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SPEKTROSKOPISCHE QUANTIFIZIERUNG VON MOLEKÜLEN DES HUMANEN HÄM-METABOLISMUS

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Veröffentlichungen in begutachteten Fachmagazinen:

- Lang, Alexander; Heckl, Christian; Vogeser, Michael; Stauch, Thomas; Homann, Christian; Hennig, Georg; Sroka, Ronald; Stepp, Herbert; *Rapid spectrophotometric quantification of urinary porphyrins and porphobilinogen as screening tool for attacks of acute porphyria;* J Biomed Opt 23(5), 1-12 (2018)
- Paal, Michael & Lang, Alexander; Hennig, Georg; Buchholtz, Marie-Luise; Sroka, Ronald; Vogeser, Michael; *A second-derivate fitting algorithm for the quantification of free hemoglobin in human plasma;* Clin Biochem 56, 62–69 (2018)
- Sroka, Ronald; Dominik, Nikolas; Eisel, Max; Esipova, Anna; Freymüller, Christian; Heckl, Christian; Hennig, Georg; Homann, Christian; Höhne, Nicolas; Kammerer, Robert, Kellerer, Thomas; Lang, Alexander; Markwardt, Niklas; Pohla, Heike; Pongratz, Thomas; Schmedt, Claus-Georg; Stepp, Herbert; Ströbl, Stefan; Ulaganathan, Keerthanan; Zimmermann, Wolfgang; Rühm, Adrian; *Research and developments of laser assisted methods for translation into clinical application*; Frontiers of Optoelectronics 10(3) 239-254 (2017)
- Meidert, Agnes S.; Lang, Alexander, Hennig, Georg; Bernasconi, Patricia; Peraud, Aurelia; Briegel, Josef; Hüttl, Tanja; *Falsely low values of oxygen saturation measured by pulse oximetry in a boy treated with Chinese herb tea*; J Clin Monit Comput. 31(2), 481-484 (2016)

Veröffentlichungen in „Conference Proceedings" nach mündlichem Vortrag:

- Lang, Alexander; Stepp, Herbert; Homann, Christian; Hennig, Georg; Brittenham, Gary M., Vogeser, Michael; *Rapid screening test for porphyria diagnosis using fluorescence spectroscopy* Proceedings of the SPIE 9537, 953706 (2015)

weitere Konferenzbeiträge:

- Mündlicher Vortrag: *Point-of-care porphyria screening by fluorescence spectroscopy of blood plasma* auf der Photonics West, BIOS, Optical Diagnostics and Sensing XVI: Toward Point-of-Care Diagnostics, San Francisco, USA, 2016
- Mündlicher Vortrag: *Point-of-care screening for acute porphyria by fluorescence and absorption spectroscopy of urine* auf der European Conferences on Biomedical Optics (ECBO) ETh2C.2, Medical Laser Applications and Laser-Tissue Interactions (MLA), München, 2016

Erfindungsmeldungen:

- Hennig, Georg; Stepp, Herbert; Homann, Christian; Lang, Alexander; *Vorrichtung zur routinemäßigen Funktionsprüfung von Messgeräten zur Anregung und Erfassung von Fluoreszenz*, 2016, Aktenzeichen VIII.3 384.3.2.1, Erfindung freigegeben
- Lang, Alexander; Brittenham, Gary; Stepp, Herbert; Homann, Christian; Hennig, Georg; *Device for diagnosis of porphyria,* 2015, Aktenzeichen: IR CU16168, Erfindung freigegeben

ABSTRACT

The present dissertation covers the development of spectroscopic methods for quantitative detection of heme synthesis and heme metabolization molecules, in particular porphobilinogen, uroporphyrin I and III, coproporphyrin I and III, hemoglobin and bilirubin. The first three substances are highly specific biomarkers for the diagnosis of acute porphyria, the other two for the diagnosis of hemoglobinemia. New diagnostic tests were developed for both clinical pictures.

In the heme biosynthesis, 5-aminolevulinic acid is metabolized via porphobilinogen and porphyrinogens to protoporphyrin IX, which then forms the heme complex with divalent iron (Fe^{2+}). The steps of the heme synthesis are enzymatically catalyzed. If one of the enzymes is inhibited in its activity by a genetic disorder, the resulting condition is called porphyria. Precursors to heme such as porphyrinogens, porphyrins and porphobilinogen then accumulate in the body and are excreted with the urine. The detection of elevated levels of porphyrins and porphobilinogen in urine allows for the diagnosis of acute porphyria with very high specificity, but so far there is no rapid test for both substances. Since acute porphyria is a very rare disease and shows very unspecific but life-threatening symptoms, patients with acute porphyria are often unrecognized and undiagnosed. A suitable screening test could prevent this.

To develop a rapid test for porphyrins and porphobilinogen, a device set-up was designed and constructed allowing for spectroscopic measurements of absorption and fluorescence on urine samples. An additional heating coil integrated into the device enables controlled and rapid heating of samples and thus the condensation of porphobilinogen to uroporphyrin I and porphobilin, which can be detected by either fluorescence or absorption spectroscopy. Using this setup, three new methods have been developed that can be combined to form an innovative rapid test for acute porphyria. It enables, first, the controlled photo-oxidation of porphyrinogens to porphyrins, second, the quantification of total porphyrins in urine in the concentration range between 0.2 and 20 µmol/L by a spectral fitting algorithm on the second derivative of spectra of patient samples and third, the indirect quantitative detection of porphobilinogen by the quantification of both or either uroporphyrin I or porphobilin. The developed test allows for the execution of all three methods including sample preparation in less than 15 minutes.

The aim of the second spectroscopic method of this dissertation thesis was the quantification of free hemoglobin in blood plasma, which results from strong intravascular hemolysis. Bilirubin, a substance occurring in the metabolism of heme, is elevated in the blood plasma following severe hemolysis. Bilirubin prevents the quantification of free hemoglobin even at very low concentrations when employing the standard optical method for free hemoglobin quantification. A mathematical fit algorithm using the second derivative of plasma absorption spectra was developed and applied on 492 samples. It allows for the quantification of both free hemoglobin and bilirubin in blood plasma, and was therefore named “HEBI-Fit”. The publication also includes the algorithm as a Microsoft Excel file to enable other laboratories to easily implement the innovative method in their daily routine.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurden spektroskopische Methoden zum quantitativen Nachweis von Molekülen der Häm-Biosynthese und der Häm-Metabolisierung entwickelt, im Speziellen Porphobilinogen, Uroporphyrin I und III, Koproporphyrin I und III, Hämoglobin und Bilirubin. Die ersten drei Stoffe stellen sehr spezifische Biomarker für die Diagnose von akuter Porphyrie dar, die Anderen für die Diagnose von Hämoglobinämie. Für beide Krankheitsbilder wurden neue diagnostische Tests entwickelt.

In der Hämsynthese wird über mehrere metabolische Schritte von 5-Aminolävulinsäure ausgehend Porphobilinogen und daraus über Porphyrinogene Protoporphyrin IX erzeugt, das den Häm-Komplex mit zweiwertigem Eisen (Fe^{2+}) bildet. Die Schritte der Hämsynthese sind enzymatisch katalysiert. Falls durch eine genetische Störung eines der Enzyme in seiner Aktivität gehemmt ist, spricht man von Porphyrie. Vorläuferstoffe zu Häm wie Porphyrinogene, Porphyrine und Porphobilinogen reichern sich dann im Körper an und werden unter anderem mit dem Urin ausgeschieden. Der Nachweis von erhöhten Konzentrationen von Porphyrinen und Porphobilinogen in Urin erlaubt mit sehr hoher Spezifität die Diagnose von akuter Porphyrie, es existiert jedoch kein Schnelltest für beide Substanzen. Da akute Porphyrie eine sehr seltene Erkrankung ist und sehr unspezifische, allerdings lebensbedrohliche Symptome zeigt, wird akute Porphyrie häufig nicht erkannt und Patienten fehldiagnostiziert. Ein geeigneter Schnelltest könnte das verhindern. Zur Entwicklung eines Schnelltests für Porphyrine und Porphobilinogen wurde ein Messaufbau konstruiert, der spektroskopische Messungen der Absorption und Fluoreszenz an Urinproben ermöglicht. Ein zusätzlich in das Gerät integriertes Thermoelement erlaubt kontrolliertes und schnelles Erhitzen von Proben und damit die Kondensation von optisch nicht nachweisbarem Porphobilinogen zu Uroporphyrin I und Porphobilin, welche über Fluoreszenz- beziehungsweise Absorptionsspektroskopie nachgewiesen werden können. Es wurden mithilfe dieses Aufbaus drei Methoden entwickelt, die zu einem neuen Schnelltest für akute Porphyrie kombiniert werden können. Dieser erlaubt erstens die kontrollierte Foto-Oxidation von Porphyrinogenen zu Porphyrinen, zweitens die Quantifizierung der Gesamtporphyrine in Urin im Konzentrationsbereich zwischen 0,2 und 20 µmol/L durch ein mathematisches Näherungs-Verfahren an der zweiten Ableitung der Spektren von Patientenproben und drittens den indirekten, quantitativen Nachweis von Porphobilinogen durch die Quantifizierung von Uroporphyrin I oder Porphobilin. Das entwickelte Messverfahren ermöglicht die Durchführung aller drei Messungen inklusive Probenvorbereitung in weniger als 15 Minuten.

Ziel der anderen spektroskopischen Methode dieser Promotionsarbeit war die Quantifizierung von freiem Hämoglobin in Blutplasma, welches durch starke intravaskuläre Hämolyse entsteht. Bilirubin, ein Stoff der bei der Metabolisierung von Häm auftritt, findet sich bei schwerer Hämolyse vermehrt im Blutplasma. Bilirubin verhindert bereits in sehr geringen Konzentrationen die Quantifizierung von freiem Hämoglobin bei Messungen mit der Standardmethode. Es wurde an 492 Proben ein mathematischer Näherungs-Algorithmus („HEBI-Fit“ genannt) an der zweiten Ableitung der Plasmaabsorptionsspektren entwickelt, welcher die Quantifizierung von freiem Hämoglobin und von Bilirubin ermöglicht. Der entsprechenden Publikation wurde dieser Auswerte-Algorithmus als Microsoft Excel File angehängt, um es anderen Laboren zu ermöglichen, diese innovative Methode selbst einfach in ihre Labor-Auswertungen zu implementieren.

SPEKTROSKOPISCHE QUANTIFIZIERUNG VON MOLEKÜLEN DES HUMANEN HÄM-METABOLISMUS

Diese Arbeit wird als kumulative Dissertation vorgelegt und besteht aus zwei veröffentlichten Originalmanuskripten als Erstautor. Beide Manuskripte verbindet inhaltlich die Quantifizierung von Molekülen der humanen Häm-Biosynthese und -Metabolisierung, nämlich Porphyrinen und Porphobilinogen (PBG) in Urin [1] und Hämoglobin und Bilirubin in Blutplasma [2], mittels Absorptions- oder Fluoreszenzspektroskopie. In beiden Fällen wurde dabei ein nichtlineares, mathematisches Näherungs-Verfahren an der zweiten Ableitung der Spektren entwickelt und angewendet, wodurch Störeinflüsse, wie der stark variierende spektrale Untergrund bei Patientenproben [1, 2] und von Molekülen mit überlappender spektraler Signatur (Bilirubin) [2], eliminiert werden konnten. Die Aufgabenstellung beider Projekte motiviert sich aus dem direkten Bedarf aus der Klinik: Die Bestimmung von PBG und Gesamtporphyrinen im Urin ist als diagnostischer Test bei Verdacht auf einen Anfall von akuter Porphyrie von großer Bedeutung; die exakte Quantifizierung von freiem Hämoglobin in Blutplasma bei Anwesenheit von Bilirubin ein bisher nicht zufriedenstellend gelöstes Problem in der Routine der Labormedizin.

SYNTHESE UND METABOLISIERUNG VON HÄM

Häm ist ein organischer Komplex aus Protoporphyrin IX, bestehend aus einer ringförmigen Anordnung von vier Pyrrolringen und zwei Carboxyl-Seitenketten, und einem zweiwertigen Eisenion (Fe^{2+}) als Zentralatom [3]. Es spielt als prosthetische Gruppe von unterschiedlichen Proteinen wie Hämoglobin, Myoglobin und Zytochromen eine elementare Rolle für biologische Prozesse wie den Sauerstofftransport, die Zellatmung, als Elektronendonor und -akzeptor in Redoxreaktionen und als katalytisches Zentrum für die Metabolisierung von Medikamenten oder Toxinen [3-5]. Häm wird für die Zellatmung prinzipiell in fast jeder Zelle des Körpers produziert, ein Großteil der Produktion findet jedoch im Knochenmark und in der Leber statt [3]. Über 85% der täglichen Hämproduktion dienen der Erythropoese [3]. Die Häm-Biosynthese besteht aus acht enzymatisch katalysierten Schritten, welche in den Mitochondrien und im Zytoplasma stattfinden [3]. Aus Glycin und Succinyl-Coenzym A wird in mehreren Schritten über 5-Aminolävulinsäure, PBG und Uroporphyrinogen III Protoporphyrin IX erzeugt [3, 6]. In dieses wird durch das Enzym Ferrochelatase ein Eisenion eingefügt und bildet damit den Hämkomplex [3, 7, 8]. Bei der Erythropoese findet dieser Prozess in den Vorläuferzellen der Erythrozyten statt, welche vor dem Eintritt in den Blutkreislauf Zellorganellen wie den Zellkern, Mitochondrien und das endoplasmatische Retikulum verlieren [9]. Die Lebensdauer der Erythrozyten beträgt etwa 120 Tage [6]. Alte oder beschädigte Erythrozyten werden von der Milz aus dem Blutstrom gefiltert, phagozytiert und durch Makrophagen abgebaut [10]. Die Globinkette wird in seine Aminosäuren zerlegt und somit wiederverwertet. Häm wird aufgespalten, wobei Biliverdin entsteht [11], und das frei werdende Eisenion wird von Ferroportin und Ferritin, dem Eisenspeicherprotein, aufgenommen [12]. Biliverdin wird zu Bilirubin reduziert, welches an Albumin gebunden durch den Blutkreislauf zur Leber transportiert wird [13]. Durch Glucuronat-Konjugation wird Bilirubin dort wasserlöslich gemacht, um über die Galle in den Darm abgegeben werden zu können [13].

Der gesamte Prozess der Hämsynthese und –metabolisierung ist in Abbildung 1 gezeigt. Falls Erythrozyten platzen (Hämolyse) und Hämoglobin frei im Blutplasma treibt, können Fängerproteine (Hämopexin und Haptoglobin) im Blutplasma an das freie Hämoglobin oder Häm binden und der regulären Metabolisierung in der Leber zuführen [14]. Bei starker Hämolyse reichert sich das freie Hämoglobin zunehmend im Blutplasma an [14]. Durch den verstärkten Abbau kommt es in der Folge auch zur Anreicherung von Abbauprodukten wie Bilirubin im Blutplasma [15].

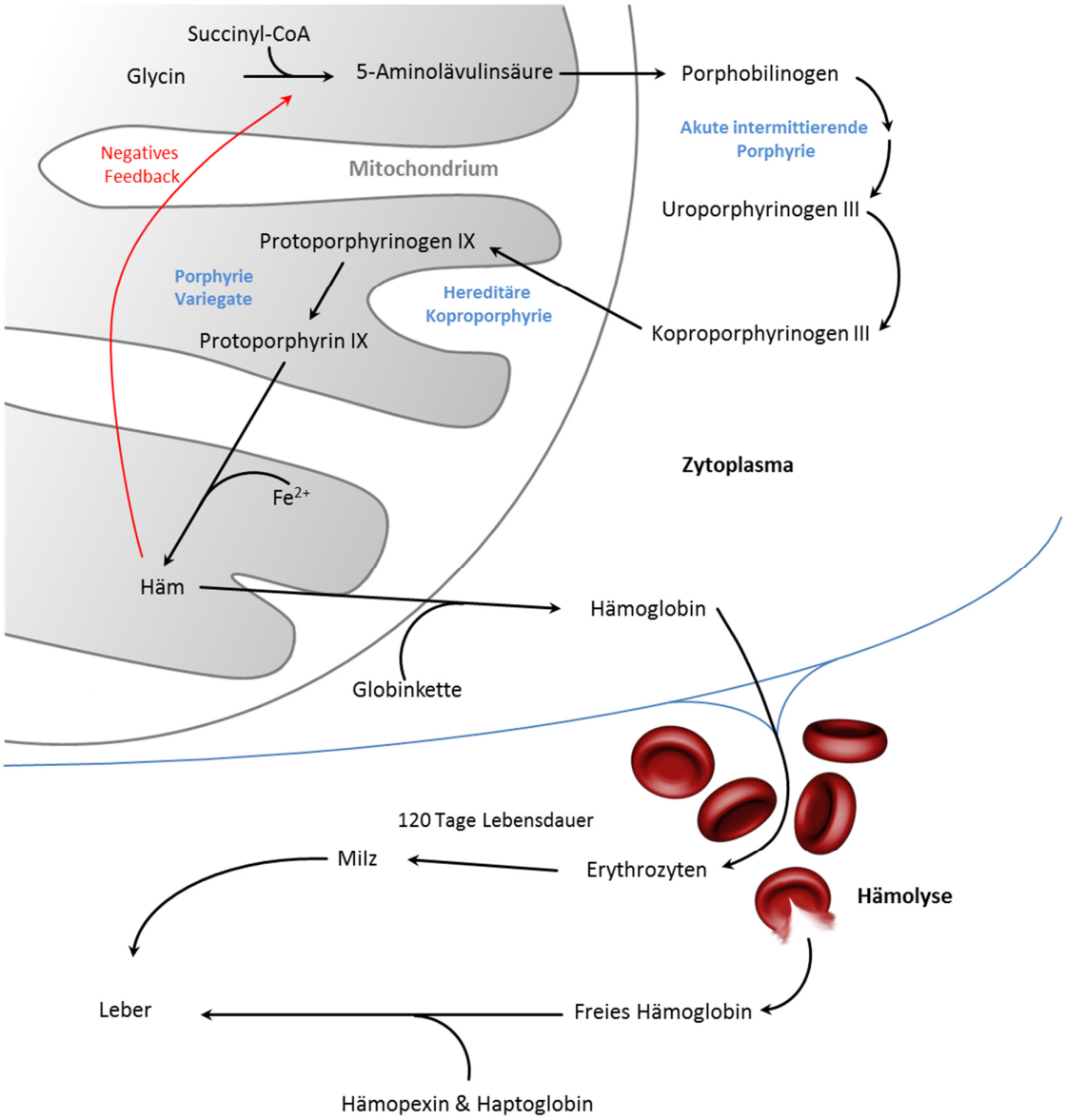

Abbildung 1: Schematische Darstellung der Hämbildung und -metabolisierung. Aus Glycin und Succinyl-Coenzym A wird über acht enzymatische Schritte, hier als Pfeile dargestellt, Häm gebildet. Start- und Endpunkt der Synthese liegt in den Mitochondrien, wo Häm regulierend die Synthese von 5-Aminolävulinsäure hemmt. Die drei akuten, autosomal-dominanten Porphyrien, welche für diese Arbeit relevant sind, sind in blau neben der enzymatischen Reaktion eingezeichnet, welche bei der entsprechenden Porphyrie beeinträchtigt ist. Hämoglobin ist hauptsächlich in den Erythrozyten für den Sauerstofftransport verantwortlich. Alte und beschädigte rote Blutkörperchen werden von der Milz wiederverwertet und die entstehenden Zwischenprodukte von der Leber metabolisiert. Dies geschieht ebenfalls, wenn Erythrozyten platzen und das Häm frei im Blutplasma treibt. In diesem Fall binden Glycoproteine an das freie Hämoglobin und führen es der Metabolisierung zu.

SPEKTRALE EIGENSCHAFTEN VON PORPHOBILINOGEN, PORPHYRINEN UND HÄMOGLOBIN

Absorbierende und nicht absorbierende Moleküle im Hämzyklus können häufig anhand ihres Suffixes im Namen unterschieden werden. Die Endung –ogen impliziert nicht absorbierende Moleküle, wie Porphobilinogen oder Porphyrinogen, wohingegen Moleküle mit Endung –in Pigmente sind, wie zum Beispiel Porphyrin oder Bilirubin. Spektren der für die Arbeit relevanten Substanzen sind exemplarisch in Abbildung 2 sowohl für Absorption als auch Fluoreszenz gezeigt. Im Folgenden wird kein Unterschied zwischen den I und III Isomeren von Uroporphyrin und Koproporphyrin gemacht, da deren spektrale Eigenschaften für die Zwecke dieser Arbeit identisch sind.

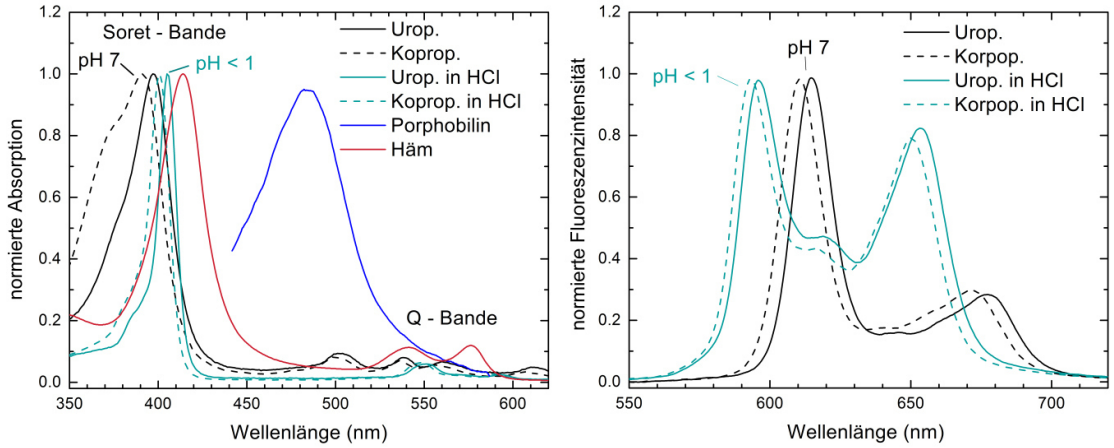

Abbildung 2: Die Absorption aller in dieser Arbeit relevanten Porphyrinmoleküle (links) weist ein deutliches Maximum im Bereich um 400 nm auf, die Soret-Bande. Die exakte Form und Position ist dabei stark von der Menge und Art von Seitenketten (vergleiche Uroporphyrin und Koproporphyrin), dem Zentralatom (siehe Häm) und dem umgebenden Medium (vergleiche Porphyrine in Salzsäure (HCl, pH<1) mit Porphyrinen in neutraler Lösung (pH 7)). Zudem sind mit geringerer Intensität die Q-Banden im Bereich um 500-620 nm erkennbar. Das aus Porphobilinogen erzeugte gelbe Pigment, Porphobilin, ist kein Porphyrin und zeigt eine deutlich anders geformte Absorption mit Maximum bei 480 nm. Die Fluoreszenz von Uroporphyrin und Koproporphyrin in neutraler und saurer Umgebung ist rechts gezeigt.

Das Porphyrinskelett besteht aus vier Pyrrolringen verbunden durch Methin. Die Struktur des Tetrapyrrol-Rings und die Bindungsabstände wurden mittels Röntgenbeugung an Porphyrinen mit einer großen Anzahl verschiedener Seitenketten gemessen und zeigt sich als sehr starr [16]. Typische Abstände der Bindungen liegen je nach Bindungswinkel zwischen 1.34 Å und 1.45 Å [16]. Porphyrine zeichnen sich durch eine ausgedehnte delokalisierte π-Elektronenwolke aus, bestehend aus elf konjugierten, alternierenden Einzel- und Doppelbindungspaaren [16]. Diese führt zu einer starken Absorption im violetten Wellenlängenbereich, der sogenannten Soret-Bande um 400 nm, und mehreren, weniger intensiven Absorptionsbanden, Q-Banden genannt, zwischen 500 nm und 650 nm. Einige Porphyrine, wie Uroporphyrin, Koproporphyrin und Protoporphyrin IX, weisen zudem nach Anregung der Soret-Bande eine starke Fluoreszenz auf, welche zwischen 600 nm und 700 nm liegt und durch zwei unterschiedlich hohe Emissionsmaxima charakteristisch für Porphyrinfluoreszenz ist. Die genaue Position der Absorptions- und Emissionsmaxima hängt stark von der Art des Porphyrins, also den Seitenketten und der Konfiguration des Zentrums, ab. So zeigt zum Beispiel Häm, und damit auch Hämoglobin, keine Porphyrinfluoreszenz [17, 18]. Grund dafür ist zum einen das zentrale Metallion, das die Wahrscheinlichkeit für Intersystem-Crossing

vom Singulett- in den Triplettzustand erhöht und damit die Relaxation des angeregten Zustands über Fluoreszenz verringert, und zum anderen die Elektronenkonfiguration des Zentralatoms. Bei Häm liegt das Eisen in der Elektronenkonfiguration „[Ar] $3d^6$“ vor, also einer nicht abgeschlossenen Elektronenschale. Die nicht an einer Bindung beteiligten Elektronen erhöhen die Wahrscheinlichkeit für Intersystem-Crossing nochmals und führen damit zu einer vollständigen Unterdrückung der Fluoreszenz [19]. Zudem beeinflusst auch das umgebende Medium die Absorption und Fluoreszenz. Im sauren Milieu unter pH 3,5 wird das Zentrum des Porphyrinrings durch zwei Wasserstoffatome protoniert und die planare Konformation des Porphyrinmoleküls ändert sich. Dadurch erhöht sich die Symmetrie des Moleküls, was zu schmaleren Soret-Banden und einer Rotverschiebung des Anregungsspektrum und Blauverschiebung der Emission führt [20, 21].

Porphyrine werden in der Hämsynthese aus vier Porphobilinogenmolekülen erzeugt. PBG ist eine farblose Substanz bestehend aus einem Pyrrolring mit zwei Carboxylseitenketten [22], welche ein sehr spezifischer Marker für akute Porphyrie ist. Da PBG keine Absorption im sichtbaren oder UV-Spektralbereich besitzt, ist ein direkter photometrischer Nachweis nicht möglich [23]. PBG ist somit nur indirekt über den Nachweis von spezifischen Reaktionsprodukten möglich und wird klassischerweise über eine rote Farbreaktion mit Dimethylaminobenzaldehyd (Ehrlich-Reagenz) nachgewiesen, wie von Mauzerall und Granick etabliert [24]. Neben der Ehrlich-Reaktion bietet die Umwandlung von PBG mittels einer enzymatischen Reaktion oder durch Heizen induzierte, spontane Kondensation zu Uroporphyrinogen die Möglichkeit, nach Oxidation von Uroporphyrinogen zu Uroporphyrin, dieses über dessen Fluoreszenz oder Absorption nachzuweisen [23, 25]. Außerdem entsteht durch Heizen von PBG Porphobilin ein gelbes Pigment mit Absorptionsmaximum bei 480 nm, welches absorptionsspektroskopisch nachgewiesen werden kann [23].

Spektrales Fitten durch Minimierung des Residuums der zweiten Ableitung

In einer ausreichend verdünnten Lösung tragen alle in ihr gelösten Stoffe additiv zur Absorption und Fluoreszenz bei; das Spektrum besteht also aus einer linearen Kombination der Einzelspektren. Das ist insofern problematisch, als oftmals nur eine Substanz von Interesse ist, andere Moleküle jedoch das Spektrum dieser Substanz überlagern und somit eine direkte Auswertung der Intensität bei einer festgelegten Wellenlänge fehlerbehaftet ist [26]. Falls die Einzelspektren aller Substanzen bekannt sind, können diese voneinander getrennt werden. Für biologische Proben mit einer Vielzahl von Substanzen ist die Auftrennung des Untergrundspektrums jedoch meist nicht möglich. Allerdings fällt das spektrale Signal solcher Proben zu längeren Wellenlängen hin ab, wodurch ein monotoner Kurvenverlauf entsteht. In Blutplasma sind dafür hauptsächlich die Transportproteine wie Albumin und Globulin sowie Bilirubin, Riboflavin und Pyridoxalphosphat verantwortlich [27]; im Urin eine Vielzahl von Molekülen, unter anderem Pyridoxinsäure und Riboflavin [28]. Bei biologischen Proben wie Blut oder Urin ist dieser Untergrund zudem nicht konstant, sondern unterliegt bereits innerhalb des Tagesverlaufs inter- und intraindividuellen Schwankungen. Das ist in Abbildung 3 beispielhaft an 6 Urinproben gesunder Spender gezeigt, welche vollkommen unterschiedliche spektrale Formen und Intensitäten aufweisen, jedoch alle im Bereich von 525 nm zu längeren Wellenlängen hin

monoton abfallen. Somit kann weder eine Konstante für den Untergrund abgezogen werden, noch dieser durch eine einfache Kurve modelliert werden. In solchen Fällen zeigt ein Verfahren, das die spektralen Beiträge über die Minimierung des Residuums der zweiten Ableitung ermittelt, große Vorteile. Das zugrunde liegende Prinzip der Verwendung der zweiten Ableitung eines Spektrums ist bereits seit vielen Jahren bekannt [29, 30]. Ausgangsbasis ist die Annahme, dass der Probenuntergrund im Bereich des Referenzspektrums, der von Interesse ist, keine starken Krümmungsänderungen aufweist. Das Reinspektrum der gesuchten Substanz (Referenzspektrum) wird von dem Spektrum der Patientenprobe subtrahiert und die zweite Ableitung des resultierenden Spektrums (Residuum) gebildet. Der spektrale Probenuntergrund ist in der zweiten Ableitung nahezu Null, da er nur geringe Krümmungsänderungen enthält, und somit stammen alle Beiträge der zweiten Ableitung des Residuums von der Substanz, die quantifiziert werden soll. Die Amplitude des Referenzspektrums, welche vom Probenspektrum abgezogen wird, wird nun solange iterativ variiert, bis das Residuum in der zweiten Ableitung minimal wird. Wenn dieser Zustand erreicht ist, entspricht die Amplitude des Referenzspektrums der tatsächlichen Amplitude der Substanz von Interesse. Die Konzentration wird dann durch den Vergleich dieser Amplitude mit einer unabhängig aufgenommenen Kalibrationskurve der Reinsubstanz quantifiziert.

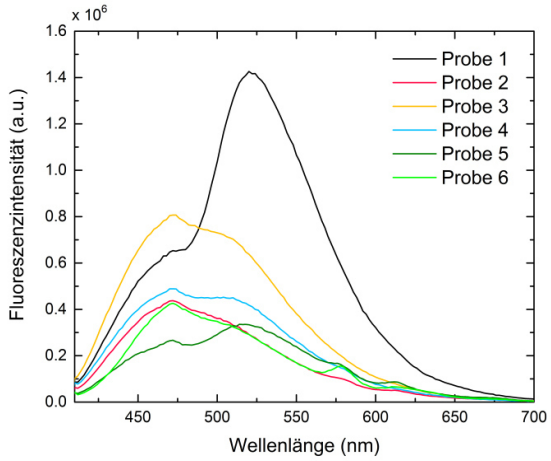

Abbildung 3: Fluoreszenzspektren von Urinproben sechs gesunder Spender zeigen bei Anregung mit 400 nm extrem unterschiedliche spektrale Verläufe, welche auf eine sowohl absolut als auch relativ zueinander schwankende Zusammensetzung unterschiedlicher Fluorophore hindeutet. Im Bereich ab 525 nm nimmt die Intensität zu längeren Wellenlängen monoton ab, außer für die beiden grünen Kurven. In diesen findet sich die spektrale Signatur von Porphyrinen, die auch bei gesunden Menschen in geringen Konzentrationen im Urin auftreten können.

PORPHYRIE ALS ERKRANKUNG DER HÄM-BIOSYNTHESE

Porphyrien sind eine Gruppe seltener, hauptsächlich genetisch bedingter Stoffwechselstörungen der Häm-Biosynthese, die jeweils durch die Fehlfunktion eines der acht enzymatischen Schritte bei der Hämbildung verursacht werden [3, 31, 32]. Durch die verringerte Aktivität des Enzyms und damit verbundene Verzögerung in der Weiterverarbeitung von Hämvorläufern entsteht ein für die Art der Porphyrie spezifisches Akkumulationsmuster von 5-Aminolävulinsäure, PBG und Porphyrinen im Blut, Urin und Stuhl [31, 33]. Die Erkrankungen können grob als akute oder chronische Porphyrie klassifiziert werden. Die vorliegende Arbeit befasst sich ausschließlich mit den akuten, autosomal-dominant vererbten Porphyrien, nämlich akute intermittierende Porphyrie, hereditäre Koproporphyrie und Porphyrie Variegate, welche mit einer kombinierten Prävalenz von ca. 1 in 10.000 untersuchten Fällen die häufigsten akuten Porphyrien darstellen [3, 33, 34]. Akute Porphyrien zeichnen sich durch latente Phasen ohne Krankheitssymptome aus, die von Anfällen mit sehr unspezifischen Symptomen unterbrochen werden. Typischerweise beginnt ein akuter Krankheitsschub mit starken Abdominalschmerzen, die unbehandelt zu schweren neuropathischen Symptomen wie Krampfanfällen, Lähmungserscheinungen und zum Tod durch Atemstillstand

führen können. Der unspezifische Charakter der Symptome führt häufig dazu, dass akute Porphyrien übersehen oder falsch interpretiert werden. Eine amerikanische Studie kommt zu dem Ergebnis, dass die korrekte Diagnose im Mittel erst 15 Jahre nach dem Einsetzen der ersten Symptome gestellt wird [35], was mit einer Vielzahl an Fehlbehandlungen, unnötigem Leid der betroffenen Patienten und Kosten für das Gesundheitssystem verbunden ist [36]. Bei einem Verdacht auf einen Anfall akuter Porphyrie ist der medizinische Konsens für die Diagnostik die Bestimmung der Konzentration von PBG und Porphyrinen im Urin [37-40]. Sind diese Werte erhöht (über 2 mg/L für PBG und über 200 nmol/L für Porphyrine) [41, 42], kann durch die Gabe von Hämsynthese-Hemmer wie Glucose oder Hämatin der Anfall therapiert werden, da diese eine negative Rückkopplung auf die Aktivität der 5-ALA-Synthase bewirken [3, 31].

STAND DER TECHNIK UND PROBLEMSTELLUNG

Die flächendeckende Verfügbarkeit eines einfach durchführbaren, schnellen und günstigen Schnelltests für PBG und Porphyrine in Urin ist entscheidend für die Diagnose von akuter Porphyrie, stellt die klinische Chemie jedoch vor große Herausforderungen [31]. Bei der Erstdiagnose müssen aus sehr vielen potentiellen Porphyrie-Patienten mit Bauchschmerzen die wenigen mit tatsächlich vorliegender akuter Porphyrie herausgefiltert werden. Es wird also ein sehr spezifischer Test benötigt. Die Kombination aus PBG- und Porphyrinbestimmung im Urin bietet diese Spezifität, Routinen für die beiden Substanzen sind aber nicht auf den großen Laboranalysegeräten implementiert. Kommerzielle Tests für PBG, die für akute Porphyrie spezifische Substanz, sind zwar verfügbar, basieren allerdings auf einem Verfahren nach Mauzerall und Granick [24], das sowohl zeit- als auch ressourcenaufwändig ist und zudem nur von erfahrenem Laborpersonal durchgeführt werden sollte [43]. Damit ist der Test für ein Notfalllabor und als Schnelltest ungeeignet. Aktuelle Fortschritte wie die schnelle Bestimmung von 5-Aminolävulinsäure, PBG und Porphyrinen mittels Hochleistungsflüssigchromatographie [44] sind kostenintensiv und in vielen Krankenhäusern fehlt es sowohl an Zeit, Expertise als auch Ausrüstung für die Durchführung chromatographischer Nachweisverfahren. Ein chemisches Testkit, welches semi-quantitativ PBG mittels eines Farbumschlags in Urin bestimmen konnte, war der bisher geeignetste Schnelltest [43]. Dieser wurde 2014 vom Markt genommen, wodurch von diesem Zeitpunkt an Krankenhäuser auf externe Labors angewiesen sind. Diese benötigen allerdings 4 bis 10 Tage, bis ein Messergebnis vorliegt [31]. Es besteht also eine hohe Nachfrage nach robusten, einfach anzuwendenden und gleichzeitig preiswerten Tests, um Porphyrine und PBG in Urin zu quantifizieren. Hierbei gilt es mehrere Herausforderungen zu überwinden:

1. Urin ist in seiner Zusammensetzung sowohl im Tagesverlauf als auch zwischen unterschiedlichen Personen höchst variabel [28]. Außerdem sind pH-Werte zwischen pH 4,5 und pH 9 möglich [45]. Dies führt zu einem variablen Fluoreszenz- und Absorptionsuntergrund, der die direkte Quantifizierung von Substanzen aus dem Spektrum stört.
2. Nicht alle relevanten Stoffe sind optisch direkt nachweisbar. In der Hämsynthese treten als Vorstufen von Protoporphyrin IX nur nicht absorbierende oder fluoreszierende Porphyrinogene auf, die bei einem Anfall akuter Porphyrie aus den Mitochondrien und den Zellen in das Blutplasma und später den Urin abgegeben werden. Diese sind diagnostisch relevant, jedoch nicht optisch aktiv. Somit muss die Probe oxidiert werden, um alle Porphyrinogene in Porphyrine umzuwandeln und dadurch den

fluoreszenzspektroskopischen Nachweis zu ermöglichen. Dieser Schritt ist unumgänglich, da in frischen Urinproben akuter Porphyrie-Patienten im Mittel 77% der diagnostisch relevanten Tetrapyrrole als Porphyrinogene vorliegen und nur 23% als Porphyrine [46]. PBG, die zweite diagnostisch relevante Substanz, besitzt weder UV/VIS Absorption noch Fluoreszenzemission, und kann daher optisch nicht direkt nachgewiesen werden. PBG muss folglich erst in einer kontrollierten, reproduzierbaren und konzentrationsabhängigen Reaktion in eine optisch nachweisbare Substanz umgewandelt werden. Obwohl die Umwandlung durch Heizen oder eine enzymatische Reaktion in vitro bereits lange bekannt ist [23, 25], gibt es bisher keine Ansätze, diese Umwandlung zur Quantifizierung von PBG zu verwenden.

3. Der Test muss bei hoher Spezifität einfach anzuwenden, schnell und kostengünstig sein, um eine tatsächliche Anwendung in der Klinik zu ermöglichen. Das bedeutet eine geringe Anzahl an Arbeitsschritten mit gebräuchlichen, ungefährlichen Chemikalien und eine Kombination aus Hardware und Software, welche die Messung und Auswertung weitgehend automatisiert.

Quantitativer Nachweis von Porphyrinen und Porphobilinogen in Urin

Für das erste der beiden Manuskripte dieser Dissertation [1] wurde ein Messaufbau entwickelt, welcher simultan das Temperieren einer Probe auf eine voreingestellte Temperatur als auch die gleichzeitige Messung von Absorptions- und Fluoreszenzspektren ermöglicht. Außerdem wurde eine Steuersoftware in der Programmiersprache LabVIEW erstellt, um sowohl einfache Messungen als auch aufwändigere Abfolgen aus Heizen, Messung von Absorptions- und Fluoreszenzspektren und die Auswertung der Spektren über den Zeitverlauf zu implementieren. Mithilfe dieses Geräts konnten drei neue Methoden entwickelt werden:

1. Die Foto-Oxidation von Porphyrinogenen zu Porphyrinen, welche mit zwei etablierten chemischen Methoden verglichen wurde, erlaubt innerhalb einer Minute die Oxidation von fast 70% der vorhandenen Porphyrinogene, was je nach Konzentration eine Beschleunigung um einen Faktor 10 bis 30 im Vergleich zu den chemischen Oxidationsverfahren darstellt.
2. Eine Quantifizierungsmethode für die Porphyrinkonzentration in Urin, basierend auf dem spektralen Fit an der zweiten Ableitung des Residualspektrums. Da die erste und zweite Methode an der gleichen Urinprobe stattfinden müssen, wurde darauf geachtet, ein Probenvorbereitungsprotokoll zu entwickeln, durch welches sowohl die Oxidation als auch die Quantifizierung der Porphyrine optimiert und gleichzeitig Störeinflüsse wie pH-Schwankungen zwischen Patientenproben eliminiert werden. Der relative mittlere Fehler und die relative mittlere Standardabweichung liegen beide unter 15% für den physiologischen Konzentrationsbereich über 200 nmol/L.
3. Die Umwandlung von PBG in Porphobilin und Uroporphyrin durch Heizen wurde quantitativ fluoreszenz- und absorptionsspektroskopisch nachvollzogen und der Nachweis von Porphobilin als geeigneter, indirekter Marker für PBG vorgeschlagen.

Für die Entwicklung der zweiten und dritten Methode wurde in mehreren Messreihen Urin gesunder Spender mit Porphyrinen oder Porphobilinogen versetzt. Für die Probensammlung von freiwilligen Spendern wurde die Erlaubnis der Ethikkomission der medizinischen Fakultät der

Universität München eingeholt (Aktenzeichen 679-15). Vorgaben zur Probenanzahl und Wahl der Konzentrationen der Richtlinie zur Evaluierung von bio-analytischen Methoden der „European Medicines Agency“ [47] wurden dabei beachtet. Die Konzentration der Porphyrin- und Porphobilinogen-Stammlösungen, die für die Erzeugung von Proben verwendet wurde, wurden im Institut für Laboratoriumsmedizin des Klinikums der Universität München in Großhadern mit der jeweiligen Referenzmethode bestimmt und das entwickelte Gerät darauf kalibriert.

Der Anteil von Alexander Lang an dieser Studie war die Entwicklung des Geräts und der Steuerungssoftware mit Unterstützung der Koautoren, die Entwicklung und Anleitung von Verbesserungen des Geräts, die Durchführung und Anleitung von Messungen, die Entwicklung und Optimierung des mathematischen, spektralen Näherungs-Algorithmus, die Auswertung der Messungen und der Vergleich zu Referenzmessungen, die Entwicklung des vorgeschlagenen Messdurchführungsprotokolls und das Verfassen des Manuskripts mit Korrekturen und Anregungen der Koautoren.

In Zukunft soll auf Basis der bisherigen Ergebnisse das bestehende Gerät weiter verbessert werden. Dazu gehören die Verwendung eines neuen Spektrometers mit höherer Auflösung, die Entwicklung eines neuen Probenhalters und die Optimierung der Anregungsgeometrie und der Detektionseffizienz, unter anderem auch durch Simulationen des Strahlengangs. Dieses Gerät soll dann in einer klinischen Studie an Patienten mit akuten Bauchschmerzen in der Notaufnahme eingesetzt werden, um die Methode an Patientenproben zu evaluieren. Die Konzentration an Porphyrinen und Porphobilinogen der untersuchten Proben sollen zudem in einem Referenzlabor für jede Probe mitbestimmt werden.

KLINISCHE BEDEUTUNG DER QUANTIFIZIERUNG VON FREIEM HÄMOGLOBIN

Freies Hämoglobin (fHb) entsteht, wenn Erythrozyten beschädigt werden oder platzen, und Hämoglobin frei im Blutplasma treibt. Grund dafür kann eine genetisch bedingte Störung in der Erythropoese (zum Beispiel Sichelzellanämie), Sepsis, bestimmte Toxine, Malaria oder mechanischer Stress an künstlichen Herzklappen oder durch extrakorporale Blutzirkulationssysteme sein [48-50]. Durch körpereigene Schutzmechanismen wird fHb gebunden und der Leber zugeführt [14]. Sind diese Mechanismen durch schwere Hämolyse erschöpft, entsteht eine Hämoglobinämie, bei der sich fHb im Blutplasma anreichert. Das oxidativ wirkende Häm im Körper fördert die Umwandlung von Lipoproteinen zu zytotoxischen Stoffen und verstärkt das Abtöten von Endothelzellen durch Leukozyten und andere oxidierende Stoffe [51], wodurch bleibenden Schäden am Gefäßsystem entstehen können [14, 51].
Die Diagnose von Hämoglobinämie und damit Hämolyse ist klinisch von großer Bedeutung. So zeigen aktuelle Studien, dass die Konzentration von fHb ein prognostischer Faktor für das Überleben bei Patienten mit schwerer Sepsis darstellen könnte. Zudem sind andere diagnostische Marker wie erhöhte Transaminasen, Bilirubin, Lactatdehydrogenase (LDH) und Kalium sowie reduziertes Haptoglobin nicht spezifisch für Hämolyse [52]. Weiterhin spielt die Bestimmung von fHb eine entscheidende Rolle, um das Alter und die Qualität von Blutproben vor der Transfusion zu bestimmen [53] oder um Patienten mit künstlichen Herzklappen [54-56] oder extrakorporalen Blutzirkulationssystemen routinemäßig auf Hämolyse zu untersuchen [57, 58].

STAND DER TECHNIK UND PROBLEMSTELLUNG

Für die Quantifizierung von fHb in Blutplasma gibt es zwei unterschiedliche methodische Ansätze. Zum einen biochemische Methoden, basierend auf einer Peroxidasen-ähnlichen Reaktion mit Benzidin- oder Benzidinderivaten [59, 60], der modifizierten Drabkin-Reaktion [61] oder Immun-Nephelometrie [62], und zum anderen optisch-spektrale Methoden, die das Signal am Absorptionsmaximum von fHb verwenden und durch lineare Interpolation zwischen zwei oder

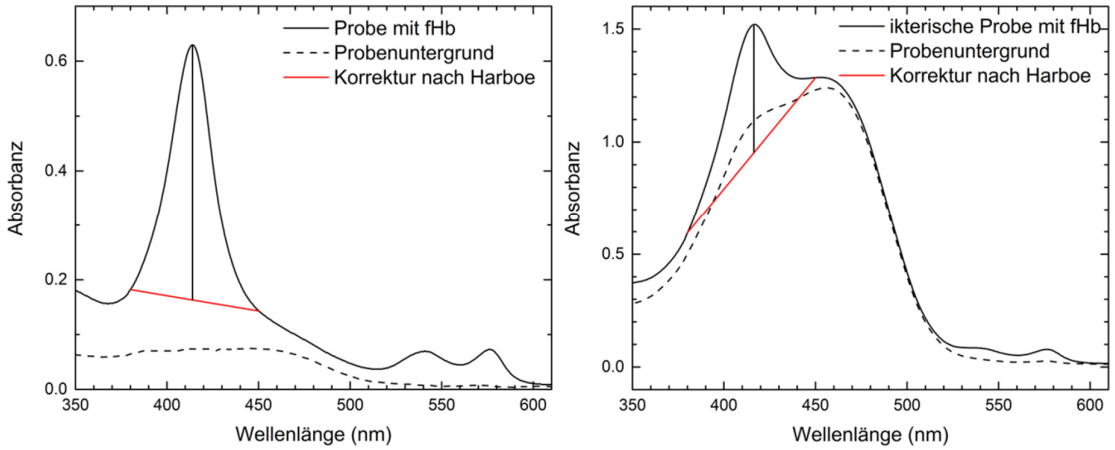

Abbildung 4: Darstellung der Methode nach Harboe an zwei 1 zu 11 verdünnten Blutplasmaproben mit erhöhtem fHb (links) und mit erhöhtem fHb und Bilirubin (rechts), wo durch lineare Interpolation zwischen 380 nm und 450 nm eine Untergrundkorrektur für die Amplitude bei 415 nm durchgeführt wird (rote Linie). Diese schlägt für Proben mit erhöhtem Bilirubin (rechts) insofern fehl, als eine zu hohe Amplitude für die fHb Absorbanz angenommen wird.

mehr Punkten den variablen Probenuntergrund annähern, um diesen Untergrund dann abzuziehen [63-66]. Die spektrale Quantifizierung von fHb ist in der klinischen Routine weit verbreitet. So verwenden 85 von 120 Teilnehmern eines deutschen Ringversuchs zur fHb-Bestimmung spektralen Methoden [67], von denen die bekanntesten jeweils nach Harboe [63], Noe [66], Fairbanks [65] und Kahn [64] benannt sind. Bei den ersten drei Methoden (Harboe, Noe, Fairbanks) findet die lineare Untergrundkorrektur um das Soret-Band-Maximum von Hämoglobin bei 415 nm statt, bei der Methode nach Kahn um das zweite Maximum der Q-Banden bei 578 nm. Eine dieser Methoden ist in den klinischen Laboren häufig in einer Microsoft Excel-Tabelle mittels einer einfachen Formel implementiert und einfach durchzuführen, was ein Grund für die weite Verbreitung der spektralen Methoden ist. Bei Methoden, welche das Hämoglobinmaximum bei 415 nm verwenden, überlappt das Spektrum von Bilirubin mit diesem, wodurch die Methode bei Vorliegen einer Hyperbilirubinämie, welche oftmals mit Hämoglobinämie koinzidiert, falsch erhöhte Werte anzeigt [63]. Das Prinzip der Korrektur nach Harboe ist in Abbildung 4 gezeigt, wo erkennbar ist, dass die lineare Interpolation zwischen 380 nm und 450 nm für ikterische Proben nicht den tatsächlichen Verlauf des Probenuntergrunds modelliert. Dies gilt ebenfalls für die anderen beiden Methoden [2, 65, 66], die das Maximum bei 415 nm verwenden, obwohl eine der beiden sogar angibt, Bilirubin zu berücksichtigen [65]. In Fällen mit Bilirubin in der Probe wird die Methode nach Kahn verwendet, da Bilirubin bei 578 nm keine Absorption zeigt (siehe Abbildung 4, rechts). Die Methode nach Kahn wird jedoch an unverdünnten Proben durchgeführt, was sie anfällig für Trübungen im Blutplasma macht (zum Beispiel durch Triglyceride). Zudem zeigt die Bestimmung von fHb mit der Methode nach Kahn schon im Originalmanuskript [64] und auch in der Routinebestimmung im Labor eine systematische Unterschätzung der tatsächlich vorhandenen fHb Konzentration gegenüber der Referenzmethode mit Benzidin [2]. Es besteht also der Bedarf nach einer verlässlichen, von Bilirubin unbeeinflussten Quantifizierungsmethode, die in der Anwendung nicht komplizierter ist als die Multiwellenlängen-Methoden, die als einfache Formel in Excel implementiert sind. Da bei der Methode nach Harboe je nach Probe eine unterschiedliche Verdünnung verwendet werden muss [68], sind weitere Herausforderungen an eine neue Methode eine Vereinfachung der Probenvorbereitung und eine direkte Korrektur des Ergebnisses je nach Verdünnung der Probe.

SIMULTANE QUANTIFIZIERUNG VON FREIEM HÄMOGLOBIN UND BILIRUBIN IN BLUTPLASMA

Zur Entwicklung einer neuen Quantifizierungsmethode wurde ein Messprotokoll nach den Vorgaben der „European Medicines Agency“ für die bioanalytische Methodenevaluierung erstellt [47], in welchem Kalibratorproben, Proben mit erhöhtem fHb und Proben mit erhöhtem fHb und gleichzeitig erhöhtem Bilirubin vermessen wurden und diese gegen die Methoden von Harboe, Fairbanks und Noe verglichen wurden. Diese Probensets umfassten 492 Einzelproben, anhand welcher der mathematische Näherungs-Algorithmus an der zweiten Ableitung sowohl für Spektren normaler, hämolytischer als auch ikterischer und hämolytischer Proben optimiert wurde. Der relative mittlere Fehler und die relative mittlere Standardabweichung betragen dabei für nicht ikterische Proben weniger als 3% und für ikterische Proben je nach Konzentration zwischen 20% und 1,9%. Die anderen Methoden überschätzen die Konzentrationen von fHb in ikterischen Proben jedoch teils um einen Faktor 8-9, was die deutliche Überlegenheit der hier vorgestellten Methode für ikterische Proben demonstriert. Da die Quantifizierung von fHb mittels der

Minimierung der zweiten Ableitung den spektralen Anteil von Hämoglobin berechnet, kann das ursprüngliche Spektrum um diesen Anteil korrigiert werden. Das verbleibende Spektrum kann danach direkt am Absorptionsmaximum von Bilirubin ausgewertet werden, wodurch zusätzlich der Anteil von Bilirubin in der Probe quantifiziert werden kann. Zusätzlich wurde der Einfluss von Methämoglobin und Methämalbumin auf die neu entwickelte Methode untersucht, da freies Hämoglobin im Körper nach wenigen Stunden zu Methämoglobin und Methämalbumin umgeformt wird, welche beide ein verschobenes Absorptionsspektrum aufweisen. Es wurde ein linearer Zusammenhang mit der Konzentration von freiem zweiwertigen Hämoglobin und dem Fit-Ergebnis festgestellt, wodurch belegt wurde, dass die Auswerteroutine oxidierte Häm-Protein Komplexe bei der Quantifizierung vernachlässigt.
Die resultierende Methode wurde „HEBI-Fit“ (HEmoglobin - BIlirubin-Fit) genannt und in Microsoft Excel in ein Makro implementiert, wodurch jeder potentielle Anwender ein gemessenes Spektrum seiner Proben in Excel einfügen, den Verdünnungsfaktor der Probe eingeben und mit dem Betätigen einer Schaltfläche nicht nur die Auswertung mit dem entwickelten Fit erhält, sondern zusätzlich die Ergebnisse entsprechend den Auswerteverfahren nach Harboe und Kahn. Das Makro wurde der Publikation als „Supplementary File“ angehängt, um die Methode einem möglichst breiten Publikum bereitzustellen.
Der Anteil von Alexander Lang an dieser Studie war zu 50% die Planung der Probenzahl und des Messablaufs, zudem die Entwicklung und Optimierung des mathematischen Auswerte-Algorithmus für sowohl fHb als auch Bilirubin, die Auswertung aller Messungen und der Vergleich zu Referenzmessmethoden, die Korrektur des entwickelten HEBI-Fit Microsoft Excel Makros, sowie zu 50% das Verfassen des Manuskripts mit Korrekturen und Anregungen der Koautoren.

ORIGINALMANUSKRIPTE

RAPID SPECTROPHOTOMETRIC QUANTIFICATION OF URINARY PORPHYRINS AND PORPHOBILINOGEN AS SCREENING TOOL FOR ATTACKS OF ACUTE PORPHYRIA

Lang, Alexander; Heckl, Christian; Vogeser, Michael; Stauch, Thomas; Homann, Christian; Hennig, Georg; Sroka, Ronald; Stepp, Herbert
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Rapid spectrophotometric quantification of urinary porphyrins and porphobilinogen as screening tool for attacks of acute porphyria

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Abstract. Autosomal-dominant acute porphyria, a group of rare diseases, can lead to life-threatening neurovisceral attacks. No efficient screening test is available today. Elevated urinary porphobilinogen in addition to elevated porphyrins is highly specific for an attack of acute porphyria. This study proposes and evaluates a custom-made device, algorithm, and methods for a two-step quantification of urinary porphyrins and porphobilinogen. The first step is oxidation of the nonfluorescent porphyrinogens and subsequent fluorescence-spectroscopic determination of total urinary porphyrins (TUP) using second derivative spectral fitting. Photo-oxidation is compared with chemical oxidation methods. The second step is the quantification of porphobilinogen in case of elevated TUP. Heat-induced conversion products of porphobilinogen, namely uroporphyrin and porphobilin, are quantified by fluorescence and absorption spectroscopy. Results show that the preferred method combination is TUP quantification (lower limit of quantification: 0.2 μmol/L) after photo-oxidation with subsequent absorption-spectroscopic determination of porphobilin after heating for indirect quantification of porphobilinogen (quantification range: 0 to 20 mg/L). Urinary porphobilinogen and porphyrins of one acute porphyria patient were quantified with <10% deviation from an external reference determination. The spectrophotometric approach requires only minimal sample processing and yields a result within 15 min, thus closing the screening gap for acute porphyria. [DOI: 10.1117/1.JBO.23.5.055006]

Keywords: acute porphyria; fluorescence; absorption; spectroscopy; porphyrins; porphobilinogen; urine.

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1 Background

Porphyrias are a group of rare, mainly genetic metabolic disorders of the heme biosynthesis pathway,[1] each caused by the malfunction of one of the eight enzymatic steps in the formation of heme and each causing a specific accumulation pattern of heme precursors in blood, urine, and stool.[1] The diseases can be broadly classified as either acute or chronic porphyria.[1,2,3] This work focuses on the autosomal dominant, acute porphyrias group, namely acute intermittent porphyria, hereditary coproporphyria, and variegate porphyria, which constitute the most common acute porphyrias with a combined prevalence of ~1 in 10,000 people.[1,2] Unlike chronic porphyrias, the acute porphyrias pose the risk of life-threatening neurovisceral attacks that typically begin with neuropathic abdominal pain but, if untreated, may later progress to mental disturbances including seizures, quadriplegia, and respiratory paralysis leading to death.[1,2]

The universal recommendation for the diagnosis of an acute attack of porphyria is rapid screening for urinary porphobilinogen (PBG).[1,2,4,5,6] Urinary PBG is strongly elevated during an attack, sometimes by more than a factor of 50 compared to healthy individuals (from <2 mg/L to over 110 mg/L).[1,2] Detection of substantially elevated PBG in urine is a highly specific indicator for acute porphyria and therefore provides the required specificity for a rare disease. Automated tests for PBG on clinical chemistry analyzers are not available. Commercial tests, often based on Mauzerall and Granick,[7] are labor intensive and require substantial practical expertise.[4] This is not suitable for a simple screening setting in the emergency room or in a 24/7 short turn-around time clinical laboratory.[5] Another, more suitable commercial screening test (Thermo Fisher Scientific Porphobilinogen "Trace" kit), which provided a semiquantitative result (discriminability of PBG concentrations of <6 mg/L, 6 to 12 mg/L, 12 to 23 mg/L, or higher),[4] was withdrawn from the market in 2014. Currently, tests for PBG at external laboratories require turnaround times of 4 to 10 days.[1] This leaves most hospitals without a simple, specific, and rapid screening tool for acute porphyria in patients with neuropathic abdominal pain. The lack of widespread screening tools and the ambiguity of the symptoms of acute porphyria lead to a remarkable average delay of diagnosis of 15 years from the onset of the first symptoms.[4] This delay often results in costly misdirected medical care and progression of the disease.[1]

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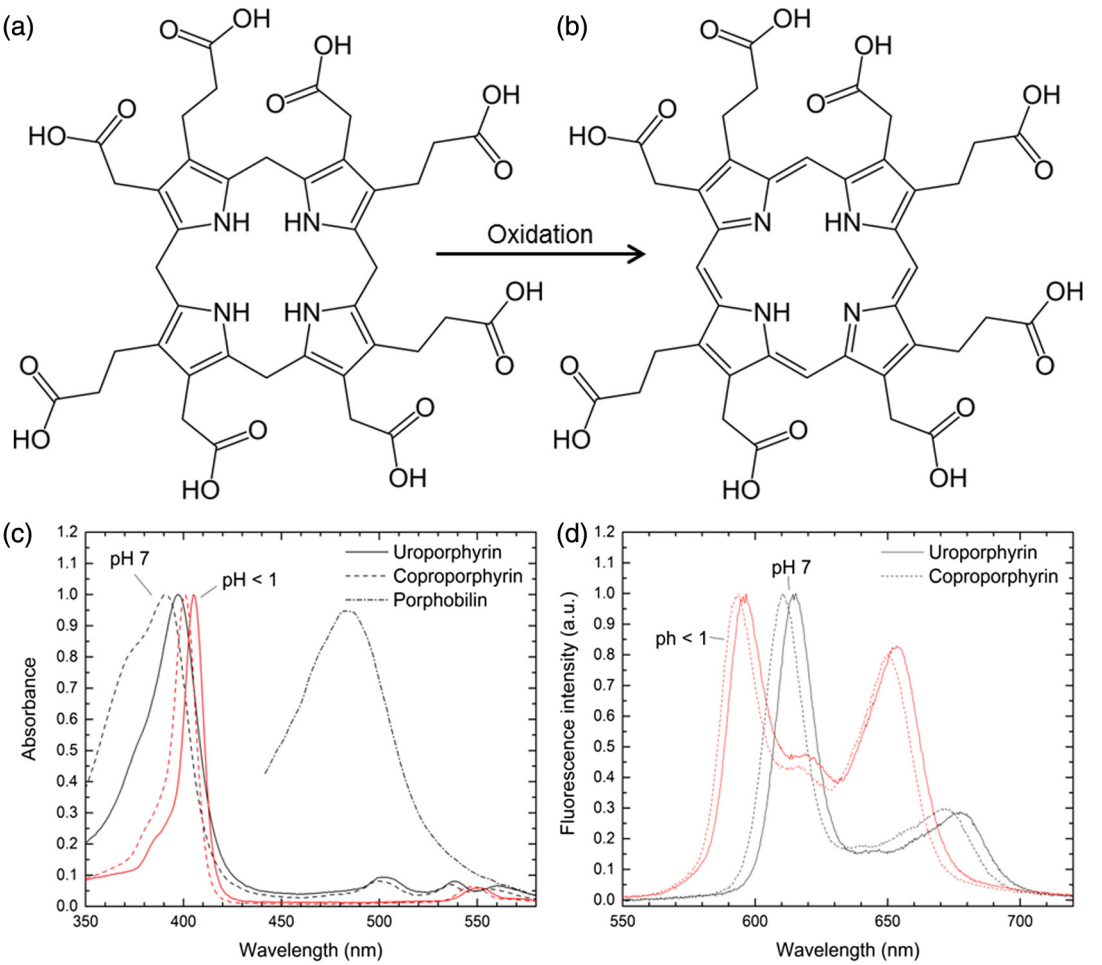

Fig. 1 (a) Structural formula of uroporphyrinogen III, (b) which is oxidized to uroporphyrin III. By losing six hydrogen atoms a conjugated system with delocalized pielectrons is formed, giving rise to strong UV/VIS absorption and fluorescence properties of the porphyrin as shown in (c) for absorption of uroporphyrin, coproporphyrin, and porphobilin as well as in (d) for fluorescence of uroporphyrin and coproporphyrin. For both graphs porphyrin spectra are shown for neutral (black) and acidic (red) environment.

Urine of patients suffering from an acute attack of porphyria contains elevated levels of porphyrins, porphyrinogens (the nonfluorescent, unoxidized form of porphyrins, Fig. 1), and PBG.[1] Porphyrinogens and porphyrins can also be elevated in urine from patients with liver disease or some other disorders (porphyrinuria), but porphyria can be excluded, if normal levels of porphyrinogens and porphyrins are found in the urine.[1] As the amount of porphyrinogens which had already auto-oxidized to porphyrins may vary greatly between patients, prior oxidation of porphyrinogens to porphyrins is required to quantitatively assess total urinary porphyrins (TUP), be it due to porphyria or porphyrinuria. We propose a two-step approach to (1) discriminate patients with elevated TUP from patients with normal TUP, and (2) quantify PBG in the group with elevated TUP. This two-step approach can be assumed to offer a very high specificity and to reduce measurement time in those with normal TUP.

For determination of TUP in the first step, the nonfluorescent porphyrinogens have to be oxidized to avoid underestimation of TUP, since porphyrinogens constitute on average 77% of TUP in fresh urine samples of acute porphyria patients.[5] In this study, we compare different oxidation procedures quantitatively. For spectroscopic PBG quantification in the second step, we propose the conversion of PBG to uroporphyrin and porphobilin by heating as reported in the 1950s,[6–8] since PBG shows no pronounced UV/VIS absorption or fluorescence emission,[6] and other tests based on Ehrlich reaction (Watson–Schwartz, Hoesch) are prone to interference.[9] We compare the subsequent detection of one of those heating products by fluorescence and absorption spectroscopy to discriminate PBG concentrations of 0, 5, 10, and 20 mg/L or higher, allowing the identification of patients with an attack of acute porphyria.

2 Material and Methods

2.1 *Urine Samples*

Spot urine of nine healthy volunteers (50 to 100 ml) was collected, anonymized, and stabilized with one part of TRIS acetate buffer with ethylenediaminetetraacetic acid (EDTA) (Rotiphorese® 10× TAE Buffer, Carl Roth, Karlsruhe, Germany) on nine parts of urine. The individual samples were pooled, aliquoted in 2 ml portions, and frozen at −20°C. Ethics approval was granted for the collection from volunteers by the Institutional Ethical Board of the Medical Faculty, Ludwig-Maximilians-University of Munich, Germany (study identifier: 679-15).

Left over urine of one acute intermittent porphyria patient was collected from the clinical laboratory after anonymization. TUP and PBG were determined using reference methods from the clinical routine. (ClinEasy® Complete Kit for Total Porphyrins in Urine, RECIPE Chemicals + Instruments GmbH, Munich, Germany, with 70% recovery and 7.2% interassay imprecision and ALA/PBG by Column Test #187-1002, Bio-Rad Laboratories, Hermes, California, with 2.6% interassay imprecision.)

2.2 *Reagents*

Porphyrins (Uroporphyrin I dihydrochloride and Coproporphyrin I dihydrochloride, Merck, Darmstadt, Germany) were dissolved in phosphate-buffered saline (PBS Dulbecco w/o $Ca^{2+}Mg^{2+}$, BioChrom, Berlin, Germany) to generate stock solutions which were quantified by absorption spectroscopy (Lambda-40, Perkin Elmer PE, Waltham, Massachusetts) using extinction coefficients [5.4×10^5 L/mol cm for uroporphyrin in 0.5-M hydrochloric acid (HCl) and 4.9×10^5 L/mol cm for coproporphyrin in 0.1-M HCl] taken from the literature,[4] resulting in concentrations of 50.4 and 72.1 μmol/L of uroporphyrin and coproporphyrin, respectively. A PBG (Merck, Darmstadt, Germany) stock solution with a concentration of 400.0 mg/L was prepared by weighing (MC1 RC 210P-0D1, Sartorius, Göttingen, Germany) and dissolving PBG in distilled water. Hydroxymethylbilane synthase protein (HMBS, His-tag protein, antibodies-online, Aachen, Germany) for generation of porphyrinogens from PBG was stored at −20°C.

2.3 *Instrumentation*

The laboratory prototype used for this investigation is shown in Fig. 2(a). Core component is an aluminum sample holder with an opening for a cylindrical, disposable 200 μl sample glass cuvette (flat bottom insert 548-0780, VWR, Darmstadt, Germany). A band heater (65 W, Acim Jouanin, Évreux, France), controlled with a relay switch (part number 194883, Conrad Electronic, Hirschau, Germany), allows heating of a sample to preset temperatures ($37.5° < T < 93°$C). All parameters of the device can be set via a LabVIEW interface (LabVIEW 2015, National Instruments, Austin, Texas) from a tablet computer. In Fig. 2(b), the optical geometry for fluorescence and transmission measurements is shown. For transmission measurements, light of an LED (425 to 700 nm, APG2C3-NW, Roithner Lasertechnik, Vienna, Austria) is guided by an optical fiber (NA = 0.22, core diameter 105 μm, type: M15L, Thorlabs, Newton, New Jersey) to the sample holder, collected after transmission by a detection fiber (NA = 0.39, core diameter 200 μm, type: FT200UMT, Thorlabs, Newton, New Jersey), and guided to the spectrometer (S2000, Ocean Optics, Dunedin, Florida). For fluorescence emission measurements, light of a laser diode (402 nm, 2 nm FHWM, 2.6 mW, SLD3134VR-31, Laser Components, Olching, Germany) is guided by an optical fiber (NA = 0.39, core diameter 200 μm, FT200UMT, Thorlabs, Newton, New Jersey) to the sample, and the fluorescence emission is collected in front-face geometry by an adjacent detection fiber [Fig. 2(b)]. Excitation light is filtered out before the spectrometer with a long-pass filter unit (435 nm INLINE-FH, Ocean Optics, Largo, Florida) integrated between two fiber pieces. All flat-top polished fiber ends were in contact with the cuvette. The excitation and detection fiber were positioned on top of each other, which minimizes the effect of the curvature of the cuvette on the detection efficiency. For the photo-oxidation of porphyrinogens, a light source (401 ± 30 nm, D-Light 20133220 with short-pass filter at 435 nm, Karl Storz, Tuttlingen, Germany) was used to illuminate the sample from the top by placing a fluid light guide (5 mm diameter) in contact with the top surface of the cuvette. The irradiance over the whole cuvette cross section at the height where the detection fiber connects to the glass cuvette was 45.5 mW/cm^2. Fluorescence spectra were normalized to the fluorescence of a rhodamine standard (1BF/RB Rhodamine, Starna Cells Inc., Atascardo, California) which was incorporated into one of the glass cuvettes whereas transmission spectra were normalized to the transmission of the LED through a water-filled cuvette. The laser diode power and calibration curves were stable over a period of several months during which all measurements were performed. The relative standard deviation

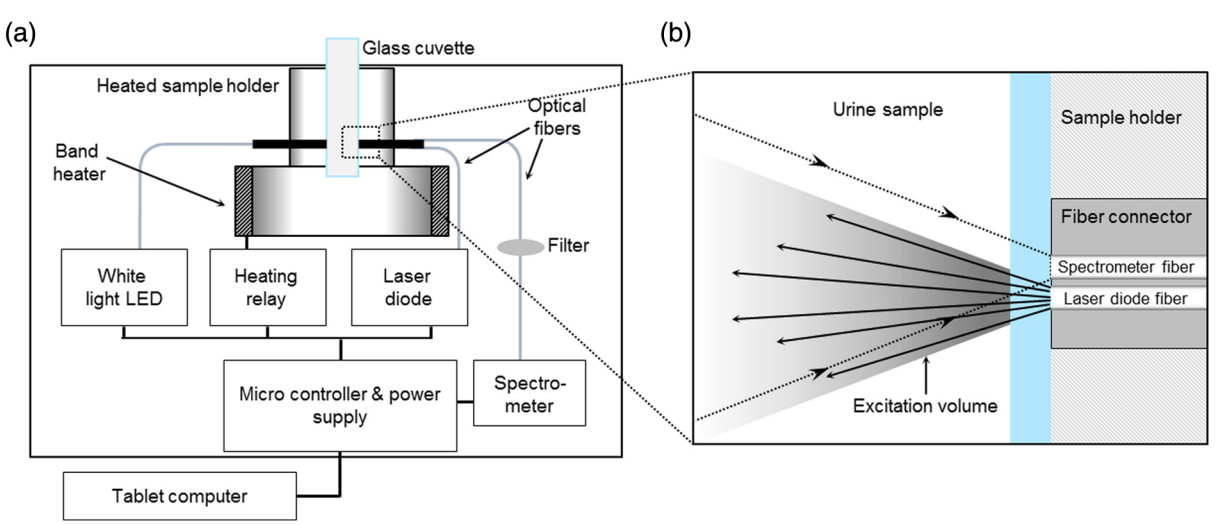

Fig. 2 (a) Schematic overview of the device for absorption and fluorescence-spectroscopic measurements on heated urine samples and the geometry of the sample holder, (b) detail of the excitation and detection of fluorescence light from the same side (180-deg geometry) using optical fibers with 200-μm core diameter and a gap of ~50 μm.

of the fluorescence standard signal was 10.9%. A blank spectrum and a fluorescence standard spectrum were recorded for normalization once per measurement day.

2.4 *Comparison of Methods for Porphyrinogen Oxidation*

Uroporphyrinogens were generated from PBG by the use of HMBS enzyme. A TAE-buffered urine sample was spiked to a concentration of 40 mg/L PBG and 12 mg/L HMBS, and incubated at 37.5°C for 1 h which resulted in an average concentration of porphyrinogens of ~25 μmol/L (quantified after complete oxidation). The sample containing porphyrinogens was then rapidly split into 12 subsamples of four varying concentrations for each of the three oxidation methods. At least 88% of porphyrins were present as porphyrinogens before oxidation, as derived from fluorescence measurements after incubation compared to the fluorescence after use of iodine. It was verified that each of the oxidation procedures stops the enzymatic reaction by performing each procedure immediately after spiking with the enzyme. In this case, subsequent incubation did not result in a detectable increase of porphyrins.

Oxidation with iodine was reported to oxidize 100% of urinary porphyrinogens after 1 h.[4] It was compared to oxidation with H_2O_2[2] and a new approach using photo-oxidation with controlled illumination in terms of speed, concentration dependence, and completeness of the oxidation.

For the oxidation with iodine, one part of urine was mixed with 0.9 parts of 1M HCl and 0.1 part of 12 g/L iodine in ethanol, and fluorescence of porphyrins was measured after waiting 1 h at room temperature.[4] For the oxidation with H_2O_2, one part of urine was mixed with four parts of 1.25M HCl and 0.04 parts H_2O_2 30%. No waiting time is reported in the literature.[2] Fluorescence spectra were recorded every 10 min over 1 h for both chemical oxidation procedures. For the controlled photo-oxidation, one part of urine was mixed with one part of 1M HCl and illuminated. Fluorescence spectra were recorded after 10, 20, 30, 60, 120, 180, and 240 s illumination time, which equals a radiant exposure of 0.46, 0.91, 1.37, 2.73, 4.10, and 5.46 J/cm^2.

In addition, blank TAE-buffered urine was subjected to the three methods and blank spectra were recorded. The blank spectra were subtracted from the fluorescence spectra acquired from the three procedures to account for possibly unoxidized porphyrinogens in the pooled urine and the resulting spectra were normalized to the rhodamine fluorescence standard. The mean peak fluorescence intensities at (595 ± 2) nm derived from the different oxidation methods were compared to each other for each of the four concentrations, using iodine oxidation as reference. The generation of porphyrinogens and subsequent oxidation and measurement was repeated independently nine times.

The fluorescence intensities were calibrated for the different oxidation methods, due to different dilutions and the use of iodine and H_2O_2. Calibration factors were derived by performing the oxidation procedures on buffered neutral urine samples which were spiked with uroporphyrin. The concentrations were chosen to yield fluorescence intensities comparable to the samples with uroporphyrin generated from PBG by HMBS. The mean calibration factors are 2.7, 5.4, and 2.4 for iodine, H_2O_2, and photo-oxidation, respectively. Most of the factor can be derived from the dilution (factor 2 for iodine and photo-oxidation, factor 5 for H_2O_2) and higher absorption of excitation and fluorescence light (for iodine).

2.5 *Generation of Calibration and Validation Sets for Total Urinary Porphyrin Quantification*

The evaluation of porphyrin quantification was performed on two sample sets, one for the calibration and one for the validation of the method according to the guideline of bioanalytical method validation of the European Medicines Agency (EMA guideline).[24]

2.5.1 *Calibration set*

Three sample-sets each consisting of eight concentrations of porphyrins (0.1, 0.5, 1, 2, 5, 10, 17.5, and 25 μmol/L) were created. Each sample was prepared in the same way as for the photo-oxidation: one part of TAE-buffered urine, spiked with the desired porphyrin concentration and one part of 1M HCl. Each of the three sample sets had a different molar ratio of uroporphyrin I to coproporphyrin I, with 25% to 75%, 50% to 50%, and 75% to 25% of total porphyrins, respectively. Each sample set was replicated four times independently from new aliquots.

2.5.2 *Validation set*

The validation set consisted of three measurement series. Each series contained 16 samples which were replicated five times independently, resulting in 240 samples. The 16 samples are generated by combining four concentrations of uroporphyrin and coproporphyrin (0.03, 0.1, 5.5, and 9 μmol/L) in every possible combination, resulting in 16 combinations ranging from 0.06 to 18 μmol/L of TUP. Samples with equal concentrations of TUP were averaged for evaluation, resulting in eight concentrations of TUP (in μmol/L: 0.06, 0.13, 0.2, 5.56 (5.53 and 5.6 averaged), 9.06 (9.03 and 9.1 averaged) 11, 14.5, and 18).

2.6 *Second Derivative Fitting Algorithm and Evaluation of the Validation Set*

The second derivative fitting algorithm is designed to quantify TUP from fluorescence spectra measured on acidic urine samples. The following description is also presented in a flowchart in Fig. 3(c). After recording a fluorescence spectrum of a sample, a previously recorded dark spectrum is subtracted and the intensity of the resulting spectrum is normalized to the rhodamine fluorescence standard at (575 ± 2) nm. Afterward, the amplitude of TUP fluorescence is quantified by the fit that minimizes the second derivative of the specimen spectrum after subtracting two iteratively varied reference spectra of uroporphyrin I and coproporphyrin I.

During the fit procedure, normalized uroporphyrin and coproporphyrin fluorescence emission spectra [see Fig. 3(a), dashed and dash dotted] are subtracted from the sample spectrum [Fig. 3(a), solid]. After subtraction [Fig. 3(a), dotted], the second derivative [Fig. 3(b)] is computed by a Savitzky–Golay filter with a window size of 41 nm and a third-order polynomial approximation.[25] Window size and order of polynomial approximation were varied on the calibration set and employed on the evaluation set until inaccuracy and imprecision were minimal. The fit varies the amplitude of the reference spectra, until the square of all points of the second derivative of the resulting residual spectrum is minimized (least squares approach),

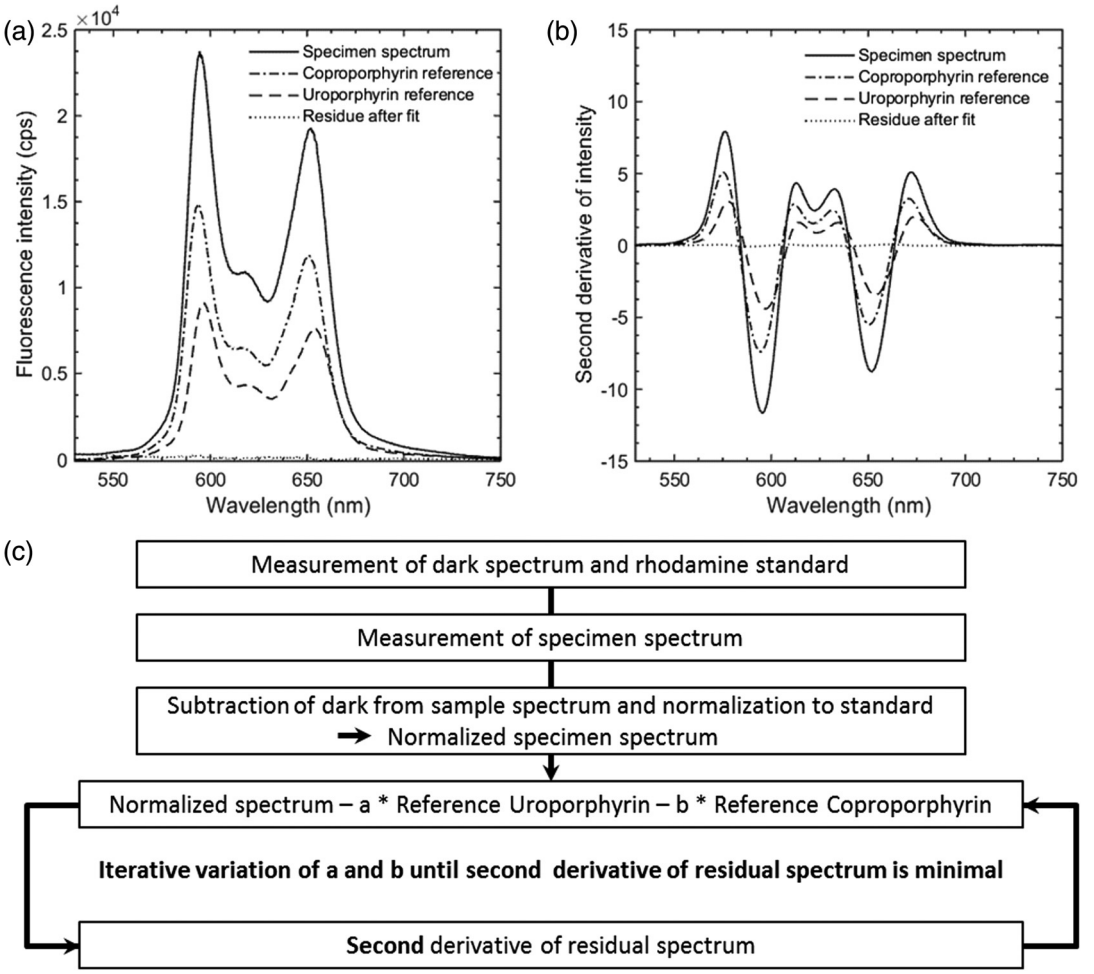

Fig. 3 During the second derivative fitting procedure, two reference spectra of uroporphyrin (a, dashed) and coproporphyrin (a, dash dotted) are subtracted from a normalized specimen spectrum (a, solid) and the second derivative of the residuum (b, dotted) is calculated. The amplitude of the reference spectra is iteratively varied by the fit until the second derivative of the residue after the fit is minimal. This allows an accurate approximation of the fluorescence amplitude while being undisturbed by the variable urine background fluorescence. A flowchart describing the steps that yield the result of the fit a (uroporphyrin amplitude) and b (coproporphyrin amplitude) is shown in (c).

which equals a minimization of the curvature of the spectrum. The sum of the resulting amplitudes of the fitted uroporphyrin and coproporphyrin reference spectra is considered the TUP fluorescence. A similar fit was applied for the quantification of free hemoglobin in blood plasma in the presence of bilirubin.[12]

When applying this fit on the calibration set, three sets of data points for the three different molar ratios of uroporphyrin and coproporphyrin are created from the TUP fluorescence intensities of the reference spectra [Fig. 4(a)]. The function of the TUP concentration C_{TUP} against the fluorescence intensity was found to be nonlinear over the concentration range. The optical system could not be described by an analytic model function due to the complex optical geometry consisting of a round cuvette and two separate fibers combined with the nonlinear change of the detection volume caused by the absorption of the fluorophore at high concentrations. Thus, the intensities derived from the validation set were evaluated by using linear interpolation between the data points of the calibration set. All measurements were corrected by the amount of porphyrins present in the pooled urine of healthy specimens before spiking.

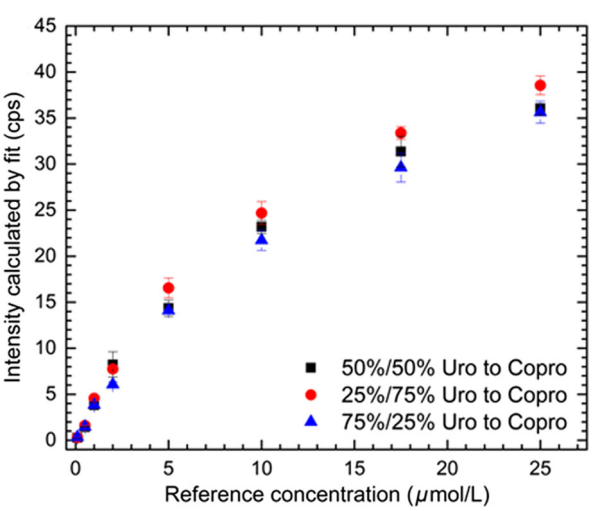

Fig. 4 The calibration curves generated from samples with different molar ratios of uroporphyrin and coproporphyrin show a nonlinear correlation between fluorescence intensity and concentration, albeit only small differences are observable between different molar ratios.

The second derivative fit was employed on all spectra generated from the validation set and the resulting fluorescence intensities were quantified using one of the three calibration curves. If the ratio of uroporphyrin to total porphyrin fluorescence in a given sample was determined by the fit to be between 0.42 and 0.58, the 50% to 50% calibration curve was used. In the other two cases, the respective calibration curve calculated from samples with 75% to 25% molar ratio or 25% to 75% molar ratio was used. The cut-offs were determined by optimizing inaccuracy and imprecision of the evaluation set.

2.7 *Comparison of Methods for Indirect Quantification of Porphobilinogen*

Nonenzymatic conversion of PBG in a mild acidic, aqueous solution to uroporphyrin and porphobilin by heating at 80°C in acidic environment (pH 5.2) was reported in the 1950s.[14,15] Based on these reports we performed measurements at pH 5.2; however, in urine, with more samples ($n = 75$) and a wider range of concentrations of PBG to assess the feasibility of a quantification method. TAE-buffered urine was spiked with PBG to concentrations of 0 (blank), 5, 10, 15, and 20 mg/L and acidified to pH 5.2 by addition of 4.8 μL of 1M HCl per 100 μL of sample volume. Since we found that higher temperatures accelerate the generation of heating products (data not shown), the samples were heated to 93°C instead of 80°C. Samples were replicated five times from fresh aliquots of PBG and urine, and each measurement series was repeated three times. The first measurement series was used as calibration set for evaluation of the following two measurement series. Fluorescence and transmission spectra were recorded every 30 s for 20 min during heating. Since heating to 93°C takes about 1 min in our setup, the first two spectra of each measurement were discarded, due to the change of fluorescence and transmission induced by the change in temperature. The third spectrum (after 1 min of heating) was subtracted from all other spectra as baseline to evaluate only the spectral change of both fluorescence and absorption (one-transmittance). The resulting fluorescence spectra were evaluated at (615 ± 5) nm for an increase in uroporphyrin fluorescence (615 nm since the sample was still close to the neutral pH, see Fig. 1) and the resulting transmittance spectra at (480 ± 2) nm for an increase in porphobilin absorption. The calibration curve for the maximum change in fluorescence was modeled by linear interpolation between the points of the calibration set, as the complex geometry of the excitation and detection geometry which was already mentioned for the quantification of urinary porphyrin fluorescence is additionally aggravated by an unknown reaction kinetic of the formation of uroporphyrin from PBG. The maximum change in absorption (one-transmittance) was modeled by an exponential decrease of transmittance derived from "Lambert Beer's law" resulting in a function for the calibration set

$$A(C_{\mathrm{PBG}}) = a - e^{(-b \cdot C_{\mathrm{PBG}})}, \tag{1}$$

with fitting parameters a (baseline transmittance of sample) and b (change of transmittance).

2.8 *Statistical Evaluation*

Results were presented as

$$\text{Inaccuracy} = \frac{\text{Mean result} - \text{reference}}{\text{reference}}, \tag{2}$$

$$\text{Imprecision} = \frac{\text{Standard deviation}}{\text{reference}}, \tag{3}$$

for the different concentration intervals, with their definition in accordance with the EMA guideline for bioanalytical method evaluation.[24]

3 Results

3.1 *Comparison of Oxidation Methods*

Urinary uroporphyrinogens were oxidized using established chemical oxidation methods and photo-oxidation with controlled illumination conditions. The fluorescence intensity at (595 ± 2) nm of oxidized porphyrins was compared for each of the methods for different concentrations of initial porphyrinogens and is shown in Figs. 5(a) and 5(b), over the course of 60 min using iodine and H_2O_2 (bottom scale) and over 4 min illumination for photo-oxidation (green and top scale). For chemical oxidation with iodine or H_2O_2, the intensity increases during the course of 60 min, flattening toward the 60-min mark. Using photo-oxidation, the fluorescence intensity reaches its maximum with a steep rise after about 1 min and stays constant thereafter for higher concentrations of porphyrins [Fig. 5(a)], whereas the intensity decreases after 1 min for lower porphyrin concentrations [Fig. 5(b)]. On average, H_2O_2 oxidation yielded 86% of the fluorescence intensity retrieved from samples oxidized with iodine after 60 min, whereas photo-oxidation yielded between 64% and 83% after 1 min. Photobleaching during 1 min of illumination for photo-oxidation did not exceed 4% over the whole concentration range, while during 4 min of illumination, 13.1% to 14.8% of porphyrins were photobleached (data not shown).

3.2 *Quantification of Urinary Porphyrins with Second Derivative Spectral Fitting*

In Fig. 4, three calibration curves for urinary uroporphyrin and coproporphyrin quantification are shown for different molar ratios. The fluorescence intensity of the validation sample set was evaluated with the second derivative fit and quantified using the respective calibration curve. The results are shown in Figs. 6(a) and 6(b), and the inaccuracy and imprecision are reported in Table 1. Both inaccuracy and imprecision were in the range acceptable according to the EMA guideline with a maximum of 15% for concentrations of 0.2 μmol/L or higher.

3.3 *Indirect Spectrophotometric Quantification of Porphobilinogen*

In Fig. 7, the change of fluorescence intensity [Fig. 7(a)] and absorption (one-transmittance) [Fig. 7(b)] is shown over the course of more than 20 min of heating at (93 ± 1.5)°C. A variation of the initial PBG concentration results in different amplitudes for both fluorescence as well as absorption. While the

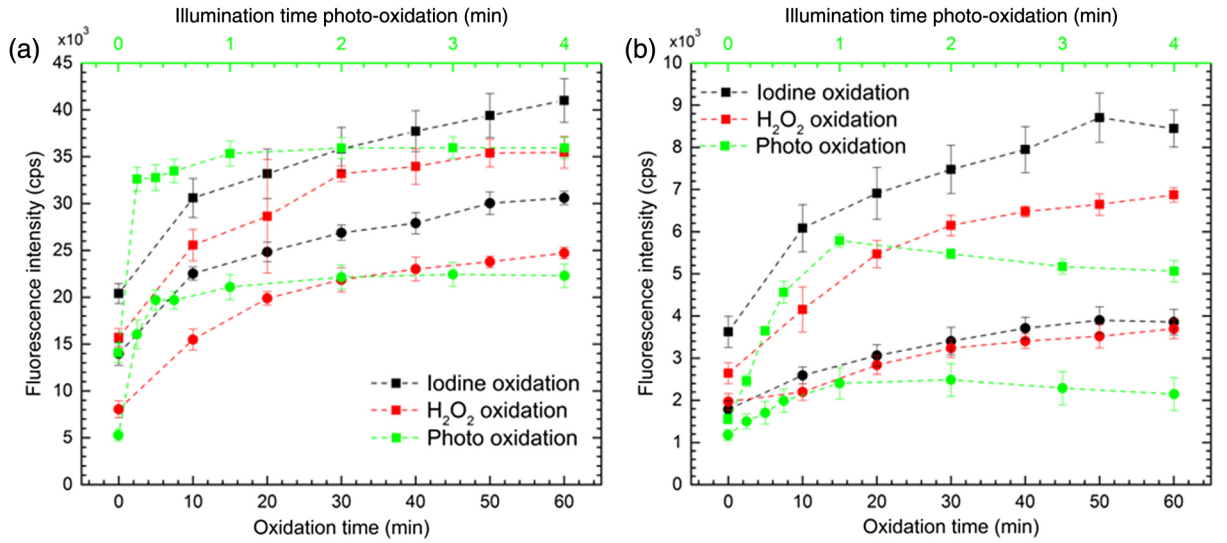

Fig. 5 The increase in fluorescence intensity at 595 ± 2 nm generated by oxidation of porphyrinogens in (a) concentrations of 25 and 12.5 μmol/L (square and round, respectively) and (b) concentrations of 2.5 and 1 μmol/L (square and round, respectively) is shown over the course of 1 h for iodine (black) and H_2O_2 (red) and for photo-oxidation (green, top axis) over the course of 4 min illumination time.

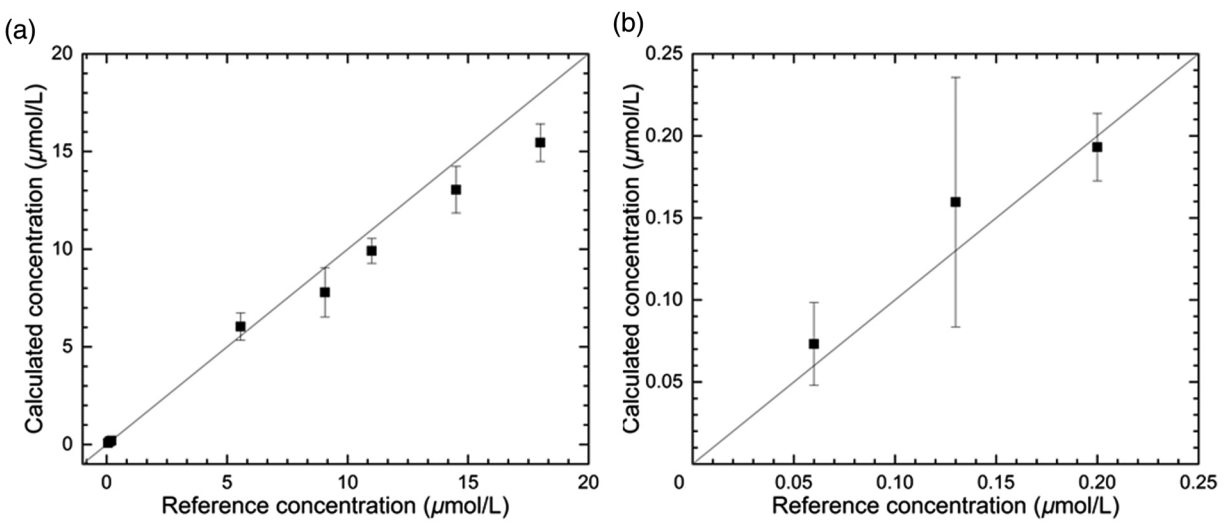

Fig. 6 (a, b) The quantification result of the independent validation sample set shows overall good recovery as described in Table 1 and high precision for concentrations of 0.2 μmol/L or higher.

maximum absorption is reached after ~9 to 10 min before decreasing, the fluorescence intensity reaches a stable level after 15 min. For both processes, the correlation between either maximal fluorescence or absorption and the concentration is nonlinear, as shown in Fig. 7(c). It is apparent that the fluorescence intensity change for concentrations of PBG of 10 mg/L or higher increases much steeper with an increase in initial PBG concentration than the change in absorption. A linear interpolation between the data points of the calibration set for the quantification of the change in fluorescence intensity

Table 1 Inaccuracy and imprecision relative to the reference concentration for the quantification of TUP in the validation sample set. For concentrations of 0.2 μmol/L or higher the limits of the EMA guideline are adhered (inaccuracy and imprecision within 15%; for the lower limit of quantification within 20% inaccuracy and imprecision).

Concentration (μmol/L)	0.06	0.13	0.2	5.56	9.06	11	14.5	18
Inaccuracy (%)	22	23	−3.4	8.6	−14	−9.9	−10	−14
Imprecision (%)	42	59	10	13	14	5.8	8.2	5.3

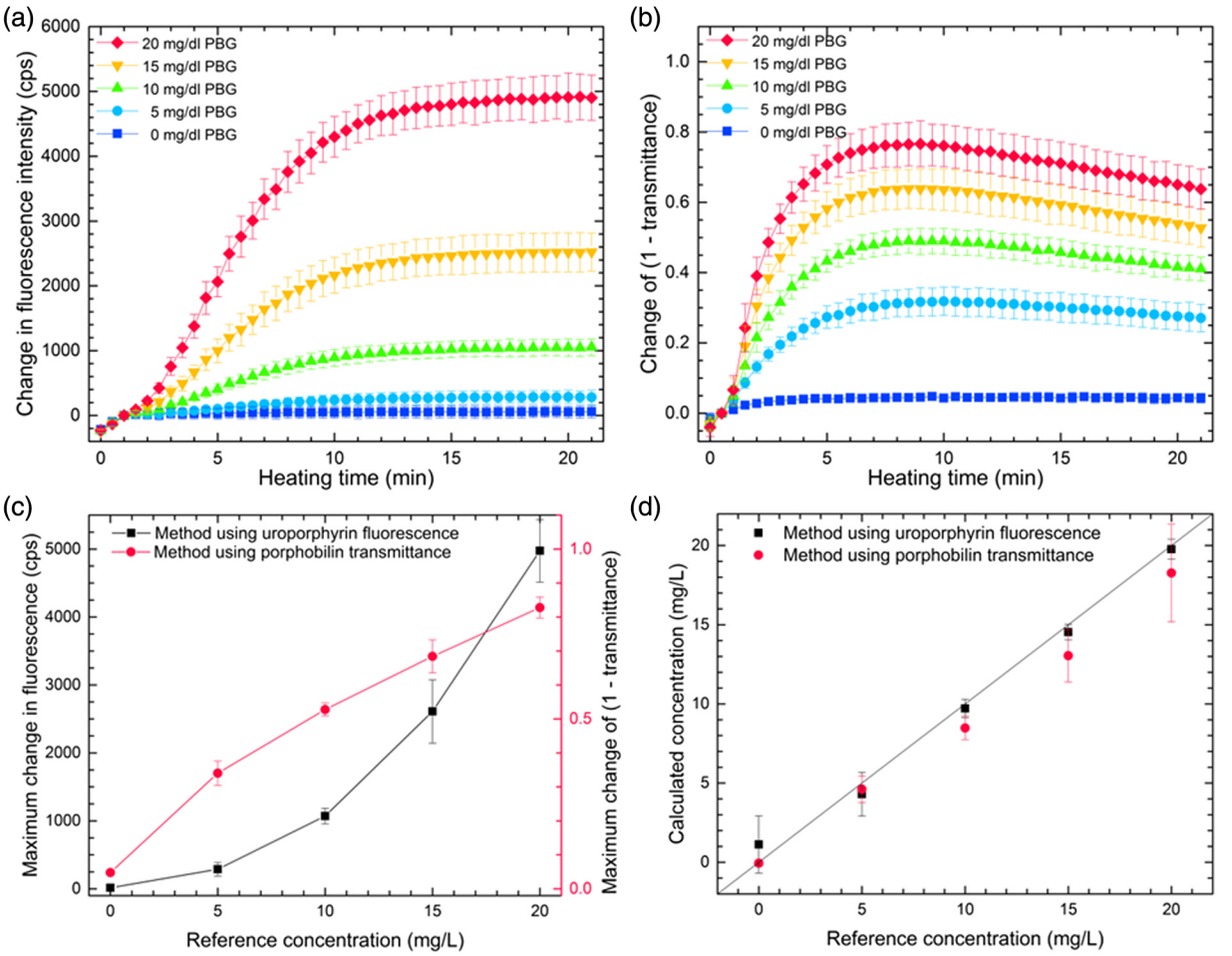

Fig. 7 The heating of PBG in urine induces the formation of uroporphyrin, detected using fluorescence emission at 620 nm and porphobilin, detected using its absorption at 480 nm. The change of (a) fluorescence intensity and (b) absorption over the course of 20 min shows a clear dependence on the initial PBG concentration. (c) The maximum change in fluorescence intensity after 20 min is shown against the initial reference concentration of PBG (black) as well as (c) the maximum change in absorption after 10 min (red).

and the derived calibration function [Eq. (1)] for the change in absorption were used for the quantification of PBG of the samples in the verification set. The resulting inaccuracy is <15% for both methods. The quantification using the porphyrin fluorescence, as shown in Fig. 7(d) and Table 2, has a high imprecision for the lowest concentration of PBG (5 mg/L), while absorption measurements also allow for the quantification of the lowest concentration with acceptable imprecision. The relative inaccuracies and imprecisions for all concentrations, except for blank samples (0 mg/L), are given in Table 2. Blank samples had a mean change in fluorescence of 50 counts with a standard deviation of 90 counts compared to a mean change of absorption of 40 counts with a standard deviation of 10 counts after 10 min. The parameters of the calibration function for the change of absorption equaled $a = 1.048$ (0.9821, 1.115; 95% confidence bounds) and 0.06916 [$(\mathrm{mg/L})^{-1}$; 0.05363, 0.08469; 95% confidence bounds].

Table 2 Comparison of inaccuracy and imprecision of the indirect determination of PBG using either fluorescence of uroporphyrin or transmittance of porphobilin.

	Fluorescence of uroporphyrin		Transmittance of porphobilin	
Concentration (mg/L)	Inaccuracy (%)	Imprecision (%)	Inaccuracy (%)	Imprecision (%)
5	−14	28	−8	17
10	−3	6	−15	7
15	−3	3	−13	11
20	−1	3	−9	15

3.4 *Proposed Method*

Based on the results, a new method is proposed for rapid screening for acute porphyria. It consists of two quantification steps as shown in Fig. 8. Samples are stabilized by adding TAE buffer. In the first quantification step, the sample is acidified and illuminated with blue light for rapid oxidation of porphyrinogens. Fluorescence spectra are recorded and the TUP concentration is determined. If TUP is not elevated, an attack of acute

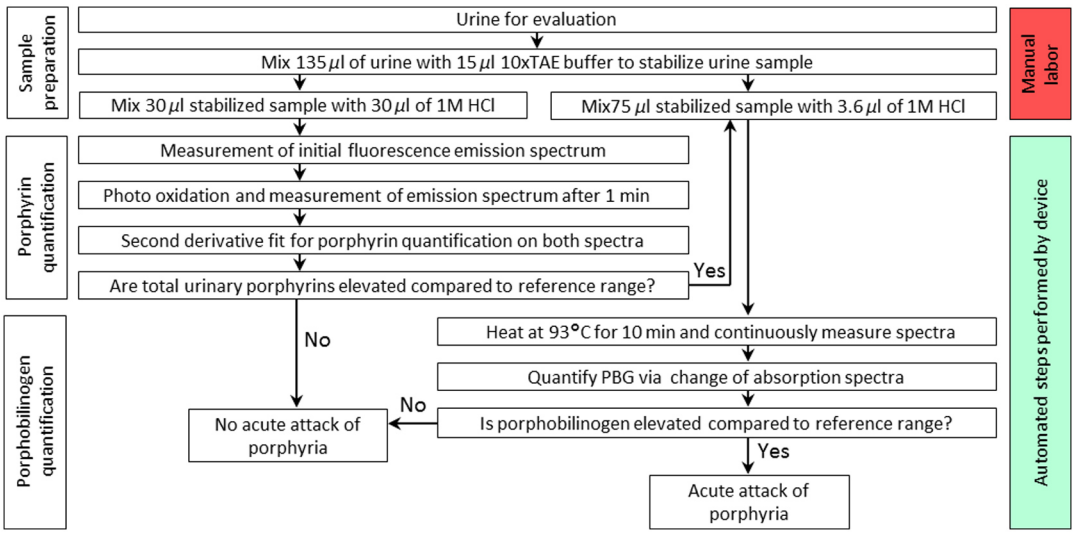

Fig. 8 Overview of the proposed method. Urine from a patient with abdominal pain is stabilized and mixed with 1M HCl. In a first step, porphyrin fluorescence of the sample is quantified before and after photo-oxidation of porphyrinogens within 1 min. The maximum amount of porphyrins found is compared to a threshold value. If porphyrins are not elevated, an acute attack of porphyria can be excluded. Otherwise, a second sample is heated for 10 min and the change of absorption induced by generation of porphobilin from PBG is quantified. If PBG is elevated, the patient is positively screened for an attack of acute porphyria. The method requires only minimal sample preparation, while quantification and illumination procedures are automated.

porphyria can be excluded. Otherwise, a second sample is mixed from the stabilized urine sample and HCl and heated for 10 min. The measurements during the heating process are completely automated, as well as the evaluation algorithm deriving the initial PBG concentration from the change of absorption introduced by the generation of porphobilin.

3.5 *Evaluation of One Acute Intermittent Porphyria Patient Sample*

The reference laboratory methods were applied to one urine sample of an acute intermittent porphyria patient, resulting in a concentration of 1.21 μmol/L TUP and 13.1 mg/L PBG. The sample was evaluated using the described methods. None of the oxidation procedures resulted in an increase in porphyrin fluorescence. The porphyrin quantification using the second derivative fit resulted in a TUP concentration of 1.43 μmol/L. During heating the sample, the change in absorption of porphobilin was quantified and yielded a concentration of 13.4 mg/L for PBG, whereas quantification of the change in fluorescence intensity of uroporphyrin resulted in a concentration of 14.0 mg/L for PBG.

4 Discussion

A device and several procedures were developed, evaluated, and compared to identify a simple and rapid test procedure to reliably quantify urinary porphyrins after oxidation of porphyrinogens and PBG. The test is aimed at the emergency setting to rapidly diagnose an attack of acute porphyria and allow for instant treatment.

The results show that oxidation of porphyrinogens with iodine is the most thorough oxidation method within the concentration range from 1 to 25 μmol/L. This is in accordance with reports in the literature,[24,25] where 100% oxidation of porphyrinogens by iodine is found and H_2O_2 oxidation yielded lower results than the use of iodine. While another study reports equal performance of iodine and H_2O_2 oxidation, the methods section lacks the details necessary for verifying the claim.[24] Chemical oxidation yields only 50% of the fluorescence intensity at 595 nm directly after the addition of chemicals (0-min value) compared to the intensity after 60 min. Since the proportion of auto-oxidized porphyrinogens may vary greatly from patient to patient, a simple correction is not possible. For example, 50% of TUP is neglected if no waiting time is adhered for a case where no porphyrinogens have auto-oxidized, but in cases where all porphyrinogens have auto-oxidized already 100% of TUP are present at 0 min. In contrast, photo-oxidation allows recording of oxidation kinetic already within 1 min, yielding information about the auto-oxidation status. If no porphyrinogens have auto-oxidized 63% of TUP are generated within 60 s of illumination, in a case where all porphyrinogens have auto-oxidized no fluorescence change is found, clearly indicating the lack of porphyrinogens. Since the porphyrin quantification after oxidation results only in a binary decision based on a threshold value, we deem controlled photo-oxidation as a sufficient measure for the oxidation of porphyrinogens in urine. After oxidation, the proposed method allows for quantification of TUP in concentrations between 0.2 and 20 μmol/L. The following hurdles had to be overcome: first, urine as a matrix has a variable pH and contains possibly interfering substances. The addition of TAE buffer allows for a pH-stabilization of urine samples in a range between pH 8.0 and 8.5 while EDTA binds free divalent metal cations such as $zinc^{2+}$, preventing the formation of zinc-porphyrins in urine. The additional acidification with molar HCl optimizes the oxidation rate.[24] Second, for samples of different individuals, urinary background fluorescence varies in intensity and spectral shape. The developed fitting algorithm minimizes the second derivative of the residual spectrum, effectively minimizing the influence of the broad and

unspecific but variable urinary background fluorescence. Third, the differences in fluorescence yield between equal molar concentrations of uroporphyrin and coproporphyrin can be minimized by choosing the optimal excitation wavelength (~402 to 403 nm, see Fig. 1, intersection of acidic uroporphyrin and coproporphyrin absorption spectra). An exact match would allow for a single calibration curve as the uroporphyrin and coproporphyrin intensity would be indistinguishable for equimolar concentrations. With the device assembled for this study, the fluorescence intensity of uroporphyrin and coproporphyrin was still slightly different, requiring the use of calibration curves with three different molar ratios. The use of only a single calibration curve with equimolar concentrations of uroporphyrin and coproporphyrin led in our case to an average increase of imprecision of 5%. Fourth, the 180 deg excitation and emission detection geometry used in the device was found to be crucial for the quantification of high porphyrin concentrations. The standard 90 deg fluorescence detection geometry commonly employed in commercial devices leads to a nonlinear correlation between fluorescence emission and concentration that is not bijective due to absorption of excitation light outside the spatial detection range of the detection fiber. This leads to a decrease of measured fluorescence intensity when a high concentration of porphyrins is further increased. The 180 deg geometry avoids this problem, since the detection volume is overlapping with the excitation volume also for high concentrations but modeling the correlation between fluorescence intensity and analyte concentration is still complex. The linear interpolation used in this study allowed for a reliable quantification of samples containing porphyrin concentrations of 0.2 μmol/L or higher. A more sophisticated model for the calibration such as a saturation function [Eq. (1)] can lower the imprecision and inaccuracy by 3% on average for higher concentrations but neglects the behavior of the correlation at lower concentrations. Another model using a bilinear approximation with an arctangent intersection

$$F(C_{\mathrm{TUP}}) = a * [\arctan(b * C_{\mathrm{TUP}})] + c * C_{\mathrm{TUP}}, \tag{4}$$

was also evaluated, as the calibration curve appears to consist of two linear functions which intersect at a concentration of 5 μmol/L. This did not show an improvement of inaccuracy which rose by 3.1% and only a slight decrease of imprecision by 1.9%.

The previously discussed optimizations for the procedure allow the quantification of TUP using fluorescence emission spectroscopy. Other spectrophotometric methods utilize fluorescence excitation or synchronous spectroscopy.[2,15,16] Although these methods may offer a higher analytical precision, they require a more sophisticated optical setup with a tunable light source, which is not available in most hospitals and the range of concentrations quantified in these studies was much smaller than in this paper. Quantifying higher concentrations is possible by these methods but requires at least one further dilution step.

The quantification of PBG is based on the detection of heating products of PBG by fluorescence or absorption spectroscopy. Using the change of absorption allows for a quantitative discriminability of concentrations of PBG <20 mg/L in spiked samples. The change of fluorescence intensity from uroporphyrin generated from PBG by heating yielded high imprecision for concentrations of 5 mg/L PBG or less. The order of reaction for both uroporphyrin and porphobilin generation is unknown. However, first and second order for the disappearance of PBG in the solution were excluded.[16] The absorption increase saturates with the derived calibration function [Eq. (1)] for increasing initial PBG. This leads to a lower imprecision for the quantification of concentrations of 20 mg/L or higher using fluorescence detection of uroporphyrin generated from PBG. The standard deviation of the results derived from the absorption determination on blank samples (0 mg/L PBG spiked) was nine times lower than that of the fluorescence quantification and the difference in mean amplitude between blank samples and samples with 5 mg/L PBG was 47% bigger for the change in absorption. Thus, the discriminability of samples with 5 mg/L PBG from blank samples was much better using the absorption of porphobilin compared to uroporphyrin fluorescence. Adjusting the pH to 5.2 for PBG quantification by heating was found to provide efficient generation of both uroporphyrin and porphobilin in preliminary experiments (data not shown) and in the literature.[16] For this reason, a different sample preparation procedure had to be used for the PBG conversion by heating than for the TUP oxidation and quantification, which required stronger acidification. Thus, the sample which is heated for PBG quantification may contain considerable amounts of porphyrinogens. The main issue for PBG quantification by measuring the increase of porphyrin fluorescence during heating is that porphyrinogens may be spontaneously oxidized by heating the sample.[8] Thus, the increase in fluorescence intensity caused by conversion of PBG to uroporphyrin cannot be distinguished from an increase generated by the spontaneous oxidation of porphyrinogens to porphyrins. Especially in samples with PBG concentrations <10 mg/L and high TUP concentrations and therefore possibly high concentrations of porphyrinogens, most of the increase in fluorescence intensity might have to be attributed to the spontaneous oxidation of previously nonfluorescent porphyrinogens. For porphobilin absorption, no interference by other substances is known. Since the relevant range for PBG quantification is <20 mg/L[4] where absorption measurements provide higher precision, we deem it reasonable to limit the method to using only absorption of porphobilin for the indirect quantification of PBG. This might additionally allow for even stronger acidification, which increases the absorbance of porphobilin formed from PBG by heating[16] and therefore could improve the sensitivity of the method.

Urinary porphyrins and PBG of a sample of an acute porphyria patient were quantified using the laboratory reference methods and the methods described in the paper. Oxidation provided no increase in fluorescence intensity, since porphyrinogens had most likely already been completely auto-oxidized during the time between sampling and measurement. PBG determination showed only 1.5% deviation from the reference method for determination with porphobilin absorption and 6.8% for uroporphyrin fluorescence.

Based on these findings, we propose a procedure to identify patients with an attack of acute porphyria from a collective of patients with abdominal pain, as shown in Fig. 8. An elevated PBG concentration indicates acute porphyria, which allows for the immediate treatment of symptoms in the emergency room and avoids false diagnoses and unnecessary time delay. Compared to the recently withdrawn screening kit, the proposed procedure has several advantages. Sample preparation requires only 2 min and cheap chemicals in low amounts. All further measurement steps can be automated. This leads to an overall low cost per test with a high usability.

Before applying the proposed screening test in the clinical routine, reference ranges for both TUP and PBG have to be determined and the necessity of normalizing to creatinine has to be studied. While literature offers an upper threshold concentration in healthy specimens at 2 to 2.5 mg/L for PBG and 0.2 μmol/L for TUP, this has to be verified on patient samples in the relevant patient collective. The reference ranges for patients with acute abdominal pain not related to acute porphyria might deviate from those of healthy subjects and the optimization of the test might require a threshold for both TUP and PBG concentrations that differs from the usual reference range. In addition, possible interfering substances may be identified present in urine samples from patients with a variety of medical conditions under various medications.

5 Conclusion

A device tailored for the rapid investigation of urine samples of patients with a suspected attack of acute porphyria was designed and evaluated. The proposed method requires only minimal sample preparation, while the measurement and data evaluation procedure can be fully automated. It therefore has the potential to close the screening gap for acute porphyria.

Disclosures

The authors have no relevant financial interests in this article and no potential conflicts of interest to disclose.

References

1. D. M. Bissell, K. E. Anderson, and H. L. Bonkovsky, "Porphyria," *N. Engl. J. Med.* **377**, 862–872 (2017).
2. D. M. Bissell and B. Wang, "Acute hepatic porphyria," *J. Clin. Transl. Hepatol.* **3**, 17–26 (2015).
3. S. Besur, P. Schmeltzer, and H. L. Bonkovsky, "Acute porphyrias," *J. Emergency Med.* **49**, 305–312 (2015).
4. Z. Karim et al., "Porphyrias: a 2015 update," *Clin. Res. Hepatol. Gastroenterol.* **39**, 412–425 (2015).
5. K. E. Anderson et al., "Recommendations for the diagnosis and treatment of the acute porphyrias," *Ann. Intern. Med.* **142**, 439–450 (2005).
6. S. Sassa, "Modern diagnosis and management of the porphyrias," *Br. J. Haematol.* **135**, 281–292 (2006).
7. M. Balwani et al., "Acute hepatic porphyrias: recommendations for evaluation and long-term management," *Hepatology* **66**, 1314–1322 (2017).
8. H. L. Bonkowsky et al., "Repression of the overproduction of porphyrin precursors in acute intermittent porphyria by intravenous infusions of hematin," *Proc. Natl. Acad. Sci. U. S. A.* **68**, 2725–2729 (1971).
9. A. L. Herrick and K. E. McColl, "Acute intermittent porphyria," *Best Pract. Res. Clin. Gastroenterol.* **19**, 235–249 (2005).
10. R. Kauppinen and M. von und zu Fraunberg, "Molecular and biochemical studies of acute intermittent porphyria in 196 patients and their families," *Clin. Chem.* **48**, 1891–1900 (2002).
11. N. Schoenfeld and R. Mamet, "Individualized workup: a new approach to the biochemical diagnosis of acute attacks of neuroporphyria," *Physiol. Res.* **55**(Suppl. 2), S103–S108 (2006).
12. D. Mauzerall and S. Granick, "The occurrence and determination of delta-amino-levulinic acid and porphobilinogen in urine," *J. Biol. Chem.* **219**, 435–446 (1956).
13. M. Vogeser and T. Stauch, "Evaluation of a commercially available rapid urinary porphobilinogen test," *Clin. Chem. Lab. Med.* **49**, 1491–1494 (2011).
14. H. L. Bonkovsky et al., "Acute porphyrias in the USA: features of 108 subjects from porphyrias consortium," *Am. J. Med.* **127**, 1233–1241 (2014).
15. P. Martasek, M. Jirsa, and V. Kordac, "Porphyrinogens in urine in various types of porphyrias," *J. Clin. Chem. Clin. Biochem.* **20**, 113–117 (1982).
16. P. E. Brockman and C. H. Gray, "Studies on porphobilinogen," *Biochem. J.* **54**, 22–29 (1953).
17. L. Bogorad and G. S. Marks, "The enzymatic synthesis of uroporphyrins from porphobilinogen. IV. Investigations on the participation of formaldehyde," *J. Biol. Chem.* **235**, 2127–2129 (1960).
18. D. Mauzerall, "The thermodynamic stability of porphyrinogens," *J. Am. Chem. Soc.* **82**, 2601–2605 (1960).
19. C. Rimington, "Spectral-absorption coefficients of some porphyrins in the soret-band region," *Biochem. J.* **75**, 620–623 (1960).
20. K. Abe and R. Konaka, "Quantification of urinary porphyrins by liquid chromatography after oxidation of porphyrinogens," *Clin. Chem.* **35**, 1619–1622 (1989).
21. A. W. van de Giessen and E. M. van Wijk, "An improved method for quantitative determination of urinary porphyrins by use of second-derivative spectroscopy," *Eur. J. Clin. Chem. Clin. Biochem.* **28**, 605–609 (1990).
22. European Medicines Agency, *Guideline on Bioanalytical Method Validation*, Committee for Medicinal Product for Human Use, London (2011).
23. A. Savitzky and M. J. E. Golay, "Smoothing and differentiation of data by simplified least squares procedures," *Anal. Chem.* **36**, 1627–1639 (1964).
24. M. Paal et al., "A second-derivate fitting algorithm for the quantification of free hemoglobin in human plasma," *Clin. Biochem.* (2018).
25. J. Westerlund, M. Pudek, and W. E. Schreiber, "A rapid and accurate spectrofluorometric method for quantification and screening of urinary porphyrins," *Clin. Chem.* **34**, 345–351 (1988).
26. M. Valcarcel et al., "Direct quantification of coproporphyrins and uroporphyrins in urine by derivative synchronous fluorescence spectroscopy," *Clin. Chem.* **33**, 1826–1831 (1987).

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A SECOND-DERIVATE FITTING ALGORITHM FOR THE QUANTIFICATION OF FREE HEMOGLOBIN IN HUMAN PLASMA

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A second-derivate fitting algorithm for the quantification of free hemoglobin in human plasma

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ABSTRACT

Background: Assessment of hemolysis *in vivo* is becoming increasingly relevant in critical care. Current methods (Harboe, 1959) for quantifying the free hemoglobin (fHb) content produce unsatisfactory results in case of hyperbilirubinemia, a frequent condition in patients at risk for intravascular hemolysis.
Methods: A novel evaluation method based on second-derivative fitting to quantify fHb content was developed. The method uses spectrophotometric data from 350 to 650 nm recorded with standard instruments as input. To evaluate the power of the new method, plasma of patients and non-icteric plasma of healthy volunteers were spiked with fHb concentrations up to 2000 mg/L and compared to methods described in the literature by Harboe, Noe and Fairbanks. All measurements were done in compliance with the bioanalytical method validation protocol from the European Medicines Agency.
Results: Both the second-derivative fitting algorithm as well as the methods of Harboe, Noe and Fairbanks quantified fHb accurately in non-icteric samples, with inaccuracy and imprecision below 10%. For icteric specimen, false high results were obtained with the established formulas for fHb concentrations below 700 mg/L. In contrast, no interference was found with the second-derivate fitting method for bilirubin concentrations up to 465 µmol/L. The lower limits of quantifications for the second-derivative fitting algorithm were specified in agreement with the EMA guideline with 25 mg/L fHb for both non-icteric and icteric specimens.
Conclusions: A user-friendly, computer-based algorithm is reported that allows the accurate quantification of fHb concentrations in the presence of high bilirubin concentrations. The new method allows for uniform sample preparation with only a single dilution step and can be readily implemented in any laboratory on standard spectrophotometers using the provided supplementary Microsoft Excel macro.

1. Introduction

Several hematologic and non-hematologic conditions can cause hemolysis *in vivo*, including autoimmune hemolysis, thrombotic microangiopathy, malaria, hereditary red blood cell disorders, paroxysmal nocturnal hemoglobinuria, the HELPP syndrome, tissue injury, sepsis and even atherosclerosis [1–3]. Especially in hematologic disorders, high amounts of free hemoglobin (fHb) are rapidly released from damaged red blood cells. When the degree of intravascular hemolysis exceeds the binding capacity of plasma scavenger proteins, fHb accumulates in the peripheral blood, and patients develop hemoglobinemia [4]. Notably, it is essential to avoid *ex vivo* hemolysis by applying appropriate pre-analytical conditions in the sampling and shipment of samples if intravascular hemolysis is to be assessed.

For the assessment of hemolysis *in vivo*, plasma fHb represents the key marker, since elevated transaminases, bilirubin, lactate dehydrogenase (LDH) and potassium, as well as reduced haptoglobin, are not specific for hemolysis and may therefore confound clinical analysis [5]. Free hemoglobin is also routinely quantified to assess the quality and usability of blood products before transfusion [6]. Due to its

Abbreviations: fHb, free hemoglobin; EMA, European Medicines Agency; HEBI-Fit, Hemoglobin Bilirubin Fit; QC, Quality control; CV, Coefficient of variation; LLOQ, Lower limit of quantification; ULOQ, Upper limit of quantification

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oxidizing nature, fHb can cause severe damage to exposed tissue, including the kidney tubular system [7]. Likewise, quantification of fHb is crucial in patients with artificial heart valves [8–10] and those supported with extracorporeal devices, where erythrocytes are mechanically stressed by shear forces [11,12].

Quantification of fHb is increasingly important, as shown by a growing number of reports. Recent studies highlight the influence of extracellular hemoglobin on adverse pathophysiological reactions associated with vascular disease, inflammation, thrombosis, renal impairment and even lung injury [13–15]. Notably, fHb as a disease-driving compound is underlined by the fact that it may serve as a predicative biomarker for poor outcomes and increased mortality in critical illness [16,17]. Therefore, various therapeutic strategies are currently investigated to target the toxicity of hemoglobin in clinical settings, such as pharmacological application of the heme scavenger protein hemopexin and heme-degrading heme oxygenase [18].

Existing methods for the quantification of fHb can be divided into two groups: a) biochemical methods, e.g., assays based on a peroxidase-like reaction with benzidine or benzidine derivatives [19,20], the modified Drabkin reaction [21], immunonephelometry [22]; and b) multi-wavelength spectrophotometric methods that are commonly used in clinical routines. This is reflected by the fact that approximately two-thirds of laboratory participants (n = 119) for the current German fHb round-robin test use direct spectrophotometry for quantification [23]. Many spectrophotometric formulas, including those of Harboe [24], Kahn [25], Noe [26] and Fairbanks [27], correct for background absorption by interpolating linearly with the Allen method (also referred to as a baseline technique), using readings at three distinct wavelengths [28]. In particular, the three-point assay of Harboe is considered one of the most reliable methods for fHb quantification, based on the porphyrin-specific absorption maximum at 415 nm (Soret band). However, as bilirubin shows a considerable overlap with the Soret band, fHb quantification with the method of Harboe is not suitable for hyperbilirubinemia [29], a condition that is often present and typically associated with severe intravascular hemolysis. In such cases, fHb determination can be accomplished by the method of Kahn using undiluted specimen (thus requiring more sample) and the absorption band at 578 nm, which is mostly undisturbed by bilirubin. However, the method of Kahn underestimated fHb compared to the benzidine method in the original work [25] and in non-icteric samples in our clinical routine when results were compared to the formula of Harboe.

No satisfying UV/VIS spectrophotometric fHb quantification method is currently available that uses a uniform sample preparation protocol, utilizes the full potential of the entire fHb spectrum and also tackles the interfering variable bilirubin adequately. The objective of the current study was therefore to develop a highly selective spectrophotometric fHb quantification method using computational processing.

2. Materials and methods

2.1. Preparation of a non-icteric plasma-pool and collection of icteric patient specimen

Citrate plasma was obtained from six healthy volunteers (the first vial was discarded to avoid carryover of hemolysate) and pooled, and aliquots were stored at −80 °C. For performance evaluation of the methods in specimen with bilirubin, leftover icteric and visually non-lipemic patient citrate plasmas from the routine coagulation laboratory were collected after anonymization. Residual whole cells were removed by centrifugation at 15000*g* for 30 s at room temperature and the supernatants were stored protected from light at −80° until further usage.

2.2. Free hemoglobin and hemin stock solutions

Free hemoglobin must be freshly extracted from biological material, as its spectral pattern differs from commercially available lyophilized methemoglobin. Free hemoglobin was therefore obtained from EDTA blood of a healthy donor by osmotic shock of erythrocytes according to Taulier et al. [30], with 8 mL distilled water and 4 ml blood volume. The hemoglobin concentration of the hemolysate was determined with a hematology analyzer (XN-1000™, Sysmex, Kōbe, Japan). Scattergram analysis and a considerably reduced erythrocyte corpuscular volume (≈25 fl) confirmed complete cell lysis. Cell debris was removed by centrifugation. Serial dilution with aqua dest yielded 10× concentrated stock solutions (up to 20,000 mg/L) that were stored at −80 °C and were later used for spiking experiments.

The influence of methemoglobin and methemalbumin on the fHb quantification was studied. Methemoglobin was obtained from extracted oxyhemoglobin within 30 min at room temperature with 1.5 M excess of potassium ferricyanide ($K_3Fe(CN)_6$, Sigma Aldrich, St. Louis, MI) over heme followed by removal of the oxidizing agent by dialysis (membrane MCWO 12–14 kDa). Free monomeric hemin, which has the same UV/VIS spectrum as methemalbumin [31–33] was obtained by dissolving hemin chloride (Sigma Aldrich, St. Louis, MI) in nitrogen purged DMSO solution (Sigma Aldrich, St. Louis, MI). For comparative analysis UV/VIS absorption spectra were recorded for equimolar heme amounts using 80 μM monomeric hemin chloride in DMSO-water (80:20, v/v), 20 μM methemoglobin and 20 μM oxyhemoglobin in aqua dest. Serial dilution with aqua dest yielded 10× concentrated methemoglobin stock solutions that were blended with equally concentrated cell-free hemoglobin solutions (obtained from EDTA blood) to yield stock mixtures with varying fHb and free methemoglobin proportions.

2.3. Preparation of samples with varying free hemoglobin concentrations

One volume of hemoglobin stock solution was admixed with nine volumes of citrate plasma to yield spiked samples with varying hemoglobin concentrations (ranging from 0 to 2000 mg/L). The control sample (admixed with distilled water) was used to measure the concentration of total bilirubin with the 2,4-dichlorophenyl diazonium method (AU5800 AU clinical chemistry analyzer, Beckman Coulter, Brea, CA, USA).

2.4. Spectral photometry

Photometric UV/VIS absorption spectra were recorded with a spectral photometer (U-1900, Hitachi High-Technologies, Tokyo, Japan) using 10-mm transmission disposable polystyrene semi-micro cuvettes (Sarstedt, Nümbrecht, Germany) and scatter-free buffer TRIS/HCl solution for sample dilution (Bioanalytic, Umkirch, Germany). The step size was set to 0.5 nm, the scan speed to 200 nm/min, and the spectral bandpass was 4 nm. Spiked plasma samples (including the control sample) were diluted 1:11 with scatter-free buffer solution, and spectra were recorded from 350 to 650 nm. Hemoglobin concentrations were calculated according to the algorithms of Harboe [24], Noe [26] and Fairbanks [27]. To apply the method of Fairbanks, the absorbance at 700 nm was replaced with absorbance at 650 nm. Comparative analysis of blind duplicates was performed with the Lambda-40 absorption spectrometer (Perkin Elmer PE, Waltham, MA, USA), in conjunction with semi-micro PMMA cuvettes (Brand, Wertheim, Germany).

2.5. Second-derivate fitting algorithm

The new algorithm is designed to quantify fHb and total bilirubin from UV/VIS-spectrophotometric raw data. It is therefore named HEBI-Fit ("Hemoglobin Bilirubin Fit").

First, the wavelength range used for the second derivate fit is determined (see Fig. 1A). If the absorbance at 460 nm is lower than at 350 nm and the absorbance at 350 nm does not exceed 0.5, there is no relevant bilirubin interference, and the fit is applied to the entire fHb absorption spectrum (380–615 nm). If interference is detected, the fit

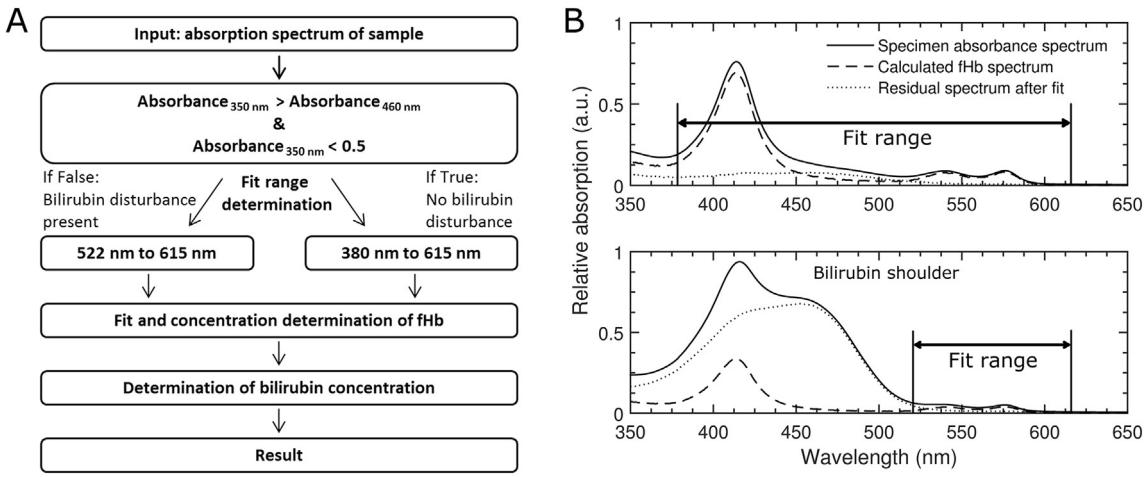

Fig. 1. A) Work-flow of HEBI-Fit algorithm. Fit wavelength range is automatically selected depending on bilirubin interference. B) From absorbance UV/VIS spectrum of non-icteric (top) or icteric (bottom) plasma specimen (solid) the by the second derivative fit calculated fHb spectrum (dashed) is subtracted, resulting in a residual spectrum with the remaining bilirubin absorbance (dotted). Bilirubin maximum absorption signal at 460 nm is used to quantify the bilirubin concentration.

range for the sample is limited to the fHb absorption maxima between 522 nm and 615 nm.

The amplitude of fHb absorbance is quantified by a fit that minimizes the second derivative of the specimen spectrum [34] after subtracting an iteratively varied reference fHb spectrum. During the fit procedure, a normalized hemoglobin absorbance spectrum (see Fig. 1B, dashed) is subtracted from the sample spectrum (Fig. 1B, solid). After subtraction (Fig. 1B, dotted), the second derivative is computed by a Savitzky-Golay filter, with a window size of 14.5 nm and a third-order polynomic approximation. The amplitude of the subtracted fHb spectrum is iteratively varied by the fit until the sum of squares of all points in the second derivative of the residual spectrum is minimal (method of least squares), within the specified wavelength range (see Supplementary Figure). The normalized fHb spectrum used for the fit is an absorption spectrum of 1000 mg/L fHb in distilled water (prepared from full blood hemolysate) normalized to the maximum absorbance at 415 nm. Scatter-free buffer was used as a blank.

Then, the bilirubin concentration is quantified. The previously determined fHb absorbance is subtracted from the specimen spectrum. From the resulting residuum spectrum, the intensity at 650 nm multiplied by 4.01 is subtracted to correct for background fluorescence. The bilirubin value is determined from the amplitude at 460 nm ± 1.5 nm divided by 0.003903. The factors were derived from a bilirubin calibration set (see Method validation, sample set 2) by minimizing the least mean square deviation from the reference bilirubin concentration.

All data analysis and fitting were performed using Microsoft Excel (version 2010, Microsoft, Redmond, WA, USA). An Excel spreadsheet macro is provided as a Supplementary Material that accompanies the online version of this article, which is able to perform not only the described HEBI-Fit but also quantifications according to the algorithms of Harboe and Kahn. Accordingly, HEBI-Fit can be run on any standard consumer computer.

The rationale for using the second-derivative fitting method is that each patient sample has a different unknown absorption background. Unlike the analytes fHb and bilirubin, this background absorption exhibits no pronounced spectral signatures. Calculation of the second derivative removes the smooth background, while the pronounced signatures of the analytes are preserved. Commonly known second-derivative methods calculate the concentration of analytes by comparing the amplitude of the second derivative at two different wavelengths, thereby omitting most spectral information [35–37]. The proposed fitting routine employs all the spectral information for the evaluation. The minimization of the second derivative of a spectrum can be summarized as a minimization of the curvature of the spectrum, while the curvature is mostly introduced by fHb. The resulting amplitude of the subtracted fHb-specific spectrum reflects the amount of fHb present in the sample. This principle was described and evaluated comprehensively by Holler et al. [38] and has been successfully adapted to other spectroscopy-based quantification methods, such as the non-invasive detection of erythrocyte zinc protoporphyrin in the lower lip [39].

2.6. Method validation

The analytical method was comprehensively validated according to the guideline of bioanalytical method validation from the European Medicines Agency (EMA), 21 July 2011 [40]. The guideline demands independent calibration of the method, which was performed on sample sets 1 and 2 for fHb and bilirubin, respectively. The conversion factors given below for fHb and bilirubin absorbance can be used to implement the method.

Set 1: Hemoglobin calibration: Eight calibrators (25, 50, 100, 250, 500, 1000, 1500, 2000 mg/L fHb) were analyzed by replicate analysis (n = 6) on four days with one measurement series per day (total 192 samples). Calibration resulted in an absorbance of 0.0007163(53) (95% confidence interval) per mg/L fHb at 415 nm for 1:11 diluted samples.

Set 2: Bilirubin calibration: Icteric patient plasma samples (n = 6, bilirubin up to 428 μmol/L) were blended in seven ratios with the non-icteric plasma pool, which had been spiked to 1000 mg/L fHb (total 42 samples). The calibration resulted in an absorbance of 0.003903(461) (95% confidence interval) per μmol/L bilirubin at 460 nm for 1:11 diluted samples.

Furthermore, an independent quality control set is required to evaluate both inaccuracy—expressed as relative mean error of the retrieved concentration—and imprecision—expressed as coefficient of variation (CV). According to the EMA guideline for biomedical method evaluation, inaccuracy and imprecision may not exceed 15% for the quality control samples, except for the lower limit of quantification where it should not exceed 20%. Additionally, the Model three of the “Milan Consensus” for analytical performance specifications is applied [41]. It requires performance of an assay to be better than current standard methods (state-of-the-art), which is evaluated in the present manuscript by comparing against results obtained using the method of Harboe. Sample set 3 was evaluated for the fHb quantification in non-icteric plasma, while sample set 4 was used to evaluate simultaneous fHb and bilirubin quantification.

Set 3: Non-icteric quality control samples: Inaccuracy and imprecision (both intra- and inter-assay), were examined by replicate analysis

(n = 6) of five different spiked concentrations (25, 50, 100, 700, 1125 mg/L fHb) on three consecutive days (total 90 samples).

Set 4: Icteric quality control samples: The influence of increasing bilirubin on imprecision and inaccuracy was examined in patient samples (n = 28, 6 samples with bilirubin < 34.2 μmol/L, 22 samples with bilirubin ≥ 34.2 μmol/L) that were each spiked with different fHb concentrations (0, 25, 50, 100, 700, 1100 mg/L fHb, overall 168 samples), covering low, medium and high concentration ranges. Bilirubin concentrations after fHb spiking ranged from 8.55 to 465 μmol/L.

To fulfill the guideline's demands for stability tests, hemoglobin stock solutions with a concentration of 1000 mg/L in aqua dest were stored at −80 °C for 180 days and UV/VIS spectra were recorded over this period. No alteration of the absorption spectrum or amplitude was observed.

2.7. Correction for pre-existing free hemoglobin

For the non-spiked plasma pool of healthy donors, the background fHb concentration was verified to be near zero using the method of Harboe. For icteric specimens (sample sets 2 and 4), the method of Kahn was used to identify samples exceeding the generally accepted plasma reference value of 20 mg/L [24]. These samples were discarded, as increased concentrations of pre-existing fHb would prevent determination of the lower limit of quantification by spiking additional fHb. For the samples with fHb concentrations < 20 mg/L, background fHb was determined by HEBI-Fit on 1:11 diluted plasma samples. The resulting fHb concentration was subtracted not only from the HEBI-Fit result of each corresponding spiked sample but also from the results derived with the method of Harboe, Noe and Fairbanks. The method of Kahn was not used for this correction because it yielded large determination errors in this low fHb concentration range. Even “negative fHb concentrations” of −18 mg/L were calculated. Additionally, the method of Kahn systematically underestimated the fHb concentration by > 7% over the entire concentration range compared to the method of Harboe or HEBI-Fit for sample set 1, even though no bilirubin interference was present.

3. Results

3.1. Quantification of free hemoglobin

Calculated *versus* reference fHb concentrations are depicted in Fig. 2. For the non-icteric quality control sample set 3 (Fig. 2A), the HEBI-Fit algorithm and spectrophotometric algorithms of Harboe, Noe and Fairbanks complied with the EMA guideline, and neither inaccuracy nor imprecision exceeded 2.3% for HEBI-Fit and 7.8% for the other algorithms (see Table 1 for a list of the results). For the icteric quality control sample set 4 (Fig. 2C), the inaccuracy of the HEBI-Fit algorithm did not exceed 3.1% for concentrations as low as 25 mg/L. The coefficient of variation (CV) was with 20% for 25 mg/L and with < 15% for higher concentrations within the requirements proposed by the EMA guideline. The examined spectrophotometric baseline-corrected algorithms (Harboe, Noe, Fairbanks) calculated fHb concentrations that were too high with increasing concentrations of total bilirubin. In Fig. 2B and D, deviations from the reference concentrations are shown as boxplot diagrams after evaluation using the HEBI-Fit- and Harboe-algorithms. However, for concentrations as high as 700 mg/L fHb, the influence of bilirubin was consistently negligible for the Fairbanks algorithm only (see Table 1). The method of Noe and Harboe also exceeded the acceptable limits for inaccuracy and imprecision for high fHb concentrations. It must be emphasized that for the Harboe algorithm, total bilirubin concentrations as low as 15.4 μmol/L already introduced considerable false high quantification errors for fHb of up to 100% for the low fHb concentration of 25 mg/L and 54% for 50 mg/L. When limiting the fit range to 522–615 nm for non-icteric sample set 3, inaccuracy increased to 27% and imprecision to 14% for 25 mg/L, which emphasizes that use of the entire spectral information yields higher precision and accuracy if no bilirubin interference is present.

For smoothing and derivation of the spectrum, a combination of running mean for smoothing and derivation by calculating differences of adjacent points was compared to a Savitzky- Golay smoothing filter, which yields the second derivative from the coefficients of the polynomial [42]. The input parameters, namely the window size and order of polynomic approximation of the Savitzky-Golay filter, were optimized using the non-icteric sample set 3. The use of Savitzky-Golay yielded only improvements of smaller than 0.5% for inaccuracy and imprecision of non-icteric samples. The inaccuracy and imprecision of the icteric sample set 4 was lowered for samples with a low concentration of fHb. For 25 mg/L fHb, the inaccuracy and imprecision was improved to −2% and 20%, respectively, for a window size of 14.5 nm (29 steps) and third-order polynomic smoothing compared to −5.9% and 23% for the use of a running mean.

3.2. Quantification of bilirubin

For all icteric plasma samples in sample set 4, the bilirubin concentration was calculated by HEBI-Fit. In Fig. 3, the results are shown in comparison with the biochemically measured total bilirubin. The implementation of the Fairbanks algorithm AII yielded linear results, but these values were off by a factor of fourteen compared to HEBI-Fit and reference results. After correction by this multiplication factor, both methods performed equally well for bilirubin concentrations above 85.5 μmol/L, as shown in Table 2. For smaller concentrations, Fairbanks had a higher CV of 22% (17.1–85.5 μmol/L) and 63% (8.55–17.1 μmol/L), compared to 10% and 27% for the HEBI-Fit algorithm, respectively.

It was also verified that the HEBI-Fit method can be used with different spectrophotometers when analyzing blind duplicates by means of comparative measurement (Hitachi U-1900 *vs.* Lambda-40). For fHb amounts above the lower limit of quantification, the relative deviation between measurements did not exceed 10%.

3.3. Spectrophotometry of monomeric hemin, methemoglobin and oxyhemoglobin

UV/VIS absorption spectra are shown in supplementary Fig. 1. In agreement with previous reports monomeric hemin has the same spectral pattern as methemalbumin shown by the prominent absorption maxima at 403, 500, and 623 nm. The spectrum of methemoglobin, with absorption maxima at 405, 500 and 632 nm [43], is close to identical to methemalbumin in the fit range of HEBI-Fit between 380 and 615 nm. The quantitative analysis was therefore carried out only on methemoglobin.

3.4. Assessment of free hemoglobin quantification in presence of free methemoglobin

For samples spiked with both fHb and methemoglobin, the accuracy of fHb quantification was assessed for combined free hemoglobin and methemoglobin of 100 mg/L and 500 mg/L. (Table 3). Inaccuracy was < 10% except for the samples where methemoglobin accounted for 85% of the combined free hemoglobin, where fHb was slightly overestimated by HEBI-Fit. In contrast, the method of Harboe neither quantified fHb correctly in the presence of methemoglobin, nor the sum of both, if the methemoglobin content exceeded 20% (Table 4).

4. Discussion

The mathematical method described here innovatively allows accurate quantification of fHb in icteric human plasma. It is validated as a computer-driven method that uses absorption spectra recorded from

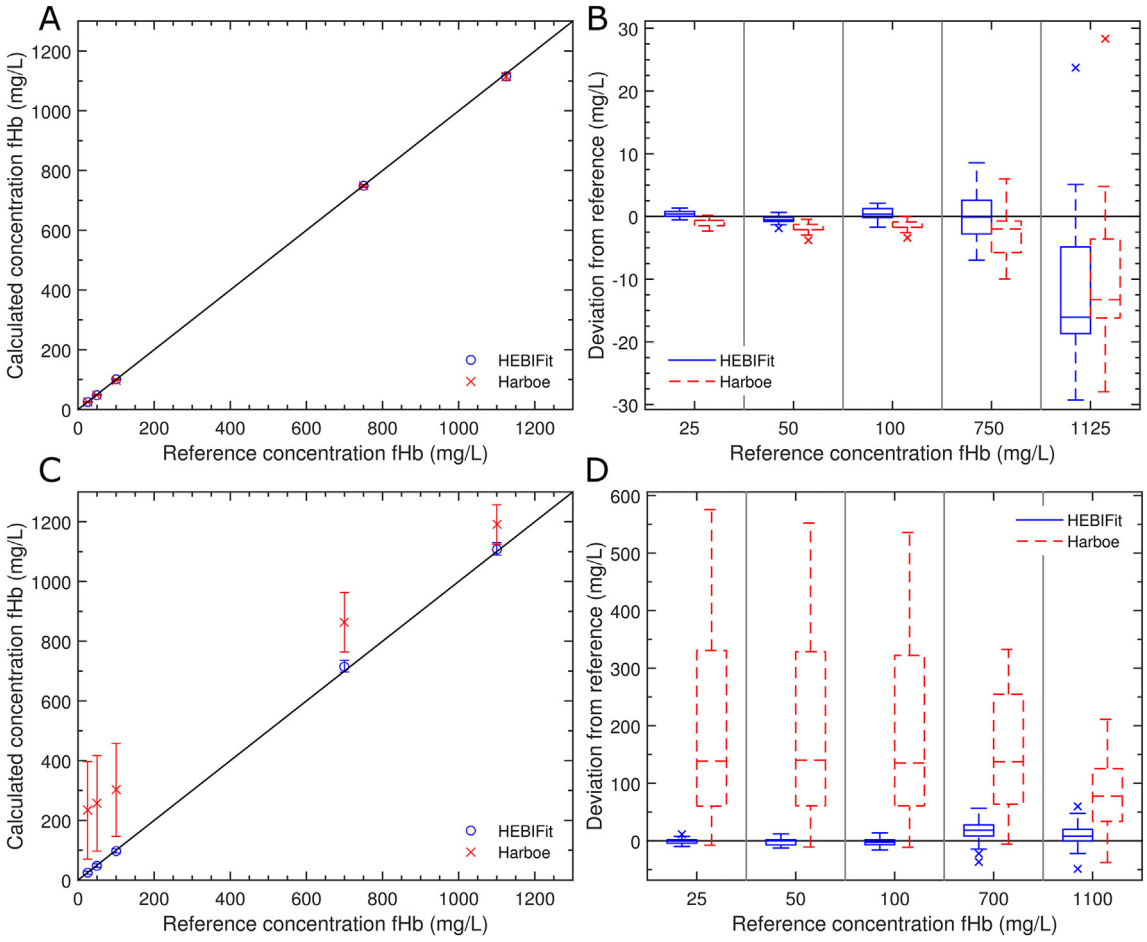

Fig. 2. Free hemoglobin concentrations calculated using HEBI-Fit and Harboe algorithms, compared to reference concentration for non-icteric samples in A) and B) and icteric samples in C) and D). Error bars denote standard deviation, line represents the median, and whiskers represent minimum/maximum range of points within 1.5 times the interquartile range in the box.

uniformly diluted human plasma to quantify fHb concentrations with a second-derivate fitting algorithm, irrespective of whether the specimen is icteric. Additionally, the total bilirubin concentration can be derived from the spectrophotometric raw data. The quantification method is therefore named HEBI-Fit ("Hemoglobin Bilirubin Fit"). The algorithm exploits all information from a recorded spectrum by subtracting a known fHb spectrum and iteratively minimizing the second derivative of the resulting residual spectrum over the entire spectral range. The HEBI-Fit-method offers essential advantages over existing direct spectrophotometric approaches and therefore fulfills the criterion of the "Milan Consensus" requiring a new method to be an improvement compared to the state-of-the-art [41]. For the first time, fully automated accurate quantification of free plasma hemoglobin and bilirubin in patients with significant hyperbilirubinemia is possible. Quantification requires only a single dilution step with scatter free diluent, and it can be performed within minutes. A corresponding Microsoft Excel macro is provided in the Data supplement that accompanies the online version of this article. The macro permits fHb and bilirubin quantification with HEBI-Fit, comparing fHb quantification results to the direct spectrophotometric algorithms of Harboe and Kahn.

The current computational software is based on derivate UV/VIS spectrophotometry, a powerful technique that allows quantification of a

Table 1
Comparison of inter-assay inaccuracy (observed – reference, relative to reference) and imprecision (CV) in observed concentrations for quantification with HEBI-Fit and three baseline correction formulas for non-icteric (3 runs with 5 concentrations and 6 replicates per concentration per assay) and icteric samples (one run with 5 concentrations for 28 specimens).

	Concentration (mg/L)	HEBI-Fit inaccuracy (%)/imprecision (%)	Harboe inaccuracy (%)/imprecision (%)	Noe inaccuracy (%)/imprecision (%)	Fairbanks inaccuracy (%)/imprecision (%)
Non-icteric (n = 90)	25 (LLOQ)	−1.0/1.1	−0.9/1.1	−1.4/1.1	−1.3/1.1
	50	−0.0/0.5	−0.3/0.6	−1.0/0.6	−0.8/0.6
	100	0.5/1.0	−1.5/0.9	−2.3/1.0	−2.7/0.9
	750	−1.1/1.3	−3.8/1.9	−4.5/1.8	−6.5/1.6
	1125 (75% ULOQ)	1.6/2.3	−3.5/3.0	−4.8/3.3	−7.8/.7
Icteric (n = 140)	25(LLOQ)	−2.0/20	840/656	941/761	253/301
	50	−3.1/12	417/322	467/374	126/145
	100	−2.3/6.9	204/156	229/182	59/70
	700	2.3/2.8	24/14	27/18	3.3/10
	1100 (73.3% ULOQ)	0.8/1.9	8.4/6.1	11/7.5	−4.1/11

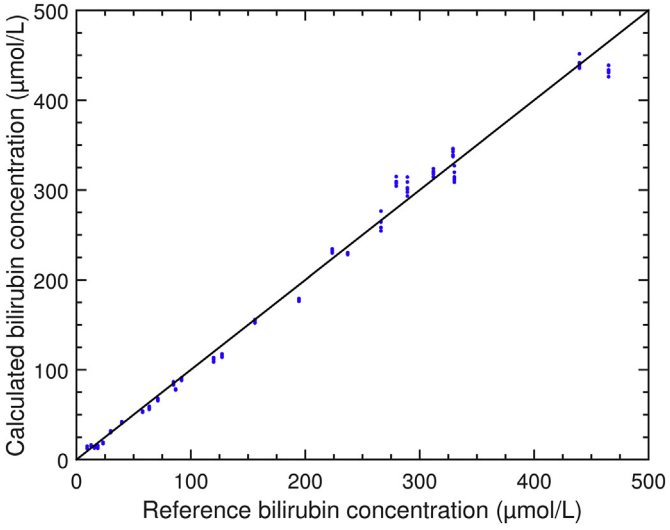

Fig. 3. Bilirubin calculated by HEBI-Fit versus reference shows satisfactory quantification performance. Imprecision and inaccuracy are reported in Table 2.

Table 2
Comparison of bilirubin quantification using HEBI-Fit and Fairbanks formula AII.

Bilirubin concentration (µmol/L)	HEBI-Fit inaccuracy (%)/imprecision (%)	Fairbanks inaccuracy (%)/imprecision (%)
8.55–17.1 (n = 16)	16/27	−11/63
17.1–85.5 (n = 48)	−7.5/10	−1.6/22
85.5–479 (n = 102)	−0.5/8.0	2.8/10

Table 3
Mean quantification error of HEBI-Fit for samples with 100 mg/L (top rows) and 500 mg/L (bottom rows) of combined free hemoglobin and free methemoglobin in dependence of the portion of fHb.

Concentration of fHb (mg/L)	100 (100%)	95.8	91.5	78.8	57.5	15 (below LLOQ)
Mean bias for non-icteric sample	4%	0%	1%	3%	8%	83%
Mean bias for icteric sample	1%	4%	2%	0%	5%	40%
Concentration of fHb (mg/L)	500 (100%)	479	458	394	285	75
Mean bias for non-icteric sample	0%	−5%	−3%	−4%	0%	40%
Mean bias for icteric sample	1%	−3%	−3%	−1%	0%	19%

Table 4
Mean quantification error of the method of Harboe for samples with 100 mg/L (top rows) and 500 mg/L (bottom rows) of combined free hemoglobin and free methemoglobin in dependence of the portion of fHb. Icteric samples are not shown since the method of Harboe is flawed for icteric samples.

Concentration of fHb (mg/L)	100 (100%)	95.8	91.5	78.8	57.5	15 (below LLOQ)
Mean bias for non-icteric sample	5%	5%	13%	30%	39%	318%
Concentration of fHb (mg/L)	500 (100%)	479	458	394	285	75
Mean bias for non-icteric sample	0%	0%	6%	25%	33%	286%

specific analyte in the presence of interfering substances [34]. Second-derivate spectra were used in the 1990s for quantification of fHb and bilirubin, but the technique did not gain acceptance for clinical routine use [35–37], most likely due to the additional effort in implementation and evaluation compared to three-point spectrophotometry. Since the introduction of the Fairbanks formula AII in 1992 [27] no considerable improvements in UV/VIS spectrophotometry have been implemented.

The mathematical algorithm was validated according to the guidelines of bioanalytical method validation from the European Medicines Agency with real patient samples only, which leaves little room for unforeseen matrix effects. Accordingly, HEBI-Fit allows quantification of fHb levels as low as 25 mg/L in non-icteric and icteric specimens. Although fHb reference values as low as 20 mg/L are reported in the literature for plasma [24], hemoglobin values ≥50 mg/L are considered relevant for the diagnosis of hemolysis in our experience.

The influence of bilirubin on direct spectrophotometry has been discussed by other groups [24–27]. Most interestingly, our data indicates calculation errors for samples with bilirubin concentrations as low as 15.4 µmol/L when using the formula of Harboe, although Bednar et al. report interference for concentrations higher than 34.2 µmol/L only [29]. Our observation is in agreement with the work of Wians et al. [44], where 23.9 µmol/L total bilirubin caused an apparent 1.5-fold increase in plasma spiked with 100 mg/L fHb. The work of Noe and coworkers is occasionally cited to give correct results when bilirubin is present, but only concentrations of 5.13 µmol/L total bilirubin or lower were evaluated. These concentrations are treated by our algorithm as "no bilirubin interference" present. It should therefore be emphasized that fHb concentrations should be reviewed carefully when multi-wavelength direct spectrophotometry is applied for samples that are considered almost icteric.

Although we provide a "pure" fHb spectrum in the supplementary information, each photometer should be calibrated with a fHb solution that is either freshly prepared from full blood or stably stored at −80 °C, as slight alterations in the spectral pattern can cause considerable falsification. In this regard, rapid sample processing or stable storage (preferably at −80 °C) is essential. Methemalbumin and methemoglobin have a different spectral pattern than fHb with the Soret band at 403 and 405 nm. The presence of one or both substances introduces a blue shift of the Soret band absorption maximum of hemoglobin. The results show that fHb is correctly identified by HEBI-Fit under these conditions, while both methemalbumin and methemoglobin are not considered. This can lead to an underestimation of the combined concentration of fHb and methemalbumin and/or methemoglobin. For comparison, the current reference method (Harboe) for fHb determination does not accurately determine fHb if methemoglobin is present and also underestimated the sum of both, if no bilirubin is present. This is in agreement with earlier observations by Fairbanks et al. [27] where spectrophotometric fHb quantifications were altered by the presence of methemalbumin or methemoglobin.

In the clinical routine, we emphasize the need to visually examine the spectrum for a blue shift of the Soret band to identify samples with relevant methemalbumin content. The HEBI-Fit macro displays a grey, vertical auxiliary line at 415 nm, simplifying the identification of peak shifts. Since the robustness of the method was only evaluated using bilirubin and methemoglobin, it should also be validated on samples from patients with active hemolysis, where methemalbumin (rather than methemoglobin) may also be present.

We also stress that, currently, no commercial quality control samples are available for HEBI-Fit, as the available lyophilized hemoglobin spectrum might deviate from physiologic oxyhemoglobin. The results with HEBI-Fit should therefore be evaluated in conjunction with a quantification method established in-house (*e.g.* algorithms of Harboe and Kahn).

In summary, the presented HEBI-Fit method combines state-of-the-art UV/VIS spectrometry with fully automated user-friendly spectrophotometric evaluation, being a convenient tool for a far more detailed

assessment of plasma fHb. The provided Microsoft Excel macro can be readily implemented in any laboratory on a standard spectrometer. Thus, UV/VIS spectrometry, which is almost considered antiquated in laboratory medicine, can reach unforeseen strength when combined with readily available computing power and an innovative algorithm.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2018.04.007.

Authors' disclosures or potential conflicts of interest

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Consultant or advisory role

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Expert testimony

None declared.

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References

[1] I.A. Tabbara, Hemolytic anemias, Diag. Manage. Med. Clin. NA 76 (3) (1992) 649–668.
[2] J.J. Robertson, E. Brem, A. Koyfman, The acute hemolytic Anemias: the importance of emergency diagnosis and management, J. Emerg. Med. 53 (2) (2017) 202–211.
[3] V. Jeney, G. Balla, J. Balla, Red blood cell, hemoglobin and heme in the progression of atherosclerosis, Front. Physiol. 5 (2014) 379.
[4] D.J. Schaer, P.W. Buehler, A.I. Alayash, J.D. Belcher, G.M. Vercellotti, Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins, Blood 121 (8) (2013) 1276–1284.
[5] W. Barcellini, B. Fattizzo, Clinical applications of hemolytic markers in the differential diagnosis and Management of Hemolytic Anemia, Dis. Markers 2015 (2015) 635670.
[6] S.O. Sowemimo-Coker, Red blood cell hemolysis during processing, Transfus. Med. Rev. 16 (1) (2002) 46–60.
[7] J. Balla, G.M. Vercellotti, V. Jeney, A. Yachie, Z. Varga, H.S. Jacob, J.W. Eaton, G. Balla, Heme, heme oxygenase, and ferritin: how the vascular endothelium survives (and dies) in an iron-rich environment, Antioxid. Redox Signal. 9 (12) (2007) 2119–2137.
[8] G. Mecozzi, A.D. Milano, M. De Carlo, F. Sorrentino, S. Pratali, C. Nardi, U. Bortolotti, Intravascular hemolysis in patients with new-generation prosthetic heart valves: a prospective study, J. Thorac. Cardiovasc. Surg. 123 (3) (2002) 550–556.
[9] J. Laflamme, R. Puri, M. Urena, L. Laflamme, H. DeLarochelliere, O. Abdul-Jawad Altisent, M. del Trigo, F. Campelo-Parada, R. DeLarochelliere, J.M. Paradis, E. Dumont, D. Doyle, S. Mohammadi, M. Cote, P. Pibarot, V. Laroche, J. Rodes-Cabau, Incidence and risk factors of hemolysis after transcatheter aortic valve implantation with a balloon-expandable valve, Am. J. Cardiol. 115 (11) (2015) 1574–1579.
[10] K. Markakis, T.H. Westhoff, N. Pagonas, Aortic valve replacement as a trigger of atypical hemolytic uremic syndrome, Ann. Thorac. Surg. 104 (3) (2017) e255-e256.
[11] J.R. Neal, E. Quintana, R.B. Pike, J.D. Hoyer, L.D. Joyce, G. Schears, Using daily plasma-free hemoglobin levels for diagnosis of critical pump Thrombus in patients undergoing ECMO or VAD support, J. Extra Corpor. Technol. 47 (2) (2015) 103–108.
[12] H.R. Omar, M. Mirsaeidi, S. Socias, C. Sprenker, C. Caldeira, E.M. Camporesi, D. Mangar, Plasma free hemoglobin is an independent predictor of mortality among patients on extracorporeal membrane oxygenation support, PLoS One 10 (4) (2015) e0124034.
[13] M.T. Gladwin, T. Kanias, D.B. Kim-Shapiro, Hemolysis and cell-free hemoglobin drive an intrinsic mechanism for human disease, J. Clin. Invest. 122 (4) (2012) 1205–1208.
[14] D.J. Schaer, P.W. Buehler, Cell-free hemoglobin and its scavenger proteins: new disease models leading the way to targeted therapies, Cold Spring Harbor Perspect. Med. 3 (6) (2013).
[15] D.R. Janz, L.B. Ware, The role of red blood cells and cell-free hemoglobin in the pathogenesis of ARDS, J. Intens. Care 3 (2015) 20.
[16] M. Adamzik, T. Hamburger, F. Petrat, J. Peters, H. de Groot, M. Hartmann, Free hemoglobin concentration in severe sepsis: methods of measurement and prediction of outcome, Crit. Care 16 (4) (2012) R125.
[17] K. Plewes, H.W.F. Kingston, A. Ghose, R.J. Maude, M.T. Herdman, S.J. Leopold, H. Ishioka, M.M.U. Hasan, M.S. Haider, S. Alam, K.A. Piera, P. Charunwatthana, K. Silamut, T.W. Yeo, M.A. Faiz, S.J. Lee, M. Mukaka, G.D.H. Turner, N.M. Anstey, L. Jackson Roberts, N.J. White 2nd, N.P.J. Day, M.A. Hossain, A.M. Dondorp, Cell-free hemoglobin mediated oxidative stress is associated with acute kidney injury and renal replacement therapy in severe falciparum malaria: an observational study, BMC Infect. Dis. 17 (1) (2017) 313.
[18] S. Immenschuh, V. Vijayan, S. Janciauskiene, F. Gueler, Heme as a target for therapeutic interventions, Front. Pharmacol. 8 (2017) 146.
[19] G.E. Hanks, M. Cassell, R.N. Ray, H. Chaplin Jr., Further modification of the benzidine method for measurement of hemoglobin in plasma; definition of a new range of normal values, J. Lab. Clin. Med. 56 (1960) 486–498.
[20] E.C. Elson, L. Ivor, N. Gochman, Substitution of a non-hazardous chromogen for benzidine in the measurement of plasma hemoglobin, Am. J. Clin. Pathol. 69 (3) (1978) 354–355.
[21] G.L. Moore, M.E. Ledford, A. Merydith, A micromodification of the Drabkin hemoglobin assay for measuring plasma hemoglobin in the range of 5 to 2000 mg/dl, Biochem. Med. 26 (2) (1981) 167–173.
[22] M. Lammers, A.M. Gressner, Immunonephelometric quantification of free haemoglobin, J. Clin. Chem. Clin. Biochem. 25 (6) (1987) 363–367.
[23] Instand: Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e.V. Ringversuch: Hämatologie 16 - Freies Hämoglobin, (2017), p. 236.
[24] M. Harboe, A method for determination of hemoglobin in plasma by near-ultraviolet spectrophotometry, Scand. J. Clin. Lab. Invest. 11 (1959) 66–70.
[25] S.E. Kahn, B.F. Watkins, E.W. Bermes Jr., An evaluation of a spectrophotometric scanning technique for measurement of plasma hemoglobin, Ann. Clin. Lab. Sci. 11 (2) (1981) 126–131.
[26] D.A. Noe, V. Weedn, W.R. Bell, Direct spectrophotometry of serum hemoglobin: an Allen correction compared with a three-wavelength polychromatic analysis, Clin. Chem. 30 (5) (1984) 627–630.
[27] V.F. Fairbanks, S.C. Ziesmer, P.C. O'Brien, Methods for measuring plasma hemoglobin in micromolar concentration compared, Clin. Chem. 38 (1) (1992) 132–140.
[28] W.M. Allen, A simple method for analyzing complicated absorption curves, of use in the colorimetric determination of urinary steroids, J. Clin. Endocrinol. Metabol. 10 (1) (1950) 71–83.
[29] R.B. Bednar, P.M., Freies Hämoglobin im Plasma - Vergleich zweier spektralphotometrischer Methoden, Laboratoriumsmedizin 18 (5) (1994) 196–199.
[30] A. Taulier, P. Levillain, A. Lemonnier, Determining methemoglobin in blood by zero-crossing-point first-derivative spectrophotometry, Clin. Chem. 33 (10) (1987) 1767–1770.
[31] G.H. Beaven, S.H. Chen, A. d' Albis, W.B. Gratzer, A spectroscopic study of the haemin—human-serum-albumin system, Eur. J. Biochem. 41 (3) (1974) 539–546.
[32] M. Rosenberg, S. D.G., Methemalbumin interaction between human serum albumin and Ferriprotoporphyrin IX, J. Biol. Chem. 183 (1950) 663–667.
[33] C.A. Daniels, C. Wagner, R.C. Hartmann, D.E. Jenkins Jr., Spectral absorption characteristics of human methemalbumin, Am. J. Clin. Pathol. 47 (5) (1967) 631–637.
[34] S.L. Upstone, Ultraviolet/Visible Light Absorption Spectrophotometry in Clinical Chemistry, Encyclopedia of Analytical Chemistry, John Wiley and Sons, Ltd., 2000.
[35] M.F. Merrick, H.L. Pardue, Evaluation of absorption and first- and second-derivative spectra for simultaneous quantification of bilirubin and hemoglobin, Clin. Chem. 32 (4) (1986) 598–602.
[36] G.J. Sanderink, H.J. van Rijn, Quantitative measurement of plasma hemoglobin by second derivative spectrophotometry, Clin. Chim. Acta 146 (1) (1985) 65–73.
[37] K. Amazon, F. Soloni, A.M. Rywlin, Separation of bilirubin from hemoglobin by recording derivative spectrophotometry, Am. J. Clin. Pathol. 75 (4) (1981) 519–523.

[38] F. Holler, D.H. Burns, J.B. Callis, Direct use of second derivatives in curve-fitting procedures, Appl. Spectrosc. 43 (5) (1989) 877–882.
[39] G. Hennig, C. Homann, I. Teksan, U. Hasbargen, S. Hasmuller, L.M. Holdt, N. Khaled, R. Sroka, T. Stauch, H. Stepp, M. Vogeser, G.M. Brittenham, Non-invasive detection of iron deficiency by fluorescence measurement of erythrocyte zinc protoporphyrin in the lip, Nat. Commun. 7 (2016) 10776.
[40] A.P. Siskos, P. Jain, W. Romisch-Margl, M. Bennett, D. Achaintre, Y. Asad, L. Marney, L. Richardson, A. Koulman, J.L. Griffin, F. Raynaud, A. Scalbert, J. Adamski, C. Prehn, H.C. Keun, Interlaboratory reproducibility of a targeted metabolomics platform for analysis of human serum and plasma, Anal. Chem. 89 (1) (2017) 656–665.
[41] S. Sandberg, C.G. Fraser, A.R. Horvath, R. Jansen, G. Jones, W. Oosterhuis, P.H. Petersen, H. Schimmel, K. Sikaris, M. Panteghini, Defining analytical performance specifications: consensus statement from the 1st strategic conference of the European Federation of Clinical Chemistry and Laboratory Medicine, Clin. Chem. Lab. Med. 53 (6) (2015) 833–835.
[42] A. Savitzky, M.J.E. Golay, Smoothing and differentiation of data by simplified least squares procedures, Anal. Chem. 36 (8) (1964) 1627–1639.
[43] W.G. Zijlstra, A. Buursma, W.P. Meeuwsen-van der Roest, Absorption spectra of human fetal and adult oxyhemoglobin, de-oxyhemoglobin, carboxyhemoglobin, and methemoglobin, Clin. Chem. 37 (9) (1991) 1633–1638.
[44] F.H. Wians, C.L. Miller, J.I. Heald, H. Clark, Evaluation of a direct spectrophotometric procedure for quantitating plasma hemoglobin, Lab. Med. 3 (3) (1988) 151–155.

LITERATURVERZEICHNIS

1. A. Lang, C. Heckl, M. Vogeser, T. Stauch, C. Homann, G. Hennig, R. Sroka, and H. Stepp, "Rapid spectrophotometric quantification of urinary porphyrins and porphobilinogen as screening tool for attacks of acute porphyria," Journal of biomedical optics **23**, 1-12 (2018).
2. M. Paal, A. Lang, G. Hennig, M. L. Buchholtz, R. Sroka, and M. Vogeser, "A second-derivate fitting algorithm for the quantification of free hemoglobin in human plasma," Clinical biochemistry **56**, 62-69 (2018).
3. M. Balwani, and R. J. Desnick, "The porphyrias: advances in diagnosis and treatment," Blood **120**, 4496-4504 (2012).
4. T. Hankeln, B. Ebner, C. Fuchs, F. Gerlach, M. Haberkamp, T. L. Laufs, A. Roesner, M. Schmidt, B. Weich, S. Wystub, S. Saaler-Reinhardt, S. Reuss, M. Bolognesi, D. De Sanctis, M. C. Marden, L. Kiger, L. Moens, S. Dewilde, E. Nevo, A. Avivi, R. E. Weber, A. Fago, and T. Burmester, "Neuroglobin and cytoglobin in search of their role in the vertebrate globin family," Journal of inorganic biochemistry **99**, 110-119 (2005).
5. I. Hamza, and H. A. Dailey, "One ring to rule them all: trafficking of heme and heme synthesis intermediates in the metazoans," Biochimica et biophysica acta **1823**, 1617-1632 (2012).
6. I. M. London, D. Shemin, and et al., "Heme synthesis and red blood cell dynamics in normal humans and in subjects with polycythemia vera, sickle-cell anemia, and pernicious anemia," The Journal of biological chemistry **179**, 463-484 (1949).
7. S. Thunell, P. Harper, and A. Brun, "Porphyrins, porphyrin metabolism and porphyrias. IV. Pathophysiology of erythyropoietic protoporphyria-diagnosis, care and monitoring of the patient" Scandinavian journal of clinical and laboratory investigation **60**, 581-604 (2000).
8. H. A. Dailey, T. A. Dailey, C. K. Wu, A. E. Medlock, K. F. Wang, J. P. Rose, and B. C. Wang, "Ferrochelatase at the millennium: structures, mechanisms and [2Fe-2S] clusters," Cellular and molecular life sciences **57**, 1909-1926 (2000).
9. Z. W. Zhang, J. Cheng, F. Xu, Y. E. Chen, J. B. Du, M. Yuan, F. Zhu, X. C. Xu, and S. Yuan, "Red blood cell extrudes nucleus and mitochondria against oxidative stress," IUBMB life **63**, 560-565 (2011).
10. M. F. Cesta, "Normal structure, function, and histology of the spleen," Toxicologic pathology **34**, 455-465 (2006).
11. R. Tenhunen, H. S. Marver, and R. Schmid, "Microsomal heme oxygenase. Characterization of the enzyme," The Journal of biological chemistry **244**, 6388-6394 (1969).
12. C. Beaumont, and F. Canonne-Hergaux, "[Erythrophagocytosis and recycling of heme iron in normal and pathological conditions; regulation by hepcidin]," Transfusion clinique et biologique : journal de la Societe francaise de transfusion sanguine **12**, 123-130 (2005).
13. M. Black, and B. H. Billing, "Hepatic bilirubin udp-glucuronyl transferase activity in liver disease and gilbert's syndrome," The New England journal of medicine **280**, 1266-1271 (1969).

14. D. J. Schaer, P. W. Buehler, A. I. Alayash, J. D. Belcher, and G. M. Vercellotti, "Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins," Blood **121**, 1276-1284 (2013).
15. J. E. Maldonado, R. A. Kyle, and L. J. Schoenfield, "Increased serum conjugated bilirubin in hemolytic anemia," Postgraduate medicine **55**, 183-187 passim (1974).
16. E. B. Fleischner, "Structure of porphyrins and metalloporphyrins," Acc. Chem. Res. **3**, 105-112 (1969).
17. R. E. Hirsch, "Front-face fluorescence spectroscopy of hemoglobins," Methods in enzymology **232**, 231-246 (1994).
18. A. A. Lamola, and T. Yamane, "Zinc protoporphyrin in the erythrocytes of patients with lead intoxication and iron deficiency anemia," Science **186**, 936-938 (1974).
19. M. Gouterman, "Spectra of Porphyrins," J. Mol. Spectrosc. **6**, 138-163 (1961).
20. B. Chauvin, A. Kasselouri, P. Chaminade, R. Quiameso, I. Nicolis, P. Maillard, and P. Prognon, "Diprotonation process of meso-tetraphenylporphyrin derivatives designed for photodynamic therapy of cancers: from multivariate curve resolution to predictive QSPR modeling," Analytica chimica acta **705**, 306-314 (2011).
21. S. E. Rodrigues, A. E. H. Machado, M. Berardi, A. S. Ito, L. M. Almeida, M. J. Santana, L. M. Liao, N. M. B. Neto, and P. J. Goncalves, "Investigation of protonation effects on the electronic and structural properties of halogenated sulfonated porphyrins," J Mol Struct **1084**, 284-293 (2015).
22. G. H. Cookson, and C. Rimington, "Porphobilinogen; chemical constitution," Nature **171**, 875-876 (1953).
23. P. E. Brockman, and C. H. Gray, "Studies on porphobilinogen," The Biochemical journal **54**, 22-29 (1953).
24. D. Mauzerall, and S. Granick, "The occurrence and determination of delta-amino-levulinic acid and porphobilinogen in urine," The Journal of biological chemistry **219**, 435-446 (1956).
25. L. Bogorad, and G. S. Marks, "The enzymatic synthesis of uroporphyrins from porphobilinogen. IV. Investigations on the participation of formaldehyde," The Journal of biological chemistry **235**, 2127-2129 (1960).
26. F. Holler, D. H. Burns, and J. B. Callis, "Direct use of second derivatives in curve-fitting procedures," Appl Spectrosc. **43**, 877-882 (1989).
27. O. S. Wolfbeis, and M. J. P. Leiner, "Mapping of the total fluorescence of human blood serum as a new method for its characterization," Analytica chimica acta **167**, 203-215 (1985).
28. M. J. P. Leiner, M. R. Hubmann, and O. S. Wolfbeis, "The total fluorescence of human urine," Analytica chimica acta **198**, 13-23 (1987).
29. A. J. Owens, *Uses of Derivative Spectroscopy* (Agilent Technologies, Santa Clara, California, 1995).
30. A. T. Giese, and C. S. French, "The Analysis of Overlapping Spectral Absorption Bands by Derivative Spectrophotometry," Appl Spectrosc. **9**, 78-96 (1955).
31. D. M. Bissell, and B. Wang, "Acute Hepatic Porphyria," Journal of clinical and translational hepatology **3**, 17-26 (2015).

32. A. T. Chemmanur, and H. L. Bonkovsky, "Hepatic porphyrias: diagnosis and management," Clinics in liver disease **8**, 807-838, viii (2004).
33. U. Stolzel, T. Stauch, and M. O. Doss, "[Porphyrias]," Der Internist **51**, 1525-1533; quiz 1534 (2010).
34. S. Sassa, "Modern diagnosis and management of the porphyrias," British journal of haematology **135**, 281-292 (2006).
35. H. L. Bonkovsky, V. C. Maddukuri, C. Yazici, K. E. Anderson, D. M. Bissell, J. R. Bloomer, J. D. Phillips, H. Naik, I. Peter, G. Baillargeon, K. Bossi, L. Gandolfo, C. Light, D. Bishop, and R. J. Desnick, "Acute porphyrias in the USA: features of 108 subjects from porphyrias consortium," The American journal of medicine **127**, 1233-1241 (2014).
36. S. E. Mattern, and A. Tefferi, "Acute porphyria: the cost of suspicion," The American journal of medicine **107**, 621-623 (1999).
37. A. L. Herrick, and K. E. McColl, "Acute intermittent porphyria," Best practice & research. Clinical gastroenterology **19**, 235-249 (2005).
38. S. Besur, P. Schmeltzer, and H. L. Bonkovsky, "Acute Porphyrias," The Journal of emergency medicine **49**, 305-312 (2015).
39. Z. Karim, S. Lyoumi, G. Nicolas, J. C. Deybach, L. Gouya, and H. Puy, "Porphyrias: A 2015 update," Clinics and research in hepatology and gastroenterology **39**, 412-425 (2015).
40. H. L. Bonkowsky, D. P. Tschudy, A. Collins, J. Doherty, I. Bossenmaier, R. Cardinal, and C. J. Watson, "Repression of the overproduction of porphyrin precursors in acute intermittent porphyria by intravenous infusions of hematin," Proceedings of the National Academy of Sciences of the United States of America **68**, 2725-2729 (1971).
41. R. Kauppinen, and M. von und zu Fraunberg, "Molecular and biochemical studies of acute intermittent porphyria in 196 patients and their families," Clinical chemistry **48**, 1891-1900 (2002).
42. N. Schoenfeld, and R. Mamet, "Individualized workup: a new approach to the biochemical diagnosis of acute attacks of neuroporphyria," Physiological research **55 Suppl 2**, S103-108 (2006).
43. M. Vogeser, and T. Stauch, "Evaluation of a commercially available rapid urinary porphobilinogen test," Clinical chemistry and laboratory medicine **49**, 1491-1494 (2011).
44. J. Zhang, M. Yasuda, R. J. Desnick, M. Balwani, D. Bishop, and C. Yu, "A LC-MS/MS method for the specific, sensitive, and simultaneous quantification of 5-aminolevulinic acid and porphobilinogen," Journal of chromatography. B, Analytical technologies in the biomedical and life sciences **879**, 2389-2396 (2011).
45. J. D. Cook, K. A. Strauss, Y. H. Caplan, C. P. Lodico, and D. M. Bush, "Urine pH: the effects of time and temperature after collection," Journal of analytical toxicology **31**, 486-496 (2007).
46. P. Martasek, M. Jirsa, and V. Kordac, "Porphyrinogens in urine in various types of porphyrias," Journal of clinical chemistry and clinical biochemistry **20**, 113-117 (1982).
47. European-Medicines-Agency, "Guideline on bioanalytical method validation," (European Medicinces Agency, London, 2012).

48. V. Jeney, G. Balla, and J. Balla, "Red blood cell, hemoglobin and heme in the progression of atherosclerosis," Frontiers in physiology **5**, 379 (2014).
49. J. J. Robertson, E. Brem, and A. Koyfman, "The Acute Hemolytic Anemias: The Importance of Emergency Diagnosis and Management," The Journal of emergency medicine **53**, 202-211 (2017).
50. I. A. Tabbara, "Hemolytic anemias. Diagnosis and management," The Medical clinics of North America **76**, 649-668 (1992).
51. J. Balla, G. M. Vercellotti, V. Jeney, A. Yachie, Z. Varga, H. S. Jacob, J. W. Eaton, and G. Balla, "Heme, heme oxygenase, and ferritin: how the vascular endothelium survives (and dies) in an iron-rich environment," Antioxidants & redox signaling **9**, 2119-2137 (2007).
52. W. Barcellini, and B. Fattizzo, "Clinical Applications of Hemolytic Markers in the Differential Diagnosis and Management of Hemolytic Anemia," Disease markers **2015**, 635670 (2015).
53. S. O. Sowemimo-Coker, "Red blood cell hemolysis during processing," Transfusion medicine reviews **16**, 46-60 (2002).
54. G. Mecozzi, A. D. Milano, M. De Carlo, F. Sorrentino, S. Pratali, C. Nardi, and U. Bortolotti, "Intravascular hemolysis in patients with new-generation prosthetic heart valves: a prospective study," The Journal of thoracic and cardiovascular surgery **123**, 550-556 (2002).
55. J. Laflamme, R. Puri, M. Urena, L. Laflamme, H. DeLarochelliere, O. Abdul-Jawad Altisent, M. del Trigo, F. Campelo-Parada, R. DeLarochelliere, J. M. Paradis, E. Dumont, D. Doyle, S. Mohammadi, M. Cote, P. Pibarot, V. Laroche, and J. Rodes-Cabau, "Incidence and risk factors of hemolysis after transcatheter aortic valve implantation with a balloon-expandable valve," The American journal of cardiology **115**, 1574-1579 (2015).
56. K. Markakis, T. H. Westhoff, and N. Pagonas, "Aortic Valve Replacement as a Trigger of Atypical Hemolytic Uremic Syndrome," The Annals of thoracic surgery **104**, e255-e256 (2017).
57. J. R. Neal, E. Quintana, R. B. Pike, J. D. Hoyer, L. D. Joyce, and G. Schears, "Using Daily Plasma-Free Hemoglobin Levels for Diagnosis of Critical Pump Thrombus in Patients Undergoing ECMO or VAD Support," The journal of extra-corporeal technology **47**, 103-108 (2015).
58. H. R. Omar, M. Mirsaeidi, S. Socias, C. Sprenker, C. Caldeira, E. M. Camporesi, and D. Mangar, "Plasma Free Hemoglobin Is an Independent Predictor of Mortality among Patients on Extracorporeal Membrane Oxygenation Support," PloS one **10**, e0124034 (2015).
59. G. E. Hanks, M. Cassell, R. N. Ray, and H. Chaplin, Jr., "Further modification of the benzidine method for measurement of hemoglobin in plasma; definitionn of a new range of normal values," The Journal of laboratory and clinical medicine **56**, 486-498 (1960).
60. E. C. Elson, L. Ivor, and N. Gochman, "Substitution of a non-hazardous chromogen for benzidine in the measurement of plasma hemoglobin," American journal of clinical pathology **69**, 354-355 (1978).
61. G. L. Moore, M. E. Ledford, and A. Merydith, "A micromodification of the Drabkin hemoglobin assay for measuring plasma hemoglobin in the range of 5 to 2000 mg/dl," Biochemical medicine **26**, 167-173 (1981).

62. M. Lammers, and A. M. Gressner, "Immunonephelometric quantification of free haemoglobin," Journal of clinical chemistry and clinical biochemistry. Zeitschrift fur klinische Chemie und klinische Biochemie **25**, 363-367 (1987).
63. M. Harboe, "A method for determination of hemoglobin in plasma by near-ultraviolet spectrophotometry," Scandinavian journal of clinical and laboratory investigation **11**, 66-70 (1959).
64. S. E. Kahn, B. F. Watkins, and E. W. Bermes, Jr., "An evaluation of a spectrophotometric scanning technique for measurement of plasma hemoglobin," Annals of clinical and laboratory science **11**, 126-131 (1981).
65. V. F. Fairbanks, S. C. Ziesmer, and P. C. O'Brien, "Methods for measuring plasma hemoglobin in micromolar concentration compared," Clinical chemistry **38**, 132-140 (1992).
66. D. A. Noe, V. Weedn, and W. R. Bell, "Direct spectrophotometry of serum hemoglobin: an Allen correction compared with a three-wavelength polychromatic analysis," Clinical chemistry **30**, 627-630 (1984).
67. Instand, ": Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e.V. Ringversuch: Hämatologie " 16 - Freies Hämoglobin **(236)** (2017).
68. bioanalytic, "Freies Hämoglobin (fHb) - Bestimmung von freiem Hämoglobin 3 Wellenlängen-Methode (380/415/450 nm) nach Harboe," (bioanalytic GmbH, Freiburg, 2016).

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EIDESSTATTLICHE VERSICHERUNG

Ich erkläre hiermit an Eides statt,

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München, 28.04.2019	Alexander Lang
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