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Evaluation of the potential use of protoporphyrins as biomarkers of anemic disease in human urine from inflammatory bowel disease patients

Claudia Giménez-Campillo [a], Isabel Montoya-Méndez [a], Natalia Campillo [a], Natalia Arroyo-Manzanares [a], Blanca del Val Oliver [b], José Zarauz-García [c], Luis Sáenz [c], Pilar Viñas [a,*]

[a] Department of Analytical Chemistry, Faculty of Chemistry, University of Murcia, Regional Campus of International Excellence "Campus Mare Nostrum", Murcia E-30100, Spain
[b] Internal Medicine Service - Gastroenterology and Hepatology Section, Hospital General Universitario Rafael Méndez, 30813 Lorca, Spain
[c] Laboratory Medicine Department, Hospital General Universitario Rafael Méndez, 30813 Lorca, Spain

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ABSTRACT

Protoporphyrins are organic compounds with cyclic structure that are synthesised by a wide variety of organisms. In humans, these compounds are detected in blood and urine, with significantly higher levels in blood. Their potential as biomarkers of anemia and other diseases is currently being investigated, as their levels change according to the biochemical processes associated with the disease. The most widely used biomarker of anemia is serum ferritin, but it is unreliable in patients with inflammatory bowel disease (IBD) because its levels can be altered by acute inflammation and/or infections. There is therefore a need to look for new markers to help diagnose anemia in IBD patients. This work develops and validates a method for the determination of three protoporphyrins in human urine: protoporphyrin IX (PPIX), protoporphyrin IX complex with Zn (ZnPPIX) and protoporphyrin IX complex with Fe (II) (FePPIX), the latter also known as heme. The aim is to evaluate their potential as biomarkers of anemic disease in patients diagnosed with IBD. The proposed analytical method is based on high performance liquid chromatography (HPLC) with dual detection based on photodiode array (PDA) and fluorescence (FD). Quantification of the analytes at very low concentrations is possible due to the efficient preconcentration provided by dispersive liquid-liquid microextraction (DLLME) and the sensitivity of the detection systems. The method was validated by evaluating linearity (25–1000 ng mL^{-1}), matrix effect, sensitivity (limits of quantification were between 5 and 11 ng mL^{-1}), selectivity, accuracy, carry-over, dilution integrity, stability and precision (< 12.1 %). Finally, statistical analyses applied to the sample quantification results showed these three markers, together with five clinical markers, were significantly different between anemic and non-anemic IBD patients.

1. Introduction

The term inflammatory bowel disease (IBD) encompasses three pathologies: Crohn's disease (CD), ulcerative colitis (UC) and unclassified IBD or indeterminate colitis [1]. As the name suggests, the common feature of the disease subtypes is inflammation, which can occur at the level of the colonic mucosa in UC or transmurally, which can affect any part of the gastrointestinal tract in CD. Heredity has been shown to play an important role in the development of IBD, and people who have poor dietary habits, smoke, live in polluted environments, or suffer from high levels of stress or depression have a higher incidence of IBD [2].

One of the main symptoms of IBD is anemia. Depending on the population examined (e.g., hospitalised or ambulatory, active disease or in remission, severe or milder disease), anemia is estimated to affect 13–90 % of IBD patients [3]. For patients predisposed to suffer from anemia, such as IBD patients, early diagnosis and treatment can significantly reduce the number of hospital visits and improve their quality of life. Today, the basic test for the diagnosis of anemia is the measurement of serum levels of ferritin, transferrin saturation and C-reactive protein (CRP). If the levels found are inconclusive, vitamin B12, folic acid, haptoglobin, lactate dehydrogenase, creatinine and reticulocyte count are also tested [4]. In patients with moderate IBD or in remission, basic

* Corresponding author.
E-mail address: pilarvi@um.es (P. Viñas).

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parameters should be assessed every 6–12 months, while in patients with active disease, the same tests are recommended every three months [5]. But because ferritin and transferrin saturation indexes are affected by inflammation, their reliability is reduced in patients with IBD disease [3] and it is of great interest to look for other more reliable markers.

Several studies have confirmed that the determination of zinc protoporphyrin IX (ZnPPIX) and protoporphyrin IX (PPIX) in whole blood is useful for the diagnosis of iron deficiency or anemia [3,5–7]. Iron deficiency refers to a lack of iron, whereas anemia to a reduction in the number of red blood cells or plasma hemoglobin (HGB) levels compared to normal values, so iron deficiency can lead to anemia if erythropoiesis is impaired due to iron deficiency [5]. ZnPPIX and PPIX are abnormally elevated in iron deficiency and anemia states. PPIX accumulates when there is insufficient iron for its incorporation into the complex, and ZnPPIX also appears when the iron concentration is suboptimal, because ferrochelatase is able to use divalent Zn as an alternative substrate to ferrous iron [8]. In addition, the fact that ZnPPIX correlates with parameters of iron homeostasis independent of the inflammatory state suggests that it may become a new marker of IBD [3].

Protoporphyrins are complex compounds consisting of four pyrrole rings linked by methene bridges with substitutions at one or more positions. PPIX is the immediate precursor of the heme group or ferroporphyrin (FePPIX), which is the prosthetic group of HGB, the molecule responsible for transporting oxygen from the lungs to the different tissues of the body [9]. PPIX is widely distributed in biological systems and forms the core of molecules involved in redox reactions essential for cellular metabolism. The presence of PPIX and its derivatives in biological fluids indicates a defect in heme group synthesis, which may be due to three main causes: (1) hereditary mutations in the genes encoding ferrochelatase and ALAS2 (5-aminolevulinate synthase), (2) iron deficiency, and (3) insufficient incorporation of iron into erythroid precursors. The latter two causes are often associated with complications of gastrointestinal and liver diseases or cancer [5]. As mentioned above, iron binds to PPIX to form FePPIX, but in the absence of iron, zinc binds to PPIX forming ZnPPIX [4].

Despite the diagnostic utility of determining ZnPPIX and PPIX concentrations, their clinical use has been limited, partly due to the technical difficulty of measuring these compounds in blood. Although its complexity, blood is the preferred matrix for the determination of protoporphyrins because it contains the highest concentrations of these analytes. However, it is also possible to detect protoporphyrins in urine because they are excreted through this route when their production is excessive [10]. Other limitations in the determination of ZnPPIX are that its levels are also elevated in lead poisoning and in some porphyrias [11]. However, the low iron availability for erythropoiesis is by far the most important cause of altered erythrocyte protoporphyrin levels [8].

The techniques currently used for the detection of protoporphyrins in different matrices are based on high performance liquid chromatography (HPLC) [10–16]. Even though mass spectrometry (MS) [11,13] has been coupled to chromatographic separations, the bibliography indicates that the detectors preferred by clinical laboratories are those based on fluorescence (FD) [8,10,11], whereas in the meat industry both photodiode array (PDA) and FD are usually combined [14–16], since some protoporphyrins fluoresce at different wavelengths and the second detector improves the selectivity of the measurements. The use of FD [8, 17–19] without chromatographic separation has also been described, as well as that of hematofluorometry [7,20,21]. This last technique uses a hematofluorimeter, an instrument specifically designed to rapidly measure ZnPPIX from a blood drop, and whose main limitation is that the detected signal can be affected by background fluorescence from other matrix constituents, such as bilirubin, resulting in a falsely high ZnPPIX/heme ratio. Dual wavelength spectrofluorimetric detection is designed to eliminate this positive interference by calculating the difference between two emission spectra [8]. Fluorescence measurements have also proven effective for the analysis of other tissues such as oral mucosa, allowing *in vivo* ZnPPIX monitorization in the lower lip [22]. HPLC-FD remains the reference technique due to its high sensitivity, relatively low cost and the fact that it does not require extensive training for both data management and handling compared to MS [10]. To mention that the use of highly fluorescent metallic nanoclusters is being investigated to assess heme levels [23]. In a recent bibliographic review, the lack of innovative analytical methods with high sensitivity and specificity for the determination of protoporphyrins and their isolation from sample matrices in a cheap, simple and rapid way was observed [24], characteristics which can be attributed to the analytical methodology here developed.

The novelty of this work lies in exploring the use of three protoporphyrins (PPIX, FePPIX and ZnPPIX) as potential biomarkers of iron deficiency and anemia by optimising a urinalysis method to be applied to samples from IBD patients. Statistical processing of the data makes it possible to determine if there are significant differences between the groups of patients, classified according to whether they suffer from iron deficiency or anemia. This study introduces a novel approach by focusing on the analysis of urine samples rather than blood samples, taking advantage of the ease and non-invasive nature of their collection. In addition, due to the expected low concentration of the analytes in the selected matrix [10], a dispersive liquid-liquid microextraction (DLLME) technique will be applied for the first time to increase the sensitivity of the method. This miniaturized sample treatment is catalogued as a simple and environmentally friendly technique with high extraction efficiency and low cost, being aligned with the principles of green analytical chemistry.

2. Materials and methods

2.1. Instrumentation and software

Urine samples were analysed on an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with a quaternary pump (G1311A) and coupled to two detectors in series: a PDA system (G4212B) and a fluorimeter (G1321A). The chromatographic column used was an ACE Excel 3 C18 (15 cm × 4.6 mm, 3 μm) (Advanced Chromatography Technologies, Aberdeen, Scotland). Injection was performed by an autosampler (G1329A) using 2 mL capacity vials with 250 μL microinserts supported on polymeric feet.

Needle-free Nipro syringes of 5 mL capacity coupled with nylon filters (25 mm) of 0.2 μm (Agilent Technologies, Santa Clara, CA, USA) and vial filters of 0.2 μm (GVS, Zola Pedrosa, Italy) were used for sample treatment. Centrifugation was performed on an EBA 20 centrifuge (Hettich, Tuttlingen, Germany).

Agilent ChemStation software (Revision B.04.03 (16)) was used to process the data obtained. The quantification of the three analytes was performed using the chromatographic peak areas, both for PDA and FD, as the analytical signals and considering as reference the retention time obtained for the standards of each compound individually analysed.

To optimise the sample treatment stage, a multivariate study was programmed using the STATGRAPHICS Centurion XV software. A central composite design (CCD, 2^3 + star, face centred) was created with 17 experiments, three of which were spaced to correspond to the central point.

Statistical tests were performed using SigmaPlot (version 14.0) and PAST (version 4.3) software. Univariate tests were performed in Sigmaplot, using the one-way ANOVA test for normally distributed data and the Kruskal-Wallis test for non-normally distributed data. On the other hand, multivariate analysis was performed using the PAST software, specifically a PERMANOVA test, which allowed us to compare several variables between different groups within the same analysis.

2.2. Reagents

The water used was purified using a Milli-Q system (Millipore, Bedford, MA, USA). The chromatographic grade solvents used were

ultra-grade methanol (MeOH) from Avantor™ Performance Materials (Deventer, The Netherlands), acetone from Chem-Lab-NV (Zedelgem, Belgium) and chloroform of purity higher than 99.8 % from Sigma Aldrich (St. Louis, MO, USA). A 0.01 M aqueous solution of ammonium acetate (Fisher Scientific, Loughborough, UK) was used to prepare the mobile phase. N,N-dimethylformamide (DMF) supplied by J.T. Baker (Gliwice, Poland) and formic acid from Sigma Aldrich were also used.

All protoporphyrin standards, ZnPPIX, synthetic (reference: 691550, lot number: 3422917); hemin FeCl(PPIX), porcine (purity $\geq$ 97 %) (reference: 51280, lot number: BCCB6735) and PPIX sodium salt (purity $\geq$ 90 %) (reference: 258385, lot number: MKCM1025) were purchased from Sigma Aldrich. Individual standard solutions were prepared weekly in DMF and stored in the dark at 4 °C. For this purpose, 5 mg of the solid compound was weighed and dissolved in 5 mL of DMF so that the final concentration of each stock solution was 1000 μg mL^{-1}. The preparation of the heme standard from hemin and PPIX involved an additional step consisting of adding a few drops of 5 % v/v ammonia and 1 M hydrochloric acid, respectively, to the solid compounds to enhance their solubility in DMF. Daily dilutions of 100 μg mL^{-1} were prepared from stock solutions, also in DMF, and stored in the dark.

Benzo[*a*]pyrene, cyanocobalamin, riboflavin, hydroxocobalamin, Sudan IV and Congo red, supplied by Sigma Aldrich, were used in selectivity studies for method validation.

2.3. Urine samples: collection and storage

Urine samples provided by healthy volunteers from the Department of Analytical Chemistry of the University of Murcia were used to optimise the analytical method. The samples were collected in sterilised containers of 100 mL capacity and stored in a freezer at − 22 °C.

The clinical samples, belonging to patients diagnosed with active IBD or in remission, were provided by the Emergency Department of the Hospital Rafael Méndez (Lorca, Spain). This study was approved by the Ethics Committees of the University of Murcia (Favourable Report ID: 2908/2020) and of the Hospital Universitario Rafael Méndez (Lorca, Spain). Informed consent was obtained from all patients and samples were used according to hospital guidelines. A volume of 3–6 mL of urine was available per patient, and the samples were collected in sterile tubes, which were also stored in the freezer at − 22 °C for a maximum of one week before analysis.

As generally performed for clinical practices, the first urine in the morning was collected in all cases considering its higher concentration for all the components. The instructions to be followed during collection aimed to avoid contamination by bacteria from the commensal flora of the skin, perineum, and distal urethra, were also applied.

A total of 68 samples from patients diagnosed with IBD in the active phase (32 samples) and in the remission phase (36 samples) were analysed. Within this sample set, 30 patients were diagnosed with iron deficiency or anemia. In addition, 13 of the urine samples belonged to men and 55 to women. The age of the patients varied between 18 and 76 years, with a mean of 48 years. Iron, HGB, calprotectin, cholesterol and CRP were also measured in all clinical samples, as these are important values to assess in patients with IBD and anemia [25].

2.4. Sample treatment and analysis

Prior to the preconcentration step, the sample was thawed, and an aliquot of 3 mL was placed in a conical polypropylene tube, which was wrapped in aluminium foil to protect it from light. Subsequently, 250 μL of formic acid was added to precipitate the proteins present in the sample due to its relatively strong acidity. Next, the tube was gently inverted several times to homogenise and centrifuged at 600 *g* for 3 min. Finally, the supernatant was collected with a disposable syringe and filtered through 0.2 μm pore size filter into a conical bottomed glass tube of 15 mL capacity and the urine was diluted by adding 3.5 mL of water.

To perform the DLLME technique, 250 μL of chloroform (extractant solvent) and 2 mL of acetone (dispersant solvent) were placed in a vial and shaken by using a pipette of variable volume (1.0–5.0 mL) for about 10 s. The homogenized mixture was then vigorously added to the sample solution, followed by manually shaking the tube for about 5 s. The resulting cloudy solution was centrifuged at 600 *g* for 3 min, the extractant phase was recovered using a glass syringe and filtered using 0.22 μm nylon filters before being injected into the chromatographic system.

Analytes were separated using a mobile phase that consisted of a mixture of MeOH (solvent B) and a 10 mM ammonium acetate aqueous solution (solvent A), combined during the analysis according to the following gradient: for the first 2.5 min, the mobile phase contained 25 % ammonium acetate and 75 % MeOH, then a gradient of 0.3 min is established until 100 % MeOH is reached, maintained for 5.2 min, and then the initial mobile phase is restored in 0.4 min and maintained until 10 min [16]. The analysis started with 75 % solvent B to condition the column and ensure that the analytes did not elute in the dead time. Next, the solvent B proportion was increased from 75 % to 100 % and finally the analytes were eluted with 100 % solvent B. The gradient was chosen to obtain maximum resolution and intensity of the recorded signals. Retention times were 5.4, 5.6 and 6.0 min for heme, ZnPPIX and PPIX, respectively.

The flow rate of the mobile phase was set at 1 mL min^{-1} and the injection volume was 20 μL. Light absorption was monitored at 400 nm in the PDA equipment, while 410 and 590 nm were selected as excitation and emission wavelengths, respectively, for FD measurements. As protoporphyrins are photolabile compounds, it is important to avoid direct light on the vials in the autosampler tray. This module was therefore adequately shielded.

2.5. Method validation procedure

The validation of the proposed method was guided by the ICH guideline M10 on bioanalytical method validation and the following parameters were established: matrix effect, linearity, sensitivity, selectivity, precision, accuracy, carry-over, dilution integrity and stability [26].

To study the matrix effect two calibration curves were performed in the range of 25–1000 ng mL^{-1}, in the absence and in the presence of sample matrix. The urine matrix used in this first study was a urine provided by a healthy volunteer from the Department of Analytical Chemistry at the University of Murcia. ANOVA statistical tests were then used to compare the slopes of the calibration lines for each protoporphyrin and to check whether there were significant differences between the two calibration curves [27,28]. The next step was to check whether there were significant differences between the slopes of the calibration curves of each protoporphyrin in the different matrices of the population studied, for which two calibration curves were constructed for each group of patients studied (IBD, IBD diagnosed with anemia and IBD diagnosed with iron deficiency), being these urines provided by the Emergency Department of the Hospital Rafael Méndez (Lorca, Spain). The relative standard deviation (RSD) for each analyte of interest at six concentrations (25, 50, 100, 400, 800, 1000 ng mL^{-1}) included in the calibration range, considering the six urine samples at each concentration level was then studied for both accuracy and precision. In addition, ANOVA statistical test was performed to compare the slopes of each analyte, including the six slopes obtained from the urine samples together with the slope obtained from the calibration curve constructed on a urine sample from a healthy volunteer (previous study), in order to verify that there were no significant differences.

Linearity was studied using matrix-matched calibration by analysing a blank urine sample (obtained from a mixture of urines from volunteers of the Department of Analytical Chemistry of the University of Murcia) spiked with nine concentration levels (0, 25, 50, 100, 200, 400, 600, 800, 1000 ng mL^{-1}). Calibration curves were constructed by plotting the analytical signal obtained versus analyte concentration. As these

analytes are present in all urine samples, the analytical signal was corrected by subtracting the blank (non-fortified urine sample) from all calibration points.

A total of three calibration curves were performed to quantify the three analytes in the samples. HPLC-PDA was used to construct two curves, one for PPIX and the other for the combination of heme and ZnPPIX, since the two peaks were not completely separated. On the other hand, HPLC-FD was used to construct a curve for ZnPPIX.

For quantification purposes, the area of the chromatographic peak was used as the analytical parameter. The ZnPPIX concentration was calculated from the fluorescence signal. To avoid possible errors related to the slight overlap between ZnPPIX and heme, a total concentration of heme plus ZnPPIX was obtained in the HPLC-PDA chromatogram. Since the total concentration of ZnPPIX was determined by HPLC-FD, the heme concentration was calculated by difference.

The limits of quantification (LOQ) were set at the concentration of the analytes in the pooled blank urine sample, as the analytes could not be quantified below this level as they are endogenous compounds.

To test the selectivity of the method, several standards (benzo[*a*]pyrene, cyanocobalamin, riboflavin, hydroxocobalamin, Sudan IV and Congo red), which absorb and emit at the same wavelength as the analytes, were analysed in the presence of blank urine samples. These interferents were selected considering they could appear in urine matrices as consequence of the consumption of food and drugs (vitamins and colorants) and the exposition to polycyclic aromatic hydrocarbons contamination.

For accuracy and precision studies, six urine samples were spiked with the analytical standards at four concentration levels (25, 75, 400 and 750 ng mL^{-1}) and analyzed using the proposed DLLME with HPLC-PDA-FD method. These samples were analysed in triplicate on the same day (n = 18) and the procedure was repeated on three consecutive days (n = 54).

To evaluate the enrichment factor (EF), two calibration curves were generated, one using the DLLME technique and the other in the absence of this preconcentration step, and their slopes were compared.

Carry-over effect was assessed by analysing a non-fortified sample, followed by the highest point of the calibration (1000 ng mL^{-1}) and next the same sample again. This procedure was repeated for three different samples.

To check the dilution integrity, calibration curves were generated by diluting the matrix by three factors (1:1, 1:3, 1:5) and the slopes of the calibration lines were compared with those of the starting matrix.

Finally, stability studies of the analytes were performed both in solution and in matrix. For the first study, solutions were prepared in DMF at different concentrations (75, 400 and 750 ng mL^{-1}) in quadruplicate. Some solutions were stored in the freezer (- 20 ºC), some in the refrigerator (+ 4 ºC), some at room temperature and some were directly injected. The solutions were kept away from light at all times. Over a week, the three stored solutions were injected daily and the analytical signal was studied. Stability studies of the analytes in the matrix were then performed. For this purpose, five non-fortified samples were analysed and stored in the dark. The analysis was repeated over the following two weeks and the analytical signal variation was studied.

3. Results and discussion

3.1. Optimization of the sample treatment

Prior to the preconcentration step, precipitation of urine proteins was carried out to simplify the sample matrix. For this purpose, 250 μL of formic acid was added to 3 mL of sample, this specific volume was chosen because it corresponded to the maximum amount provided by the patients. The mixture was centrifuged and the filtered supernatant was diluted with 3.5 mL of water and subjected to DLLME. All experiments were carried out using urine sample spiked with FePPIX, ZnPPIX and PPIX at 500 ng mL^{-1}.

The efficiency of different organic extractant solvents for DLLME preconcentration was investigated. Both extractants of lower density than water (4-methyl-2-pentanone, ethyl acetate, 1-octanol and 2-octanone) and denser (chloroform, dichloromethane and 1,2-dichloroethane) were tested, in all cases by adding 600 μL of solvent previously mixed with 1.5 mL of acetone (dispersant agent) to the sample solution. Solvents lighter than water were discarded because they provided less intense signals compared to those obtained with denser solvents and, moreover, they were more difficult to recover. On the other hand, when evaluating signal intensity, symmetry and peak resolution, chloroform and 1,2-dichloroethane were preselected because they showed higher extractant power than dichloromethane but the efficacy of one over the other was not clearly defined.

Acetone, ethanol, acetonitrile and MeOH (1.5 mL) were tested as dispersing agents by mixing them with 600 μL of extractant (chloroform or 1,2-dichloroethane) and adding the mixture to the urine solution after precipitating the proteins. Acetonitrile and MeOH were discarded considering the low sensitivity for all analytes. Fig. 1 shows the peak areas obtained for the compounds using the different solvent combinations. It should be noted that the width of the chromatographic peak of ZnPPIX increased and the PPIX peak was split when ethanol was used, so it was discarded. The highest sensitivity was obtained for all three analytes with the chosen combination of chloroform and acetone.

The volumes of acetone, chloroform and the water added to the urine were then optimised. These three factors are closely related experimental variables that affect the selectivity and sensitivity of the DLLME method, so a multivariate method was developed using a response surface design using STATGRAPHICS Centurion programme. In this study, the quantitative factors are the volumes of the extractant, chloroform (0.25–1 mL); dispersant, acetone (0.5–2 mL) and water added to the urine (0–4 mL). As one of the main advantages of using DLLME is the speed with which the analyte is transferred to the acceptor phase, the variables temperature, extraction time and agitation were not considered as factors to be included in the multivariate study. A total of 17 experiments were performed and the peak areas corresponding to each analyte were considered as response variables. Data analysis generated the Pareto chart, which showed the effects of the three experimental variables studied on the extraction efficiency, measured as percentage recovery. The Pareto diagrams corresponding to the experimental design showed that the volume of extractant was the only variable studied with statistically significant effect on the recovery of the analytes.

Multiple response optimization was then performed using the desirability function, taking into account the signal of the three protoporphyrins, as a statistically significant effect was observed for all of them. The results obtained from the application of the response surface design allowed the following DLLME conditions to be established for maximum extraction efficiency: 3.5 mL water added to the urine, 2 mL acetone and 250 μL chloroform. Fig. 2 shows the three-dimensional

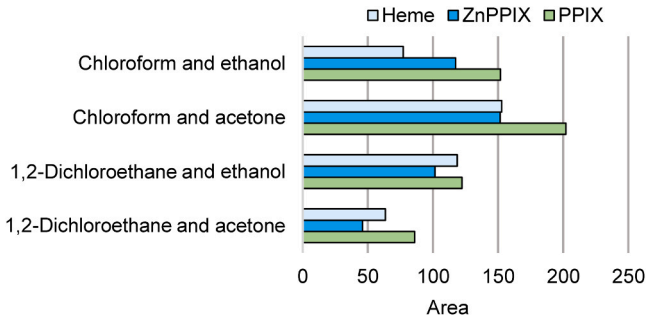

Fig. 1. Signals of three protoporphyrins (heme, ZnPPIX (zinc protoporphyrin IX) and PPIX (protoporphyrin IX)) obtained by HPLC-PDA when varying the combinations of extractant and dispersant solvents used in the dispersive liquid-liquid microextraction (DLLME) procedure.

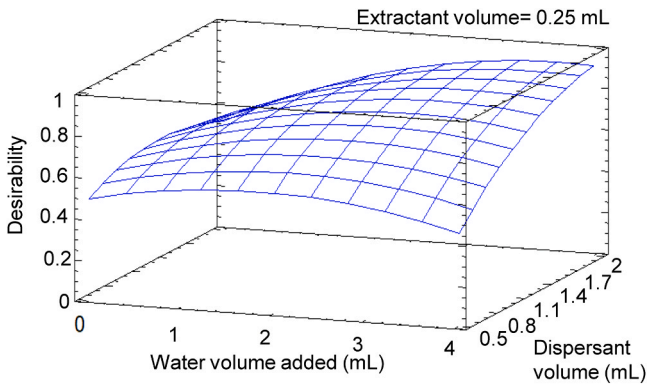

Fig. 2. Response surface obtained for the multivariate optimization of the volume of extractant (chloroform), volume of dispersant (acetone) and volume of water added to the urine in the dispersive liquid-liquid microextraction (DLLME) procedure.

response surface obtained by setting the volume of extractant at its optimum value (250 μL).

The effect of salt concentration in the aqueous phase on the microextraction efficiency was evaluated by adding NaCl at 0, 5 and 10 % m/v. The results showed that there were no significant differences in the signal area of the three analytes when salt was added, so it was decided not to use NaCl. Finally, a centrifugation step at 600 g for 3 min was found to be enough to obtain a differentiated two-phase system. After centrifugation of the sample, a volume of 80 ± 10 μL of extractant phase was recovered.

3.2. Method validation

The matrix effect was studied by obtaining the calibration curves for each analyte in the absence and in the presence of sample matrix, in all cases obtaining regression coefficient values above 0.99, indicating good linearity in the range studied. ANOVA statistical tests were used to compare the slopes of the calibration lines for each protoporphyrin, with p-values less than 0.05. This confirmed the presence of significant differences at the 95 % confidence level. The matrix effect prevented direct quantification with aqueous standards. The RSD values (Table S1) for six concentration levels studied in six different urine samples were then found to be lower than 15 % for both accuracy and precision in all cases. Furthermore, when the slopes of the calibration lines of all ~~six~~ urine matrices (six from IBD patients and one from a healthy volunteer) were compared, it was found that there were no significant differences between them (*p-value* > 0.05). A matrix-matched calibration was therefore performed to quantify the samples. Table S1 shows the mean slopes with their corresponding standard deviations and p-values of each ANOVA.

The three calibration curves were then constructed using a blank urine sample as described in Section 2.5. In all cases, regression coefficients greater than 0.99 were obtained, confirming excellent linearity over the range tested. The slopes obtained for the three calibration curves are given in Table 1. LOQs ranging from 5 to 11 ng mL^{-1} were found, with the lowest value corresponding to ZnPPIX and the highest to PPIX. Table 1 shows the LOQ for each protoporphyrin in urine samples. These LOQ values coincided with the detected concentrations of the three analytes in the pooled blank urine sample and a representation of the chromatograms obtained for this sample is shown in Fig. S1.

The selectivity studies showed that the interferent compounds tested eluted at different times than the protoporphyrins, this point checked for six different urine samples, thus not interfering with the measurement of the analytes.

Table 1 shows the results obtained in terms of RSD for the intra-day and inter-day studies, which were in all cases lower than 4.3 % and 12.1 %, respectively. On the other hand, the method accuracy studies provided recovery values between 82 % and 116 %.

Comparison of the slopes of the calibration curves obtained in the presence and in the absence of the DLLME step provided EF values for heme, ZnPPIX and PPIX of 30.0, 39.1 and 10.3, respectively (Table 1). This demonstrated the effectiveness of using DLLME to improve the sensitivity of the method.

As regards the carry-over studies, it was observed that in no case the alteration of the analytical signals from non-fortified samples was higher than 4.6 % after the analysis of the highest calibration point. For dilution integrity, no significant differences between the dilution slopes were found in any of the analyses. Both studies confirmed the reliability of the quantification of the analytes in the samples.

Finally, when analysing the stability of the analytes in DMF solution, a progressive loss of the analytical signals was appreciated for the three protoporphyrins over a week, especially in the solutions kept at room temperature. It was therefore decided to prepare the standards daily as described in Section 2.2. Stability studies of the analytes in the matrix were then carried out and it was observed that after ten days the signal of the analytes started to decrease in all cases. It was therefore decided to perform the analysis within one week from sample collection and to always treat the samples immediately prior to chromatographic analysis.

Fig. 3 shows the chromatograms obtained using the DLLME with HPLC-PDA-FD method under the selected conditions, applied to a non-fortified urine sample from a patient with IBD. In this sample the analytes were detected at 340, 522 and 1203 ng mL^{-1} for heme, ZnPPIX and PPIX respectively.

3.3. Analysis of urine samples and statistical studies

In accordance with the main objectives of this study, a total of 68 urine samples of IBD patient were analysed. In order to have more information about this variable disease, patients in different stages of the

Table 1
Validation parameters for the DLLME with HPLC-PDA-FD method.

Compound	Detector	Slope (mL ng^{-1})	EF (%)	LOQ[a] (ng mL^{-1})
Heme	PDA	0.098	30.0	10
PPIX	PDA	0.133	10.3	11
ZnPPIX	FD	0.061	39.1	5.0

	Repeatability[b] (%)				Intermediate precision[b] (%)			
Compound	QC_1	QC_2	QC_3	QC_4	QC_1	QC_2	QC_3	QC_4
Heme	1.4	1.1	0.7	0.6	7.5	6.9	5.2	5.2
PPIX	4.3	4.2	3.6	3.2	11.1	9.8	9.3	9.2
ZnPPIX	3.6	3.0	2.8	2.5	12.1	11.5	10.4	10.2

[b] intraday analysis (n = 18); EF: enrichment factor; LOD: limit of detection; LOQ: limit of quantification; PDA: photodiode array; PPIX: protoporphyrin IX; ZnPPIX: zinc protoporphyrin IX; FD: fluorescence; QC_1 = 25 ng mL^{-1}; QC_2 = 75 ng mL^{-1}; QC_3 = 400 ng mL^{-1}; QC_4 = 750 ng mL^{-1}.

[a] Corresponds to the concentration of the analyte in the pooled sample blank.

[b] Interday analysis (n = 54).

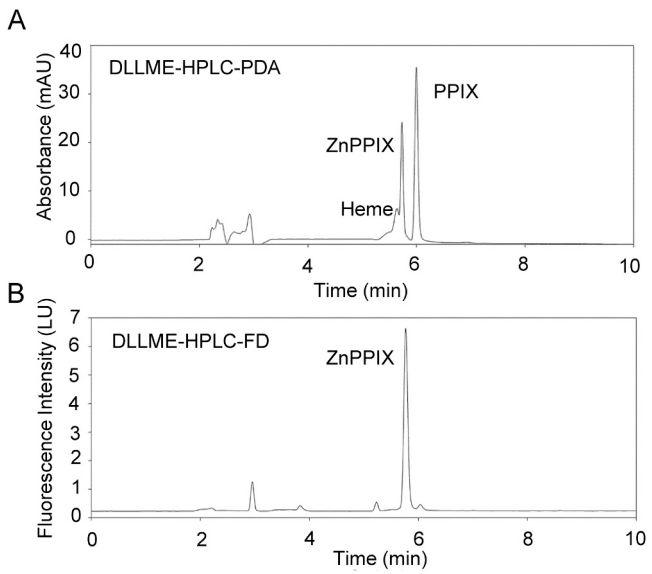

Fig. 3. Chromatograms obtained by dispersive liquid-liquid microextraction (DLLME) and high performance liquid chromatography coupled diode array and fluorescence (HPCL-PDA-FD) for a non-fortified urine sample from an IBD patient. (A) PDA and (B) FD. The figure shows the three protoporphyrins analysed in this work: heme, zinc protoporphyrin IX (ZnPPIX) and protoporphyrin IX (PPIX).

disease, with active disease (32 patients) and in remission (36 patients), were included. Specifically, a total of 18 were diagnosed with iron deficiency and 12 with anemia, while the remaining 38 patients were diagnosed as non-anemic or iron deficient.

Table 2 summarises the demographic characteristics of these three groups of patients, as well as the mean values obtained in blood tests for five clinical IBD-related markers (iron, HGB, calprotectin, CRP, and cholesterol) previously described as abnormalities in patients with IBD [4,29].

Analysis of the data provided in Table 2 shows that the number of women was significantly higher than that of men, especially among IBD patients diagnosed with iron deficiency or anemia. Data for age and cholesterol levels are normally distributed, so one-way ANOVA tests were used to evaluate differences in demographic and clinical parameters between the three groups, whereas that Kruskal-Wallis test was used in cases of non-normal distributions (Fig. S2), i.e. for iron, HGB, calprotectin and CRP levels. The *p-values* obtained for the statistical analyses of each parameter are shown in Table 2. Significant differences ($P < 0.05$) were found between the three groups for iron (Fig. S1B), HGB (Fig. S2C) and cholesterol (Fig. S2F) levels, whereas no significant differences ($P > 0.05$) were found for age (Fig. S2A), calprotectin (Fig. S2D) and CRP (Fig. S2E) levels. The specific groups between which there were significant differences in the above parameters were then identified using the Holm-Sidak method for those with a normal distribution and the Dunn method for those with a non-normal distribution. Significant differences were found in iron and cholesterol levels in patients diagnosed with anemia, as shown in Figs. S2B and S2F, where it can be seen that the group of patients with anemia had significantly lower levels of these parameters. In addition, significant differences in HGB levels were observed when comparing patients without a diagnosis of anemia or iron deficiency with those diagnosed with these diseases, with significantly higher HGB levels observed in patients diagnosed only with IBD (Fig. S2C).

Table 2
Demographic and clinical data of the IBD patients studied.

	Patients with iron deficiency and IBD (n = 18)	Patients with anemia and IBD (n = 12)	Patients with IBD and diagnosed as non-anemic or iron deficient (n = 38)	*P-value*
Gender ratio Male: Female	3:15	0:12	10:28	-
Average age in years	48.9	52.8	46.1	0.573
Mean iron (μg dL^{-1})	83.7	53.0	87.7	0.003
Mean HGB (g dL^{-1})	12.8	12.8	14.2	0.001
Mean calprotectin (μg g^{-1})	650	2790	483	0.672
Mean CRP (mg L^{-1})	0.584	5.00	0.315	0.085
Mean cholesterol (mg dL^{-1})	200	172	199	0.007

-: no data; HGB: hemoglobin; CRP: C-reactive protein.

The 68 urine samples were subjected to protoporphyrin analysis according to the DLLME with HPLC-PDA-FD method described above, and the results obtained are summarised in Table 3. Samples with concentrations above the established linear range were diluted and re-analysed to ensure accurate quantification. To account for the dilution of the concentration in urine, protoporphyrin concentrations were corrected for urinary creatinine [30]. Therefore, the final concentration was expressed as ng protoporphyrin per mg creatinine. Urinary creatinine was determined by a colorimetric method using picric acid [31] and values were in the range of 0.43–1.2 mg mL^{-1} for all urines analysed. Looking at the results in Table 3, it is difficult to determine whether there are significant differences in protoporphyrin concentrations between the different study groups without the use of statistical tools.

Due to the non-normal distribution of the data, the Kruskal-Wallis test (Fig. S3) was used to analyse whether there were significant differences in protoporphyrin concentrations between the three groups of patients, it was found that there were significant differences in the levels of heme ($P = 0.039$), ZnPPIX ($P = 0.014$) and PPIX ($P < 0.001$). Subsequently, using Dunn's method to determine between which groups these significant differences were found, it was determined that the discrepancy was between patients diagnosed with anemia and those diagnosed as non-anemic or iron deficient in the case of heme (Fig. S3A), between patients diagnosed with anemia and those with iron deficiency in the case of ZnPPIX (Fig. S3B), and between patients with anemia and the other two groups for PPIX (Fig. S3C). Correlating these results with the data presented in Table 3, it can be concluded that the presence of heme is higher in patients who have not been diagnosed with anemia or iron deficiency, with an incidence of 100 % in this group. As for ZnPPIX, its concentration is significantly higher in patients diagnosed with anemia, with an incidence of 100 % in this group. On the other hand, when comparing the PPIX values, it was noted that this protoporphyrin is not found in the urine of anemic patients, unlike those with iron deficiency or without a diagnosis of anemia or iron deficiency.

Finally, a PERMANOVA test was performed to evaluate the existence of differences between the three groups of patients, considering all the markers simultaneously. For this analysis, a matrix of 68 samples (rows) was created and the variables (columns) included the values of five clinical markers (iron, HGB, calprotectin, cholesterol and CRP) commonly monitored in IBD patients and three protoporphyrins (heme, ZnPPIX and PPIX). A *p-value* = 0.0001 was obtained, indicating significant differences between groups. When analysed pairwise, there were significant differences between the group of patients with anemia and the other two groups, as shown by the *p-values* in Table 4. This leads to conclude that in patients with IBD, where the identification of anemia is complicated by inflammation, protoporphyrins, supported by other clinical indicators commonly measured in IBD, may facilitate the diagnosis of this disease.

Table 3
Concentration of protoporphyrins found in the urine samples analyzed.

	Compound	Range[a] (ng mL^{-1})	Range[b] (ng mg^{-1})	Mean[b] (ng mg^{-1})	SD[b] (ng mg^{-1})	Incidence (%)
Patients with iron deficiency and IBD	Heme	NQ–54.8	NQ–128	40.4	36.9	88.9
	ZnPPIX	NQ–27.0	NQ–30.7	5.47	10.5	33.3
	PPIX	NQ–72.9	NQ–145	29.6	42.2	77.8
Patients with anemia and IBD	Heme	NQ–696	NQ–1054	251	444	83.3
	ZnPPIX	5.0–1252	5.0–1897	431	778	100
	PPIX	NQ	NQ	NQ	NQ	0.00
Patients with IBD and diagnosed as non-anemic or iron deficient	Heme	10–5277	10–9423	665	2086	100
	ZnPPIX	NQ–522	NQ–621	51.6	144	71.1
	PPIX	NQ–1203	NQ–1432	159	377	71.1

SD: Standard deviation calculated from the concentrations measured in all individuals in the study group; IBD: inflammatory bowel disease; NQ: not quantifiable; PPIX: protoporphyrin IX; ZnPPIX: zinc protoporphyrin IX.
[a] Concentration expressed as ng per mL urine.
[b] Concentration expressed as ng per mg creatinine.

Table 4
P-value of PERMANOVA test.

	Patients with iron deficiency and IBD	Patients with anemia and IBD	Patients with IBD and diagnosed as non-anemic or iron deficient
Patients with iron deficiency and IBD		0.0107	0.1981
Patients with anemia and IBD	0.0107		0.0005
Patients with IBD and diagnosed as non-anemic or iron deficient	0.1981	0.0005	

IBD: inflammatory bowel disease.

Although this research is very promising, it has some limitations that must be taken into account. Firstly, the method cannot distinguish between different oxidation states of the iron compound, such as hemin or heme, since both show the Soret band at the same wavelength used for spectrophotometric detection (400 nm) and elute at the same retention time. This problem was attempted to be solved by HPLC coupled to time-of-flight mass spectrometry (QTOF-MS) analysis, but it was observed that when both standards (heme and hemin) were injected, they appeared with the same retention time and with the mass of heme, since the chlorine bound to the iron of hemin was lost during ionisation. Therefore, HPLC-QTOF-MS also did not allow to distinguish the oxidation state of the iron in the compound. In addition, it is not possible to completely separate heme from ZnPPIX by HPLC-PDA analysis. However, this limitation is overcome by using HPLC-FD, which provides a unique signal for ZnPPIX and allows us to accurately quantify both analytes in the samples. Finally, it should be noted that these are very unstable analytes, so samples should be treated immediately prior to analysis and standards should be prepared daily. In addition, the whole procedure must be carried out using light-protected material and with extreme care.

4. Conclusions

The miniaturized sample treatment based on the DLLME technique coupled to HPLC with dual detection (PDA-FD) has demonstrated its suitability for the quantification of the protoporphyrins heme, ZnPPIX and PPIX in human urine. The microextraction allows efficient isolation and preconcentration of the analytes involving the use of low volumes of both sample and solvents, thus following the principles of green analytical chemistry. High sensitivity has been achieved as LOQs were in the order of ng mL^{-1} for the three analytes with high levels of precision and accuracy. The use of instrumental equipment generally available in any analytical laboratory is an added value to the developed method.

The analysis of urine samples from IBD patients revealed the presence of quantifiable levels of the three protoporphyrins studied. Statistical studies, both univariate and multivariate, indicate significant differences in urinary levels of FePPIX, ZnPPIX and PPIX, as well as five clinical markers (iron, HGB, calprotectin, cholesterol and CRP) commonly monitored in IBD patients, distinguishing anemic patients from those without a diagnosis of anemia. These results suggest that they may be promising biomarkers for the diagnosis of anemia in IBD patients, a task that is particularly challenging because the markers normally used are altered by inflammation.

CRediT authorship contribution statement

Luis Sáenz: Writing – review & editing, Resources. **Pilar Viñas:** Writing – review & editing, Visualization, Project administration, Funding acquisition, Conceptualization. **Claudia Giménez-Campillo:** Writing – original draft, Validation, Formal analysis, Data curation. **Isabel Montoya-Méndez:** Validation, Investigation, Formal analysis. **Natalia Campillo:** Visualization, Supervision, Methodology, Investigation, Conceptualization. **Natalia Arroyo-Manzanares:** Software, Investigation, Formal analysis. **Blanca del Val Oliver:** Resources. **Jose Zarauz-García:** Resources.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2024.116456.

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