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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  $\mu\text{mol/L}$ ) 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. © 2018 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.23.5.055006]

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

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## 1 Background

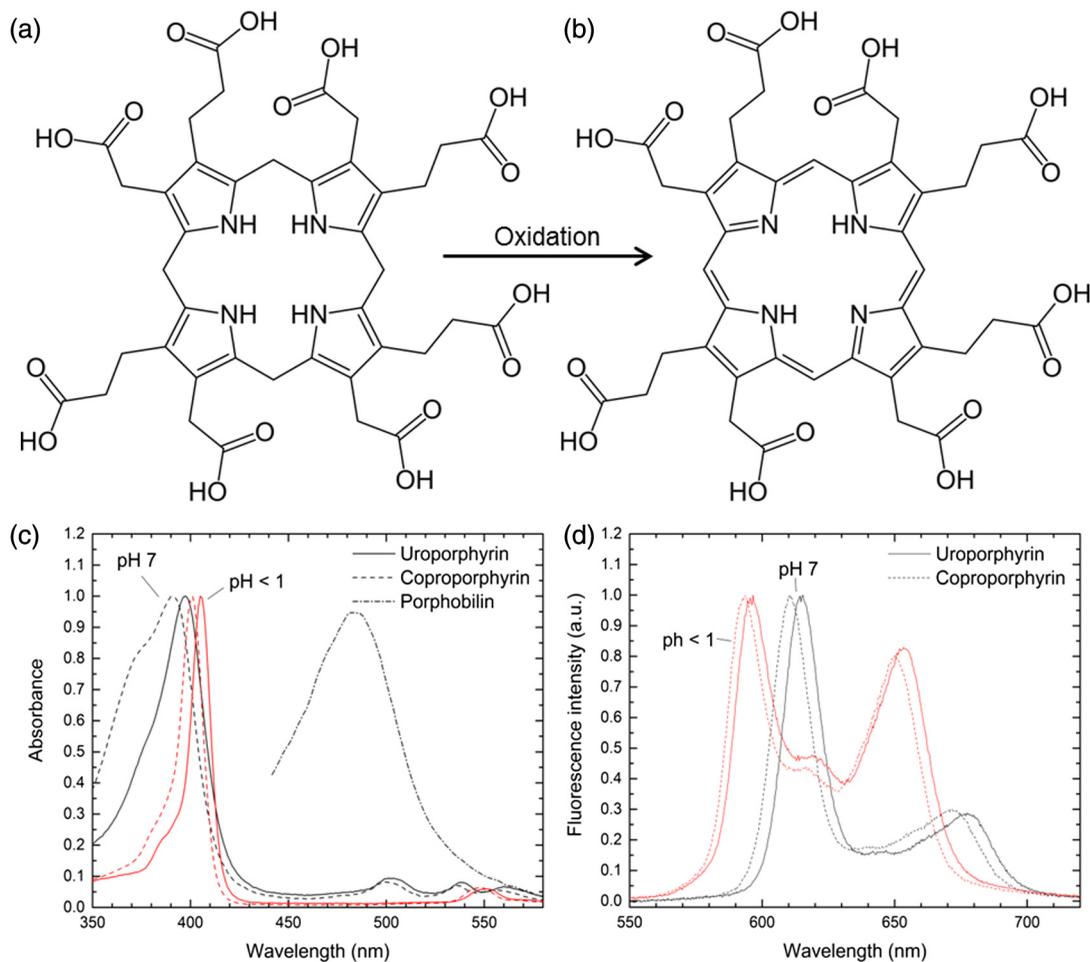
Porphyrias are a group of rare, mainly genetic metabolic disorders of the heme biosynthesis pathway,<sup>1</sup> each caused by the malfunction of one of the eight enzymatic steps in the formation of heme and each causing a specific accumulation pattern of heme precursors in blood, urine, and stool.<sup>2</sup> The diseases can be broadly classified as either acute or chronic porphyria.<sup>1,3,4</sup> This work focuses on the autosomal dominant, acute porphyrias group, namely acute intermittent porphyria, hereditary coproporphyrin, and variegate porphyria, which constitute the most common acute porphyrias with a combined prevalence of ~1 in 10,000 people.<sup>5,6</sup> 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.<sup>3,7</sup>

The universal recommendation for the diagnosis of an acute attack of porphyria is rapid screening for urinary porphobilinogen (PBG).<sup>1,3,4,8,9</sup> 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).<sup>10,11</sup> 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,<sup>12</sup> are labor intensive and require substantial practical expertise.<sup>13</sup> This is not suitable for a simple screening setting in the emergency room or in a 24/7 short turn-around time clinical laboratory.<sup>13</sup> 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),<sup>13</sup> was withdrawn from the market in 2014. Currently, tests for PBG at external laboratories require turnaround times of 4 to 10 days.<sup>1</sup> 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.<sup>14</sup> This delay often results in costly misdirected medical care and progression of the disease.<sup>1</sup>

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<sup>†</sup>This paper is part of the inaugural thesis of Alexander Lang to be submitted at the Medical Faculty of the LMU Munich, Germany



**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 non-fluorescent, unoxidized form of porphyrins, Fig. 1), and PBG.<sup>10</sup> Porphyrinogens and porphyrins can also be elevated in urine from patients with liver disease or some other disorders (porphyria), but porphyria can be excluded, if normal levels of porphyrinogens and porphyrins are found in the urine.<sup>2</sup> 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 porphyria. 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.<sup>15</sup> 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,<sup>16–18</sup> since PBG shows no pronounced UV/VIS absorption or fluorescence emission,<sup>16</sup> and other tests based on Ehrlich reaction (Watson–Schwartz, Hoesch) are prone to interference.<sup>13</sup> 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  $-20^{\circ}\text{C}$ . 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<sup>®</sup> 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, Hercules, California, with 2.6% interassay imprecision.)

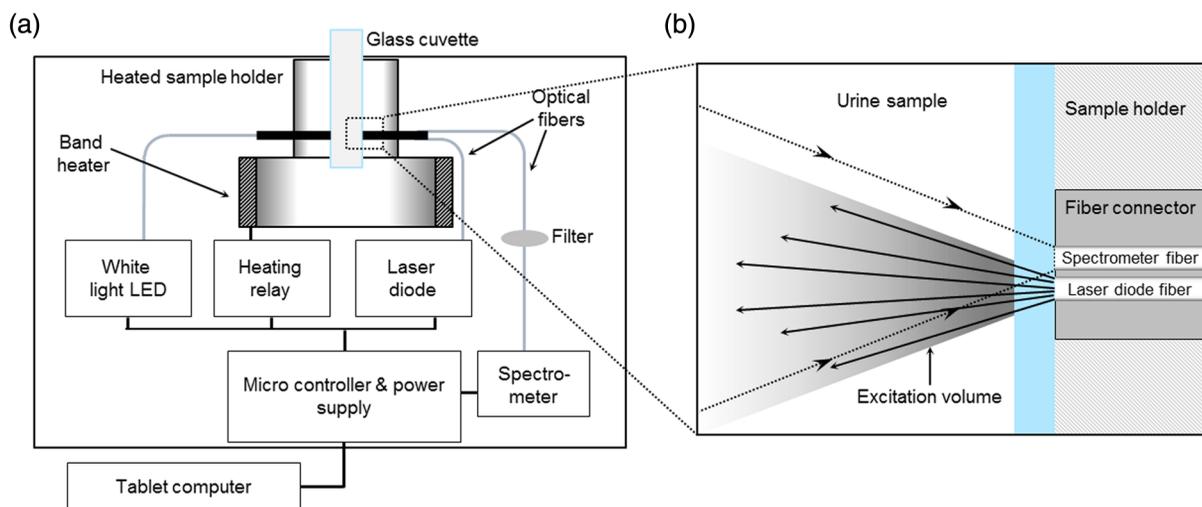
## 2.2 Reagents

Porphyrins (Uroporphyrin I dihydrochloride and Coproporphyrin I dihydrochloride, Merck, Darmstadt, Germany) were dissolved in phosphate-buffered saline (PBS Dulbecco w/o  $\text{Ca}^{2+}\text{Mg}^{2+}$ , BioChrom, Berlin, Germany) to generate stock solutions which were quantified by absorption spectroscopy (Lambda-40, Perkin Elmer PE, Waltham, Massachusetts) using extinction coefficients [ $5.4 \times 10^5$  L/mol cm for uroporphyrin in 0.5-M hydrochloric acid (HCl) and  $4.9 \times 10^5$  L/mol cm for coproporphyrin in 0.1-M HCl] taken from the literature,<sup>19</sup> resulting in concentrations of 50.4 and 72.1  $\mu\text{mol/L}$  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  $-20^\circ\text{C}$ .

## 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  $\mu\text{l}$  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 ( $37.5^\circ < T < 93^\circ\text{C}$ ). 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  $\mu\text{m}$ , type: M15L, Thorlabs, Newton, New Jersey) to the sample holder, collected after transmission by a detection fiber (NA = 0.39, core diameter 200  $\mu\text{m}$ , 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 FWHM, 2.6 mW, SLD3134VR-31, Laser Components, Olching, Germany) is guided by an optical fiber (NA = 0.39, core diameter 200  $\mu\text{m}$ , 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 ( $401 \pm 30$  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  $45.5 \text{ mW/cm}^2$ . Fluorescence spectra were normalized to the fluorescence of a rhodamine standard (1BF/RB Rhodamine, Starna Cells Inc., Atascadero, 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- $\mu\text{m}$  core diameter and a gap of  $\sim 50 \mu\text{m}$ .

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  $\sim 25 \mu\text{mol/L}$  (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.<sup>20</sup> It was compared to oxidation with  $\text{H}_2\text{O}_2$ <sup>21</sup> 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.<sup>20</sup> For the oxidation with  $\text{H}_2\text{O}_2$ , one part of urine was mixed with four parts of 1.25M HCl and 0.04 parts  $\text{H}_2\text{O}_2$  30%. No waiting time is reported in the literature.<sup>21</sup> 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<sup>2</sup>.

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  $(595 \pm 2)$  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  $\text{H}_2\text{O}_2$ . 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,  $\text{H}_2\text{O}_2$ , and photo-oxidation, respectively. Most of the factor can be derived from the dilution (factor 2 for iodine and

photo-oxidation, factor 5 for  $\text{H}_2\text{O}_2$ ) 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).<sup>22</sup>

### 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  $\mu\text{mol/L}$ ) 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.

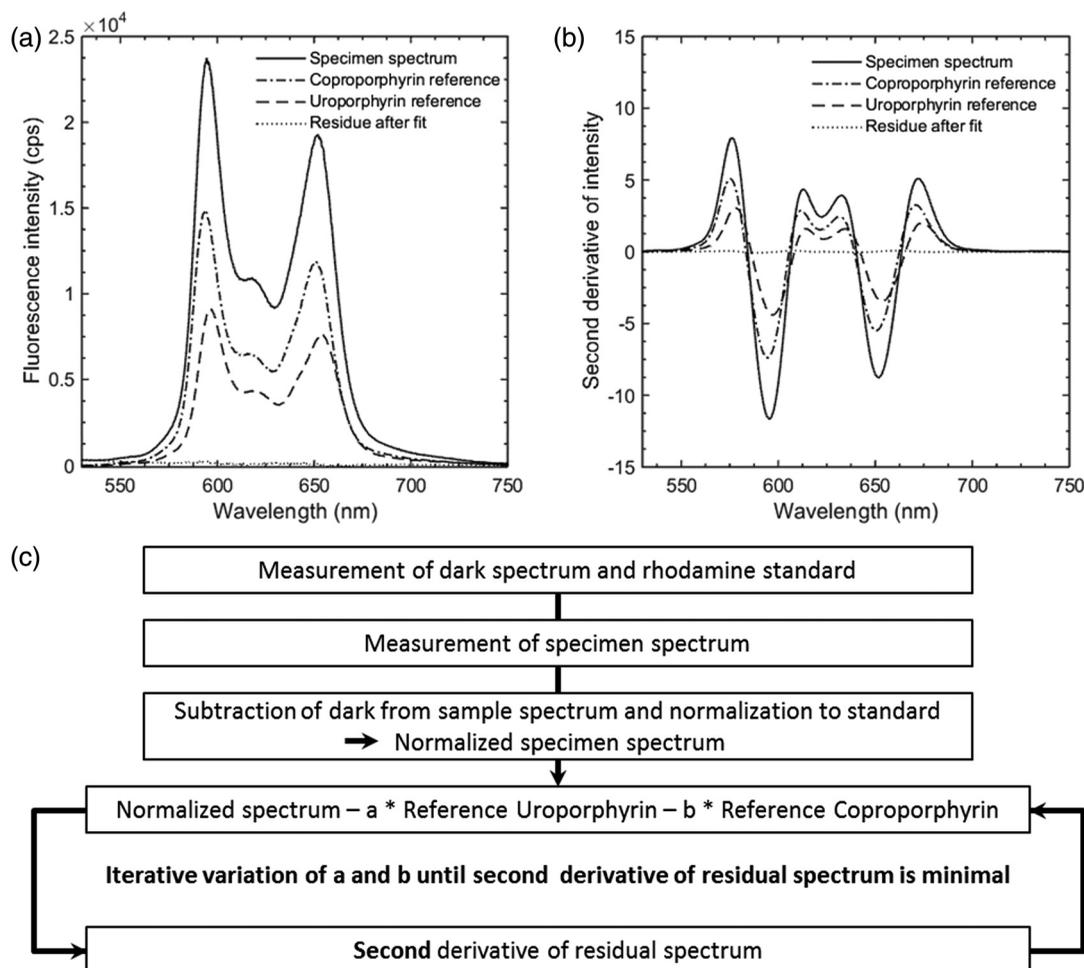
### 2.5.2 Validation set

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  $\mu\text{mol/L}$ ) in every possible combination, resulting in 16 combinations ranging from 0.06 to 18  $\mu\text{mol/L}$  of TUP. Samples with equal concentrations of TUP were averaged for evaluation, resulting in eight concentrations of TUP (in  $\mu\text{mol/L}$ : 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  $(575 \pm 2)$  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.

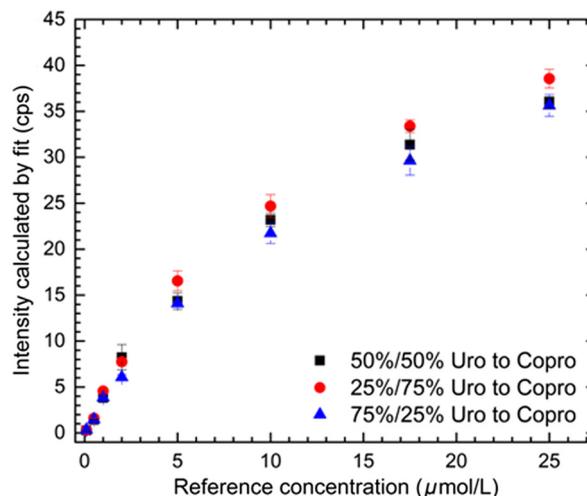
During the fit procedure, normalized uroporphyrin and coproporphyrin fluorescence emission spectra [see Fig. 3(a), dashed and dash dotted] are subtracted from the sample spectrum [Fig. 3(a), solid]. After subtraction [Fig. 3(a), dotted], the second derivative [Fig. 3(b)] is computed by a Savitzky–Golay filter with a window size of 41 nm and a third-order polynomial approximation.<sup>23</sup> 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.<sup>24</sup>

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.<sup>16,17</sup> 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  $\mu\text{L}$  of 1M HCl per 100  $\mu\text{L}$  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  $(615 \pm 5)$  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  $(480 \pm 2)$  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

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

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

## 2.8 Statistical Evaluation

Results were presented as

$$\text{Inaccuracy} = \frac{\text{Mean result} - \text{reference}}{\text{reference}}, \quad (2)$$

$$\text{Imprecision} = \frac{\text{Standard deviation}}{\text{reference}}, \quad (3)$$

for the different concentration intervals, with their definition in accordance with the EMA guideline for bioanalytical method evaluation.<sup>22</sup>

## 3 Results

### 3.1 Comparison of Oxidation Methods

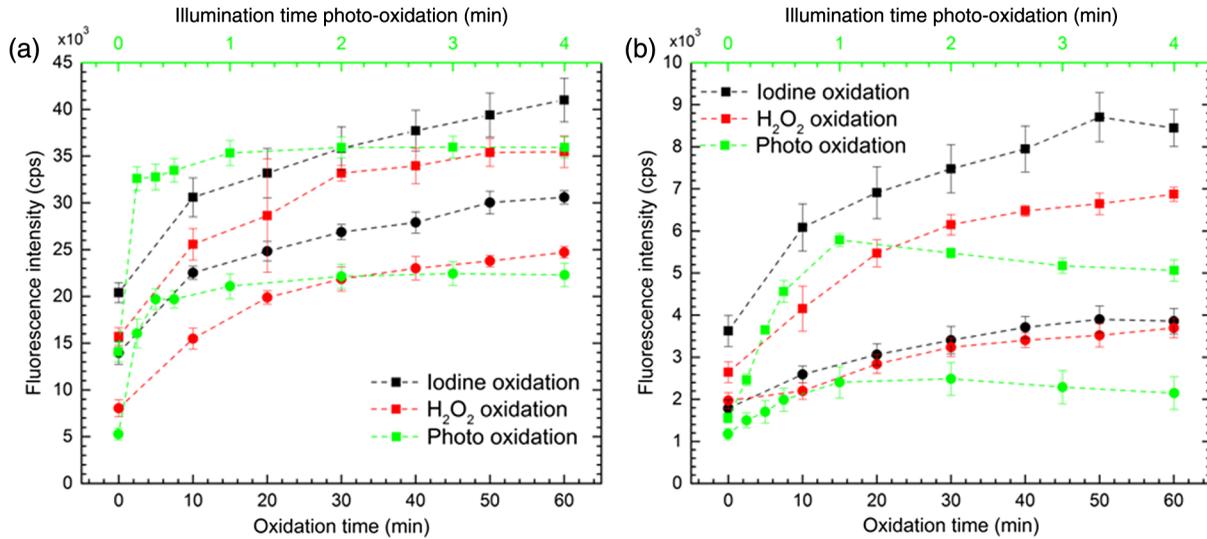
Urinary uroporphyrinogens were oxidized using established chemical oxidation methods and photo-oxidation with controlled illumination conditions. The fluorescence intensity at  $(595 \pm 2)$  nm of oxidized porphyrins was compared for each of the methods for different concentrations of initial porphyrinogens and is shown in Figs. 5(a) and 5(b), over the course of 60 min using iodine and  $\text{H}_2\text{O}_2$  (bottom scale) and over 4 min illumination for photo-oxidation (green and top scale). For chemical oxidation with iodine or  $\text{H}_2\text{O}_2$ , 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,  $\text{H}_2\text{O}_2$  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

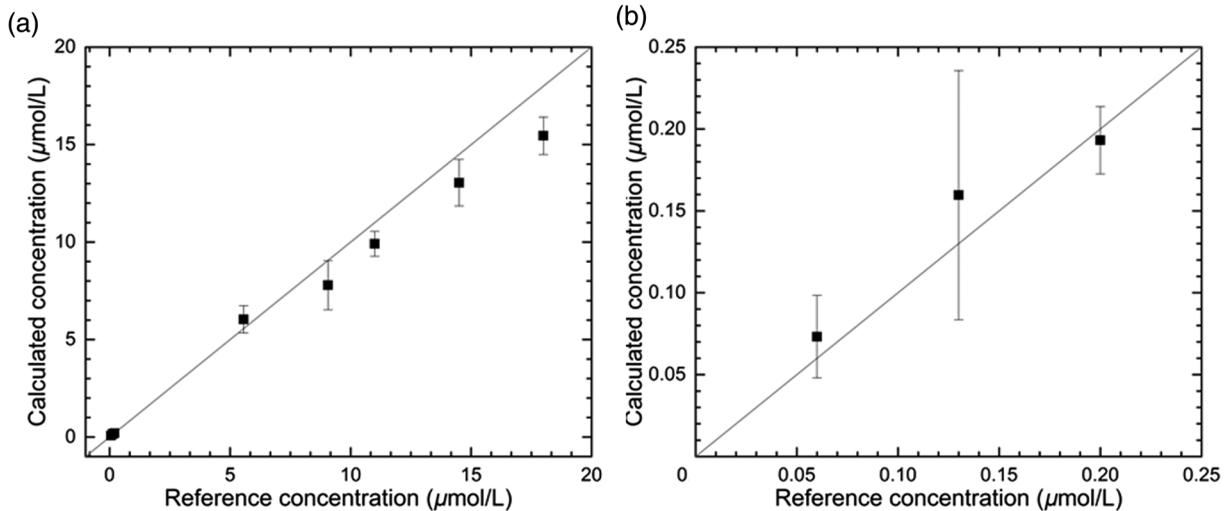
In Fig. 4, three calibration curves for urinary uroporphyrin and coproporphyrin quantification are shown for different molar ratios. The fluorescence intensity of the validation sample set was evaluated with the second derivative fit and quantified using the respective calibration curve. The results 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  $\mu\text{mol/L}$  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  $(93 \pm 1.5)^\circ\text{C}$ . 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  $595 \pm 2$  nm generated by oxidation of porphyrinogens in (a) concentrations of 25 and 12.5  $\mu\text{mol/L}$  (square and round, respectively) and (b) concentrations of 2.5 and 1  $\mu\text{mol/L}$  (square and round, respectively) is shown over the course of 1 h for iodine (black) and  $\text{H}_2\text{O}_2$  (red) and for photo-oxidation (green, top axis) over the course of 4 min illumination time.



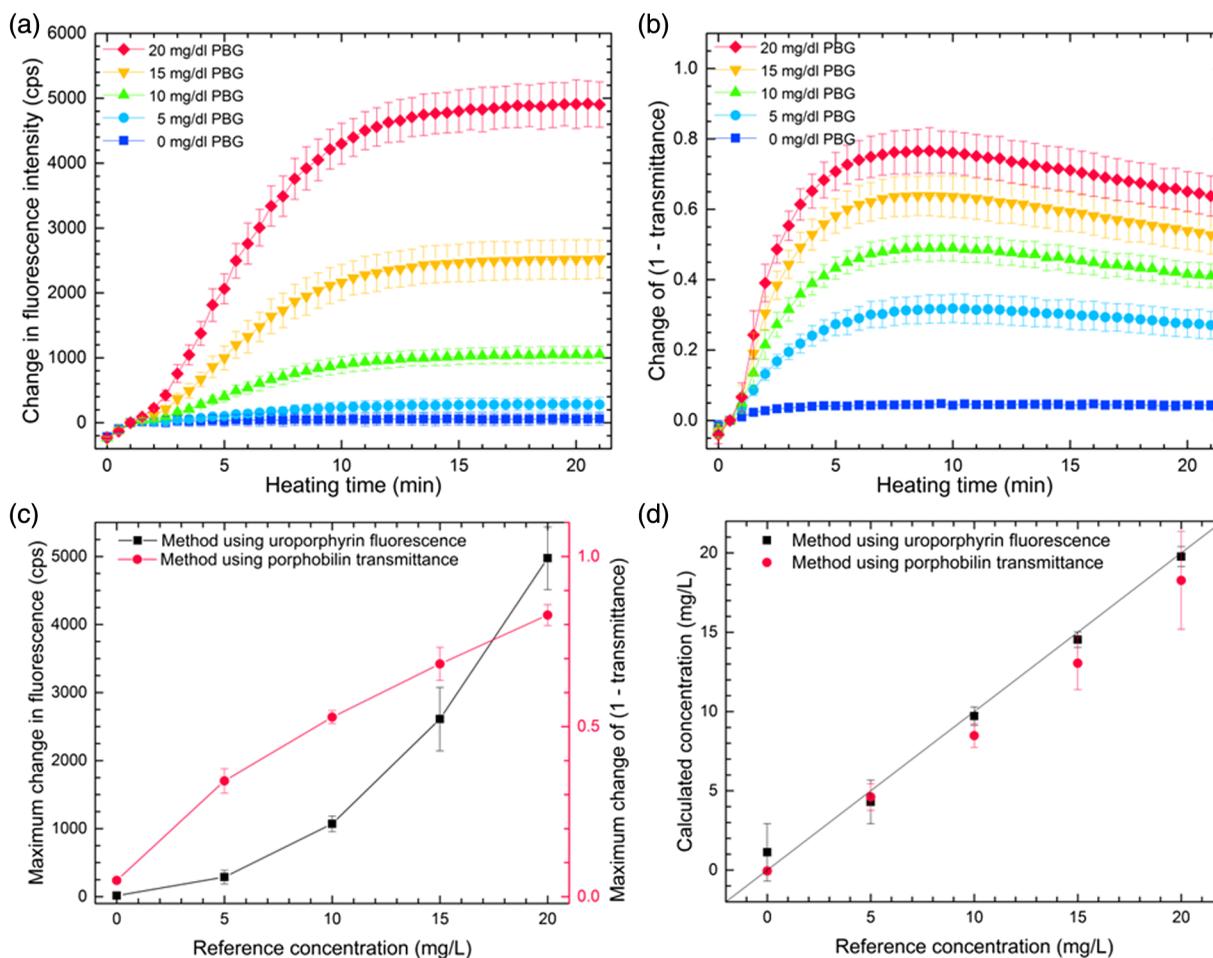
**Fig. 6** (a, b) The quantification result of the independent validation sample set shows overall good recovery as described in Table 1 and high precision for concentrations of 0.2  $\mu\text{mol/L}$  or higher.

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  $\mu\text{mol/L}$  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 ( $\mu\text{mol/L}$ )	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

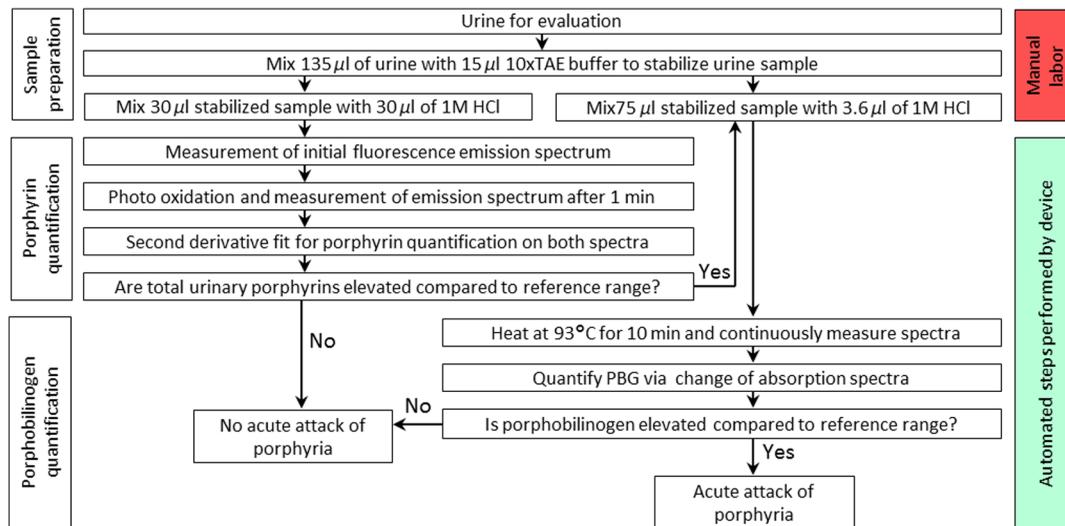
**Table 2** Comparison of inaccuracy and imprecision of the indirect determination of PBG using either fluorescence of uroporphyrin or transmittance of porphobilin.

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

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  $0.06916$  [(mg/L)<sup>-1</sup>; 0.05363, 0.08469; 95% confidence bounds].

### 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  $\mu\text{mol/L}$  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  $\mu\text{mol/L}$ . 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  $\mu\text{mol/L}$ . This is in accordance with reports in the literature,<sup>20,25</sup> where 100% oxidation of porphyrinogens by iodine is found and  $\text{H}_2\text{O}_2$  oxidation yielded

lower results than the use of iodine. While another study reports equal performance of iodine and  $\text{H}_2\text{O}_2$  oxidation, the methods section lacks the details necessary for verifying the claim.<sup>21</sup> 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  $\mu\text{mol/L}$ . 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<sup>2+</sup>, preventing the formation of zinc-porphyrins in urine. The additional acidification with molar HCl optimizes the oxidation rate.<sup>20</sup> 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  $\mu\text{mol/L}$  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

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

was also evaluated, as the calibration curve appears to consist of two linear functions which intersect at a concentration of 5  $\mu\text{mol/L}$ . 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.<sup>21,25,26</sup> 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.<sup>16</sup> 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.<sup>16</sup> 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.<sup>18</sup> 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<sup>13</sup> 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<sup>16</sup> 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  $\mu\text{mol/L}$  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.

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