org/10.1177/000456328402100212

13. Zhang X, Iraneta PC, Marszalkowski FJ, Fountain KJ. A simplified solid phase extraction (SPE) protocol for bioanalysis using Oasis HLB. Waters Application Note: 2014. http://www.waters.com/waters/library.htm?locale=de_DE&lid=134817360&cid=511436. Accessed April 26, 2021.

14. Merck KGA. Solid phase extraction: Ion exchange methodology. http://www.sigmaaldrich.com/technical-documents/articles/analytical/solid-phase-extraction/ion-exchange-methodology.html. Accessed April 28, 2021.

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