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Simple sensitive and simultaneous high-performance liquid chromatography method of glucoconjugated and non-glucoconjugated porphyrins and chlorins using near infra-red fluorescence detection

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Abstract

This paper reports, for the first time, a reversed-phase high performance liquid chromatographic method for the simultaneous determination of seven glucoconjugated and non-glucoconjugated porphyrins and chlorins, using near infra-red fluorescence detection. Chromatographic separation was performed on nucleosil-CN analytical column using an isocratic acetonitrile–0.1% (w/v) TFA at pH 1.8 (55:45, v/v) as mobile phase. Wavelength gradient was employed for sensitive detection, porphyrins derivates were monitored at $\lambda_{exc} = 440$ nm and $\lambda_{emi} = 680$ nm; and chlorins derivates at $\lambda_{exc} = 420$ nm, $\lambda_{emi} = 650$ nm. The method was validated and applied to monitor the biodegradation of a tri glucoconjugated chlorin derivative, TPC(glu)$_{3,}$ in spiked samples of human serum.
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Keywords: Porphyrins; Chlorins; Glucoconjugated; Photodynamic therapy; HPLC

1. Introduction

Photodynamic therapy (PDT) is a non-invasive method for local treatment of small and superficial tumours. PDT is based on the administration of a light-activated drug (photosensitizer), which is selectively retained by neoplasic tissues, followed by irradiation of the tumour with appropriate wavelength light, leading to the destruction of tumour cells [1].

Porphyrin derivates are a class of agents that are used as photosensitizers by PDT. Photofrin® was the first photosensitizer developed for clinical uses; it belongs to the group of haematoporphyrin derivates, but presents adverse effects such as inducing prolonged cutaneous sensitivity [2]. A second generation of photosensitizers are currently under investigation including purpurins, texaphyrins, chorins and benzoporphrins derivates [3]. Among these; 5,10,15,20-*meso*-tetra-(*meta*-hydroxyphenyl) chlorin (*m*-THPC, Foscan®) [4], received regulatory approval in 2002 in the European Union as an agent for palliative treatment of head and neck cancers [5,6]. Recently, a methodology has been developed for the synthesis of various glycoconjugated tetraphenylporphyrins [7] and tetraphenylchlorins [8]. Glycoconjugation appears as an effective mean to modulate the amphiphilicity of tetrapyrrolic compounds that has been demonstrated to play an important role in PDT efficacy [7b,9,10]. In a recent study, it has been reported that a triglucoconjugate of Foscan® was four times more photoactive in vitro than Foscan® itself [8].

Traditionally, high-performance liquid chromatography (HPLC) has been employed for the separation of free porphyrins [11–13], anionic porphyrins [14], cationic porphyrins [15], metalloporphyrins [16,17], porphyrins and metalloporphyrins [18], but data about the simultaneous determination of glucoconjugated derivates of porphyrins and chlorins by

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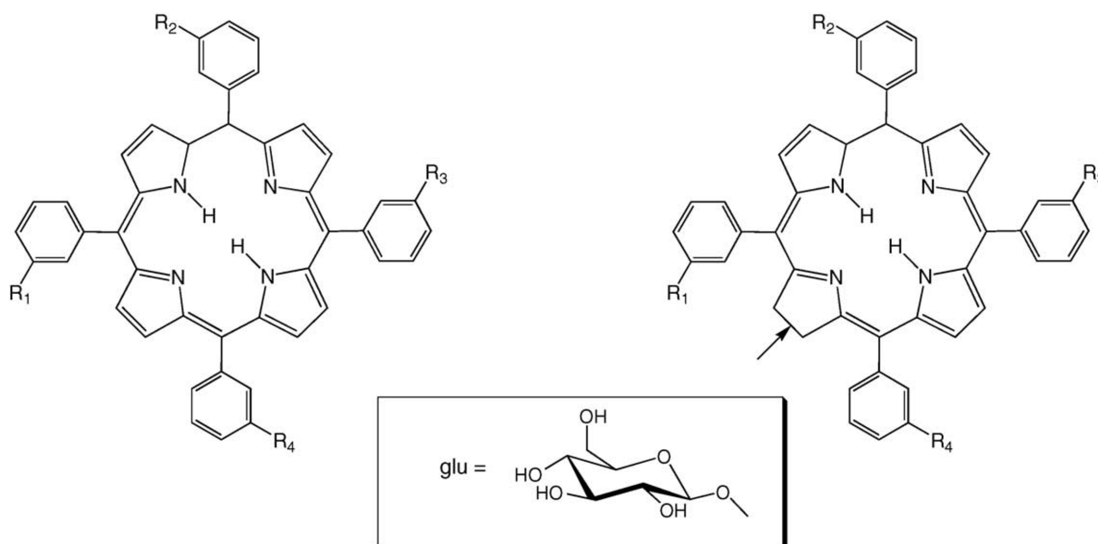

Porphyrins (1, 2, 3, 4) Chlorins (5, 6, 7)

Compound number	*Meso*-Substituent				Name used
	R_1	R_2	R_3	R_4	
1	OH	OH	OH	OH	***m***-THPP
2	glu	H	glu	H	TPP(glu)$_2$
3	glu	Glu	glu	H	TPP(glu)$_3$
4	glu	Glu	glu	glu	TPP(glu)$_4$
5	OH	OH	OH	OH	***m***-THPC
6	glu	Glu	glu	H	TPC(glu)$_3$
7	glu	Glu	glu	glu	TPC(glu)$_4$

Fig. 1. Molecular structures of the studied glucoconjugated and non-glucoconjugated porphyrins and chlorins. The arrow in the formula indicates the difference between porphyrins and chlorins.

chromatography methods are not available in the literature to date.

In the last time, capillary electrophoresis (CE) has also proven to be an effective technique for the separation and determination of porphyrins [19–21], coproporphyrin isomers [22] and metalloporphyrins [23]. Recently, our group has developed an electrophoretic method using non aqueous capillary electrophoresis (NACE) combined with a stacking technique [24] for simultaneous separation and detection of these compounds. Although the sensitivity was enhanced 100-fold by employing a pseudo transient isotachophoresis preconcentration procedure, LODs reached ($\sim 1 \times 10^{-8}$ M) were not high enough to quantify trace amounts of these analytes in serum samples after PDT.

The aim of this work was to develop a selective and sensitive analytical method, using reverse phase high performance liquid chromatography (RP-HPLC) with near infra-red (NIR) fluorescence detection, for the simultaneous determination of seven glucoconjugated and non-glucoconjugated porphyrins and chlorins synthesised as PDT new photosensitizers, all exhibiting very close structures (Fig. 1). Due to its simplicity, rapidity and increased sensitivity, this technique could be very useful for pharmacokinetic, metabolic and biodistribution studies as well as for in vitro experiments analysing chlorins, where the corresponding porphyrins have been demonstrated to be also present as a photodegradation product [25,7c].

Finally this method was validated in terms of linearity, limit of detection and reproducibility and was applied to the analysis of a highly photoactive triglucoconjugate of Foscan®, TPC(glu)$_3$, in human plasma samples.

2. Experimental

2.1. Chemicals

All chemicals and solvent were of analytical grade. Trifluoroacetic acid (TFA) was Aldrich products (Germany). Sodium phosphate and phosphoric acid were obtained from Sigma (St. Louis, MO, USA). Methanol was obtained from Carlo Erba (France). Acetonitrile and dimethyl sulfoxide (DMSO) were obtained from Prolabo (Paris, France). Ultra

pure water was provided by a Millipore Milli-Q system (Millipore, Bedford, MA, USA).

Porphyrins, 5,10,15,20-*meso*-tetra-(*meta*-hydroxyphenyl) porphyrin [abbreviated *m*-THPP (**1**)], 5,15-*meso*-di-(*meta*-*O*-β-D-glucosyloxyphenyl)-10,20-diphenyl porphyrin [abbreviated TPP(glu)$_2$ (**2**)], 5,10,15-*meso*-tri-(*meta*-*O*-β-D-glucosyloxyphenyl)-20-phenyl porphyrin [abbreviated TPP(glu)$_3$ (**3**)] and 5,10,15,20-*meso*-tetra-(*meta*-*O*-β-D-glucosyloxyphenyl) porphyrin [abbreviated TPP(glu)$_4$ (**4**)]; and chlorins, 5,10,15,20-*meso*-tetra-(*meta*-hydroxyphenyl) chlorin [abbreviated *m*-THPC (**5**)], 5,10,15-tri(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-20-phenyl-2,3-chlorin and 5,10,15-tri(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-20-phenyl-7,8-chlorin [abbreviated TPC(glu)$_3$ (**6**)] and 5,10,15,20-*meso*-tetra-(*meta*-*O*-β-D-glucosyloxyphenyl) chlorin [abbreviated TPC(glu)$_4$ (**7**)] were synthesized according to previous works [7,8,26,27]. TPC(glu)$_3$ (**6**) was obtained as an inseparable mixture of two isomeric forms (50/50) [8].

Stock solutions of porphyrins and corresponding chlorins were prepared in methanol and kept at +4 °C in the dark for less than a month. Previous studies have demonstrated that a 50% (v/v) methanol content is necessary in order to prevent self-assembly and ensure compound solubility [28].

2.2. Liquid chromatography

The HPLC studies were performed with a system consisting of a LC-10 AS pump (Shimadzu, Kyoto, Japan), equipped with a Rheodyne® 7125 injection valve (Berkeley, CA, USA) fitted with a 20 μL sample loop. Data acquisition and instrument control were carried out using Kromasystem 2000 software. Separations were carried out on three different columns: Uptisphere ODB, 150 mm × 4.6 mm, with 5 μm particle size (Interchrom), ODS2 and CN analytical columns, both of 5 μm particle size, 250 mm × 4.6 mm (Waters-Spherisorb). The analytical columns were maintained at 30 ± 0.5 °C with a thermostatic oven (Shimadzu®). A Shimadzu RF-10AXL fluorescence detector was employed. A flow rate of 1 mL min^{-1} was employed throughout the experiments. Different mobile phases have been used in this study: (i) acetonitrile–water in different proportions and (ii) a mobile phase containing acetonitrile–0.1% (w/v) TFA at pH 1.8 (55:45, v/v) which allowed the separation of the analytes.

The following wavelength gradient was used for compound detection at optimized conditions: 0–4.5 min: λ_{exc} = 420 nm, λ_{em} = 650 nm; 4.5–8.0 min: λ_{exc} = 440 nm, λ_{em} = 680 nm; 8.0–10.0 min: λ_{exc} = 420 nm, λ_{em} = 650 nm; 10.0–25.0 min; λ_{exc} = 440 nm, λ_{em} = 680 nm.

2.3. Preparation of plasma sample

The human plasma samples were spiked with standard solution of TPC(glu)$_3$ and homogenized by vortexing. The extraction procedure was performed as reported by Whelpton et al. [29]. Briefly, 500 μL of a solution DMSO–MeOH (1.20:3.80, v/v) were added to 50 μL plasma sample. The mixture was stirred and centrifuged (10 min at 4000 rpm) and 200 μL of the supernatant were then dissolved in 800 μL of mobile phase before injection.

3. Results and discussion

Fluorescence emission characteristics of the studied compounds show strong pH dependence. At neutral pH, all compounds exhibit very similar spectral distribution, with λ_{exc} maxima at 420 nm and λ_{em} maxima at 650 nm, while they present strong differences in their protonated forms, with λ_{exc} maxima at 440 nm and λ_{em} maxima at 680 nm and λ_{exc} maxima at 420 nm and λ_{em} maxima at 650 nm for porphyrins and chlorins, respectively. Indeed, it has been demonstrated that chlorin protonation in acidic media induces quenching of the fluorescence emission contrary to porphyrins that are more fluorescent in their protonated form [28]. In Fig. 2 are shown fluorescence emission spectra of *m*-THPP and *m*-THPC (other compounds show the same spectral distribution than the corresponding porphyrin or chlorin), in the solvents used as mobile phase for the chromatographic anal-

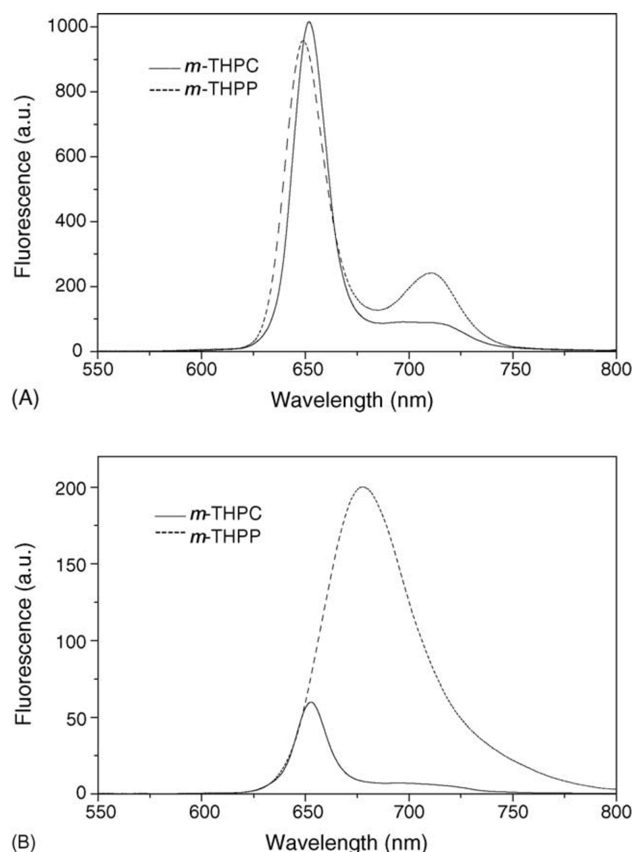

Fig. 2. Fluorescence emission spectra of the *m*-THPP and *m*-THPC. (A) *Solvent*: acetonitrile–water (50:50, v/v), λ_{exc} = 420 nm. (B) *Solvent*: acetonitrile–0.1% (w/v) TFA at pH 1.8 (55:45, v/v), λ_{exc} = 420 nm (*m*-THPC) and λ_{exc} = 440 nm (*m*-THPP). Analyte concentration: 1 × 10^{-6} M.

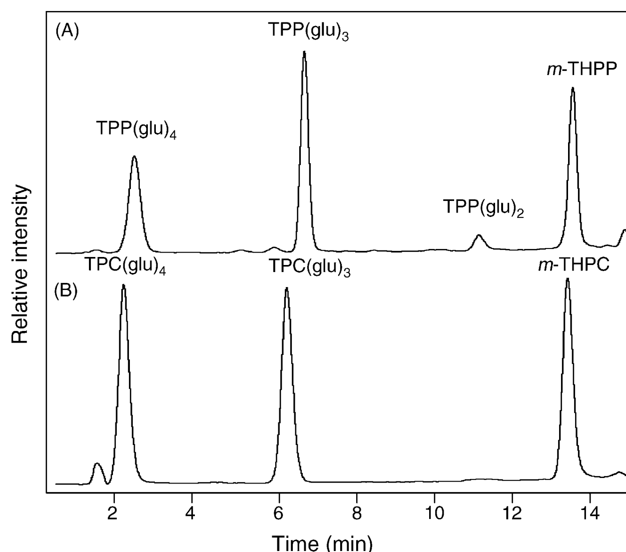

Fig. 3. Separation of (A) porphyrin derivates and (B) chlorin derivates. Using a reversed-phase HPLC column (C-18); mobile phase gradient: acetonitrile–water: starting from 42% acetonitrile increasing the acetonitrile percentage to 100% in 12 min and running isocratically for 15 min; flow rate: 1 ml min^{-1}; column temperature: 30 °C; detection: λ_{exc} at 420 nm, λ_{em} at 650 nm; analyte concentration: 1×10^{-8} M.

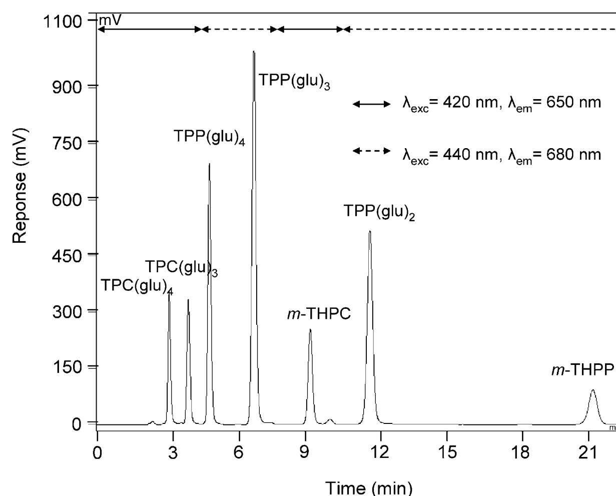

Fig. 4. Simultaneous separation of glucoconjugated and non-glucoconjugated porphyrins and chlorins using a reversed-phase HPLC column CN; isocratic mobile phase: acetonitrile–0.1% (w/v) TFA at pH 1.8 (55:45, v/v). The detection program is illustrated by arrows. Other conditions as in Fig. 3.

ysis: acetonitrile–water (50:50, v/v) and acetonitrile–0.1% (w/v) TFA at pH 1.8 (55:45, v/v).

In a first time, the separation of the seven glucoconjugated and hydroxylated porphyrins and chlorins (**1**–**7**) at neutral pH on a RP-18 (Uptisphere ODB 5 μm, 150 mm × 4.6 mm) analytical column was attempted. Different proportions of acetonitrile–water were tested as mobile phase but, due to affinity of compounds, especially, *m*-THPP and *m*-THPC, towards the stationary phase, it was necessary to use an elution gradient to elute the analytes in less than 20 min. Only the separation of mixtures containing either, porphyrins or chlorins was achieved (Fig. 3). This is due to similar polarities of glucoconjugated porphyrins and chlorins derivates which have the same structure except that one of the double bonds of the tetrapyrrolic ring is saturated in the case of chlorins (Fig. 1). After that, we have afforded the separation of the compounds with a more polar CN analytical column (Waters-Spherisorb 5 μm, 250 mm × 4.6 mm). The same operated mode was followed using different acetonitrile–water percentage as mobile phase. As said, the stationary phase CN is less hydrophobic that the precedent one used, for this reason compounds present a lower affinity for this stationary phase and consequently the retention time decreased. Nevertheless, with this approach, separation of porphyrins and chlorins tri- and tetra-glycosylated was no longer possible.

Thus, the possibility to separate the analytes by ion pairing was tested, using trifluoroacetic acid (TFA). In reversed-phase ion-pair chromatography (RP-IPC), pH should be selected to achieve maximal ionization of analyte molecules to favour the formation of ions-pairs [30]. According to previous studies on the physicochemical properties of these glucoconjugated and hydroxylated porphyrins and chlorins [27], it is known that at the ground state, the porphyrins present, whatever the substitution, the same mean apparent ionization constant (pK_a = 2.7), corresponding to two undistinguishable steps of protonation of tetrapyrrolic nitrogens. With regard to chlorins, only one protonation process is observed for the amino-group of pyrrole, which exhibits an apparent pK_a of 3.0. Hydroxylated compounds present a second ionization, corresponding to the phenol group, pK_a = 10.5.

For the analyses of these compounds an isocratic mixture containing acetonitrile and 0.1% (w/v) TFA at pH 1.8 was selected as mobile phase. At this pH, all compounds were mainly protonated. The effect of the percentage of acetonitrile in the mobile phase on the separation was then studied by varying it from 30 to 60% (v/v). An optimum mobile phase: acetonitrile–0.1% (w/v) TFA (55:45, v/v) was chosen as a good compromise between resolution of whole compounds and time of analysis (Fig. 4). In Table 1 chromatographic parameters such as resolution ($Rs_{n/n-1}$), peak width-length medium (w), and retention factor (k) are summarized.

We checked that the separation of compounds is due to the formation of ion-pairs. For this, we tested the separation the mixtures of compounds using a mobile phase of acetonitrile–50 mM phosphate buffer at pH 1.8 (55:45, v/v), devoid of ion pairing agent. No convenient separation of the mixture was attained and the more polar compounds [TPC(glu)$_4$, TPC(glu)$_3$, TPP(glu)$_4$ and TPP(glu)$_3$] were coeluted; the other analytes were eluted in 4.5 min. In Table 1, are summarized the obtained k for each compound using this mobile phase. A comparison of k values obtained with the two mobile phases was performed. The variation

Table 1
Chromatographic parameters obtained on CN column used in RP-HPLC mode

Analytes	$TPC(glu)_4$	$TPC(glu)_3$	$TPP(glu)_4$	$TPC(glu)_3$	THPC	$TPP(glu)_2$	THPP
$Rs_{n/n-1}$	–	3.13	3.03	5.56	5.83	5.45	>10
ω	0.120	0.148	0.155	0.194	0.207	0.270	0.423
k (acetonitrile–TFA, pH 1.8)	2.56	3.50	4.53	6.72	9.39	12.33	23
k (acetonitrile–phosphate buffer, pH 1.8)	1.42	1.42	1.42	1.42	3.27	2.09	2.86

For details see text.

of *k* for a given compound, suggests different chromatographic mechanisms are involved with each mobile phase. The ions-pair associate has a greater affinity for the non-polar stationary phase, which causes a stronger retention of the analyzed compound, adsorbing as uncharged ion-pair [30], leading to high *k* values. As seen, the retention is higher with RP-IPC than with classical partition RP-HPLC at a same pH value, whatever the compound. This strongly suggests the occurrence of an ion pairing mechanism. Indeed, in RP-IPC method, porphyrins are more retained than chlorins, having the same glucosylation degree, this behaviour is due to the different compounds charges: porphyrins (+2) and chlorins (+1).

Separation of compounds by RP-IPC using a C-18 analytical column (Waters-Spherisorb 5 μm, 250 mm × 4.6 mm) was also tested. The same mobile phase that used for separation on CN column was employed [acetonitrile–0.1% (w/v) TFA at different percentages]. However, under these conditions, no separation was obtained. It may be explained by the role of TFA, which acts as a non-classic counter-ion. One might suggests that TFA exhibiting no aliphatic chains shows a higher affinity towards short chain stationary phase as CN.

On the other hand, we have noted the importance of the injection solvent polarity. First analyses were achieved using 100% (v/v) methanol. In this case, we obtained a double peak for $TPC(glu)_4$. After that, different injection media were tested: methanol–water at different proportions, 100% (v/v) acetonitrile, acetonitrile–water in different proportions and mobile phase, acetonitrile–0.1% (w/v) TFA (55:45, v/v). We found that for methanol–water environment the peak remained shouldered, in contrast to solvents including acetonitrile only one peak was obtained (Fig. 5).

In the optimized conditions, the chromatogram of a mixture of the seven compounds is displayed in Fig. 4, the detection program used, in order to get the best sensitivity for all analytes, is also shown. Separation is very satisfactory, a baseline resolution of all peaks (Rs > 1.5) and symmetrical peak shape were obtained. All the analytes were eluted in 22 min.

3.1. Method validation

Under the optimized conditions, calibration graphs were obtained by preparing five calibration standards at concentrations ranging from 3.0×10^{-9} to 3.0×10^{-7} mol L^{-1} for *m*-THPC, *m*-THPP, $TPC(glu)_3$ and $TPC(glu)_4$ and from 3.0×10^{-10} to 3.0×10^{-8} mol L^{-1} for $TPP(glu)_2$, $TPP(glu)_3$ and $TPP(glu)_4$. Samples were injected in triplicate and average peak areas were used to plot the calibration curves. The linearity was checked according to standard statistical procedures using *F* test (ANOVA; $n = 3$, 5 concentrations). In all cases, existence of a significant slope and the validity of the adjustment were confirmed (95% confidence interval). The intercepts were not significantly different from 0 (Student's *t*-test). The detection limit (LOD) was considered as the minimum analyte concentration yielding a signal-to-noise equal to three. The quantification limit (LOQ) was adopted as the lowest analyte concentration yielding a signal 10 times greater than the noise and that could be reliably determined. The detection limits found were between 5×10^{-10} and 5×10^{-12} mol L^{-1}. The results obtained are listed in Table 2.

The precision of the method was determined by the measure of repeatability (intraday) and intermediate precision (interday). Repeatability was evaluated by the R.S.D. of replicate experiments ($n = 7$) of seven analytes with a concentration of 1×10^{-8} mol L^{-1}. The intermediate precision was assessed by measuring the R.S.D. of replicate experiments ($n = 7$) carried out at different days. The results, summarized in Table 3, show that the R.S.D. of intra and interday repeatability of retention times were less than 0.3% indicating a very satisfactory precision of retention times. The intra and interday precision of peak areas ranging from 0.52 to 4.98% were also satisfactory.

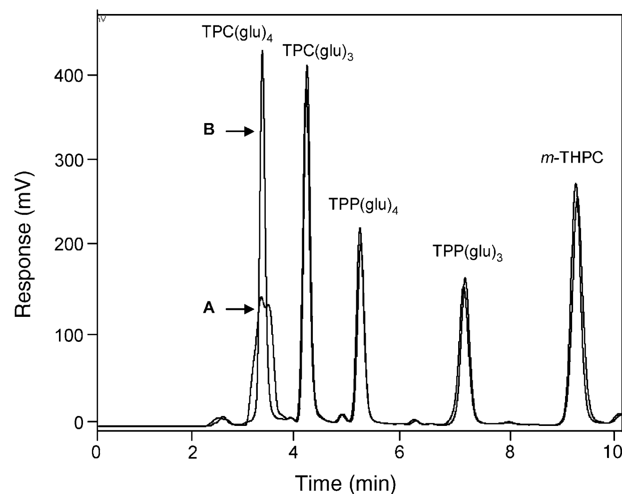

Fig. 5. Separation of glucoconjugated and non-glucoconjugated porphyrins and chlorins using different solvent injection: (A) methanol and (B) mobile phase (acetonitrile–0.1% (w/v) TFA at pH 1.8 (55:45, v/v). Detection: λ_{exc} at 420 nm, λ_{em} at 650 nm; analyte concentration: 1×10^{-8} M.

Table 2
Linearity parameters and limits of detection and quantification obtained in method validation

Analyte	Linearity range ($mol\,L^{-1}$)	Calibration parameters					
		Intercept	Slope ($\times 10^9$)	Correlation coefficient (*r*)	Linearity[a]	LOD ($mol\,L^{-1}$)	LOQ ($mol\,L^{-1}$)
m-THPC	3×10^{-9}–3×10^{-7}	−1.30	0.62	0.9991	99.5	1×10^{-10}	3×10^{-10}
TPC(glu)$_3$	3×10^{-9}–3×10^{-7}	−0.50	0.63	0.9983	99.3	1×10^{-10}	3×10^{-10}
TPC(glu)$_4$	3×10^{-9}–3×10^{-7}	−0.06	0.53	0.9998	99.6	1×10^{-10}	3×10^{-10}
m-THPP	3×10^{-9}–3×10^{-7}	−0.02	0.28	0.9998	99.5	5×10^{-10}	2×10^{-9}
TPP(glu)$_2$	3×10^{-10}–3×10^{-8}	−0.05	1.57	0.9999	99.6	2×10^{-11}	5×10^{-11}
TPP(glu)$_3$	3×10^{-10}–3×10^{-8}	0.11	4.71	0.9996	99.3	5×10^{-12}	2×10^{-11}
TPP(glu)$_4$	3×10^{-10}–3×10^{-8}	0.40	5.81	0.9998	99.5	5×10^{-12}	2×10^{-11}

[a] 95% confidence interval of the Fisher test.

Table 3
Precision for the retention time and peak area, 1×10^{-8} M of each compound

Analyte	Retention time R.S.D (%) (*n* = 7)		Peak area R.S.D (%) (*n* = 7)	
	Intraday	Interday	Intraday	Interday
m-THPC	0.08	0.11	1.99	1.56
TPC(glu)$_3$	0.01	0.01	2.63	1.65
TPC(glu)$_4$	0.07	0.07	3.48	3.67
m-THPP	0.30	0.30	2.34	0.52
TPP(glu)$_2$	0.03	0.05	2.68	4.51
TPP(glu)$_3$	0.01	0.01	2.52	3.65
TPP(glu)$_4$	0.02	0.02	4.98	2.69

3.2. Application to plasma sample

The optimized method was used to monitor chlorin degradation in human plasma (ex vivo stability). Tri-glucoconjugated derivative (TPC(glu)$_3$) was chosen as an example. In a recent study [8] we have demonstrated that this compound was about four times more photoactive in vitro than the non glucoconjugated analogue *m*-THPC, in contrast with the tetra-glucoconjugated one [TPC(Glu)$_4$] that was poorly active.

Plasma samples were spiked with TPC(glu)$_3$ leading to final concentration of $1.8\times10^{-8}\,mol\,L^{-1}$. This value corresponds to mean concentration levels found in samples after extraction during preliminary pharmacokinetics studies of glucoconjugated and non-glucoconjugated analogues. The extraction yields were found very high, as it has already been mentioned by Desroches [31]. In Fig. 6 two chromatograms are plotted: one corresponding to the spiked plasma sam-

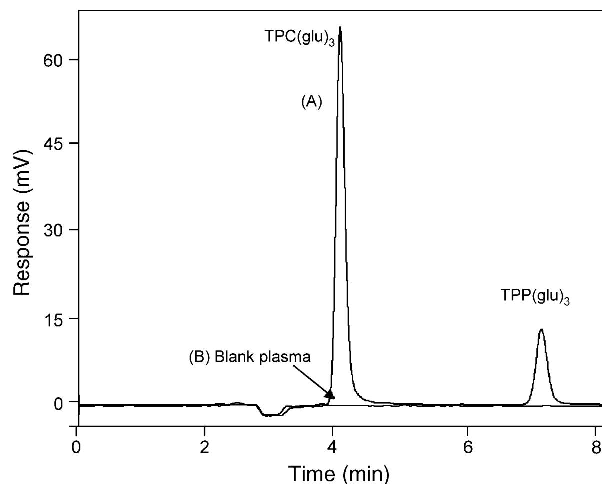

Fig. 6. Chromatograms of samples obtained after an extraction from human plasma: (A) blank plasma and (B) plasma spiked with 1.8×10^{-8} M TPC(glu)$_3$. Other conditions as in Fig. 4.

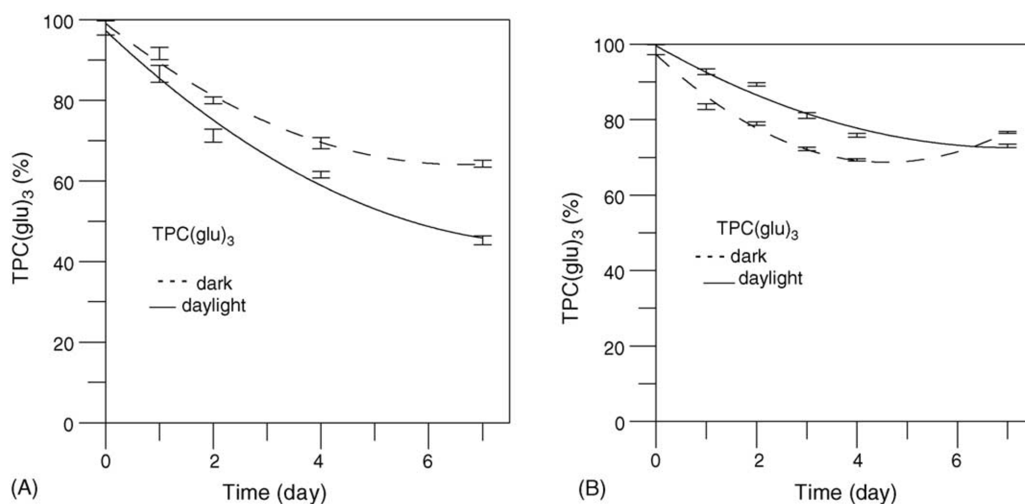

Fig. 7. Study of porphyrins stability in plasma: (A) TPC(glu)$_3$ degradation vs. time and (B) TPP(glu)$_3$ degradation vs. time. Other conditions as in Fig. 4.

ple, treated as detailed in the experimental section and the other one, corresponding to the plasma blank, processing in the same way that plasma spiked sample. We can observe that there is no-interference peak from the plasma matrix. In the chromatogram corresponding to spiked sample we can observe one peak at 4.04 min, belong to TPC(glu)$_3$ and weaker one, at 7.10 min belonging to TPP(glu)$_3$. The existence of the peak of TPP(glu)$_3$ from the first time is because in the TPC(glu)$_3$ synthesis, it rest about 10% of porphyrin, TPP(glu)$_3$, that cannot be separated [8].

Moreover, two ex vivo stability studies were achieved. For this purpose, spiked plasma samples were placed in two glass tube, one tube was stored in a dark place and the other one was at daylight, both were maintained at room temperature. Each plasma sample (daylight and protected from light) was injected daily for triplicate during one week and the evolution of the fluorescence intensity of both peaks [TPC(glu)$_3$ and TPP(glu)$_3$], was studied. In Fig. 7A is shown the fluorescence intensity evolution of the peak belonging to TPC(glu)$_3$ in both plasma sample. When the plasma is exposed to light, the decrease of fluorescence signal is higher than in the plasma protected from light. Therefore TPC(glu)$_3$ degradation is enhanced with light. With regard to intensity evolution of TPP(glu)$_3$ peak (Fig. 7B) at the first days the intensity decrease in the same way that TPC(glu)$_3$, and after four days the intensities reach a plateau, due to the TPC(glu)$_3$ oxidation to give, among other compounds, its analogue porphyrin [TPP(glu)$_3$] [25].

4. Conclusion

The method described is a simple and sensitive HPLC method that allows all seven glucoconjugated and non-glucoconjugated porphyrins and chlorins to be simultaneously separated with baseline resolution. Due to its high sensitivity, this method would be of particular interest for clinical quantitative analysis, to monitor degradation and metabolisation of such compounds in biological samples.

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