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Enhanced detection of seven glucoconjugated and hydroxylated porphyrins and chlorins by nonaqueous capillary electrophoresis combined with stacking

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Abstract

The nonaqueous capillary electrophoresis mode which includes a preconcentration step based on a transient pseudo-isotachophoresis to the simultaneous separation of seven glucoconjugated and hydroxylated porphyrins and chlorins, exhibiting very close structures, is reported. A high methanol content, of the buffer solution, was necessary in order to prevent self-assembly of the compounds and to enhance their solubility during separation. With the addition of 66% (v/v) methanol and 1% (w/v) NaCl in the aqueous sample solution, large volumes could be injected (44% capillary volume) without a loss in resolution. Sensitivity of detection was therefore improved by a 100-fold factor with regard to the method employing normal injection (2% capillary volume). Optimum electrophoretic conditions, in terms of sensitivity and performance, were obtained by using 20 mM phosphoric acid buffer, pH 2.2 and 50% methanol. The method was validated and applied to qualitative analysis of glucoconjugates in serum samples.

Keywords: Porphyrins; Chlorins; Nonaqueous capillary electrophoresis; Isotachophoresis

1. Introduction

Photodynamic therapy (PDT) is a modality for local treatment of small cancers that involves administration of a light-activated drug (photosensitizer) which is selectively retained in tumour tissues. To initiate the photochemical process, wavelengths in accordance to the absorption spectrum of the different photosensitizers have to be selected for the excitation. Following excitation, the generation of reactive oxygen species, mainly singlet oxygen ($^1O_2$), leads to irreversible destruction of the treated tissues. However, the mechanism of action is still not well understood [1–3].

Photofrin®, was the first photosensitizer developed for clinical uses, it belongs to the group of haematoporphyrin derivates but presents some adverse effects such as inducing prolonged cutaneous sensitivity [4]. A second generation of sensitizers has been developed such as purpurins, benzoporphyrins, texaphyrins, and chlorines [5]. Meso-tetraarylporphyrins are the most easily accessible synthetic porphyrins having favourable photophysical properties [6]. Among these, 5,10,15,20-*meso*-tetra(meta-hydroxyphenyl)chlorin (*m*-THPC, Foscan) seems to be a promising candidate [7], and it received regulatory approval in 2002 in the European Union for human palliative treatment [8,9]. In the last years, a methodology has been developed for the synthesis of glucoconjugated tetraphenylporphirins [10] and tetraphenylchlorins [11]. These structural modifications, induced by glucoconjugation of the tretrapyrrole group, appear as an effective mean to modulate the amphiphilicity of these compounds. Indeed, amphiphilicity of sensitizers has been shown to increase photoefficacy. In a recent study, it has been reported that a triglucoconjugate of Foscan, was four times more photoactive in vitro than Foscan itself [11].

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Traditionally, HPLC has been the most widely used method for the separation of porphyrins [12], but recently, capillary electrophoresis (CE) has been shown to be an alternative for the determination of these species [13]. Micellar electrokinetic chromatography (MEKC) was used to separate non charged polyhaematoporphyrin (PHP) derivates [14] and to separate coproporphyrin isomers [15]. MEKC has been also applied for the chiral separation of benzoporphyrin derivate using bile salts as chiral selector [16]. A limit of detection (LOD) around 4×10^{-6} M was attained using laser-induced fluorescence (LIF). NACE (nonaqueous capillary electrophoresis) was reported for the analysis of photofrin®, used in PDT [17]. In this method, Brij 35 was added to buffer electrolyte as a complexation reagent to adjust the selectivity and improve the efficiency of the separation. Under these conditions a partial separation of 60 porphyrin derivates was obtained in 30 min. Cationic porphyrins atropoisomers have been separated by capillary zone electrophoresis (CZE), exploiting the different electrophoretic mobilities of the compounds afforded by different shapes and dipole moments of the atropoisomers [18]. Metalloporhyrins were also analysed by CE [19]. In this work, the sensitivities of inductively coupled plasma mass spectrometry (ICP-MS) and UV detection were compared. It was concluded that LODs were slightly better with ICP-MS. However, for both detection methods, LODs were still in the picogram range.

One serious problem of CE arises from the low sensitivity, associated with the limited optical pathway of on-capillary photometric detection and the small sample volumes that can be injected [20]. In the last decade, a considerable interest to improve concentration sensitivity in capillary electrophoresis has been developed. Two basic approaches can be distinguished to reach this goal: either special detection methods more sensitive than UV detection, such as LIF and electrochemical mode or an increased analyte mass loading [21]. Among the pre-concentration methods, stacking has been extensively described in the literature and has emerged as an efficient method to increase sensitivity in CE [21–23]. One preconcentration method can be performed by including organic solvents in the sample. This type of stacking occurring with many organic solvents is greatly enhanced by the presence of high concentrations of different inorganic ions in the sample [24–26]. The mechanism of organic solvent stacking, is similar to that of isotachophoresis (ITP) where the salts (in the sample) act as leading ions while the acetonitrile and others alcohols give the high field strength similar to the action of the terminating ions but without the rigid requirements of pH, concentration or counter ions necessary in ITP [23]. Recently, Shihabi suggested that this method could be termed transient-ITP or better, transient "pseudo-ITP" [27]. So et al. have demonstrated the effectiveness of this preconcentration method for the detection of coproporphyrin isomers by MEKC [15].

During the last few years, nonaqueous capillary electrophoresis (NACE) has grown in popularity and their benefits have been described in recent reviews [28–31]. NACE is a very useful technique for the separation of hydrophobic compounds and substances which are difficult to separate in aqueous buffer. In addition, as the physicochemical properties of organic solvents (e.g. dielectric constant, viscosity, autoprotolysis) differ widely, these solvents may enhance or modify selectivity [32]. Because of the low conductivity of most of the organic solutions, stacking approaches are quite limited. Therefore, there is a great need for methods allowing injection of large volumes of sample while inducing at the same time stacking, which are compatible with this mode of separation. Shihabi has obtained enhancement in sensitivity in NACE by the introduction of transient isotachophoresis (t-ITP). In this method, triethanolamine (TEA) located at the anode, acted as a terminating ion while sodium served as leading ion in the t-ITP process [33].

The aim of the present study was to develop a sensitive method for the simultaneous determination of seven glucoconjugated and hydroxylated porphyrins and chlorins, with poor water solubility and exhibiting very close structures (Fig. 1). Metabolism of tetra and triglucoconjugated chlorins [TPC(glu)$_4$ and TPC(glu)$_3$] in biological medium results, by action of cellular glucosidases, in the formation of partially or totally deglucosylated compounds and in the formation of porphyrins by oxidation of chlorins macrocycles [34]. Thus all these compounds, may be found in biological fluids after a PDT therapy based on administration of glucoconjugated porphyrins or chlorins. Successful separation of glucoconjugated derivates of *m*-THPC and *m*-THPP have not been reported so far.

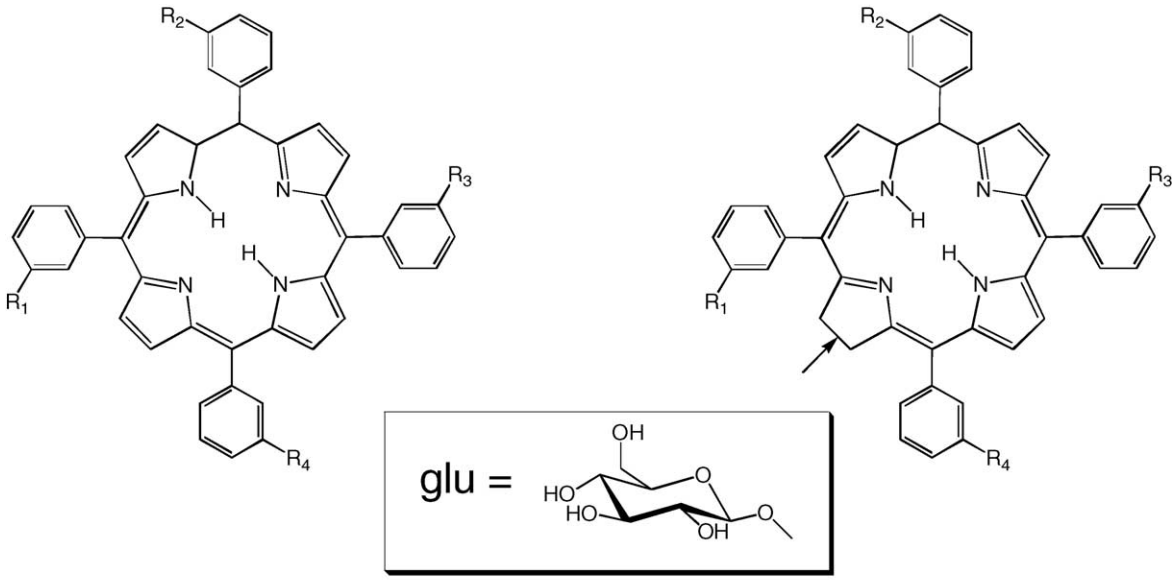
With the aim of monitoring the analytes in plasma samples, we have investigated a preconcentration method for NACE based on the stacking approach developed by Shihabi, pseudo-transient ITP [27]. To optimize the CE method, the effects of experimental parameters, such as apparent pH, ionic strength and methanol content in the buffer, the amounts of salt (NaCl) and methanol in the sample matrix and volume injection, have been studied.

Finally this method was validated in terms of linearity, limit of detection and reproducibility and was applied to the analysis of TPP(glu)$_3$ in rat plasma samples.

2. Experimental

2.1. Chemicals

All chemicals and solvent were of analytical grade. Sodium cholate (SC), sodium taurodeoxycholate (STDC), sodium docecyl sulfate (SDS), 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS), sodium tetraborate, sodium phosphate, hydrochloric acid and phosphoric acid were obtained from Sigma (St. Louis, MO, USA). Sodium deoxycholate (SDC) was obtained from Acros Organics (New Jersey, USA). Sodium chloride was obtained from Merck



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 hydroxylated porphyrins and chlorins. The arrow in the formula indicates the difference between porphyrins and chlorins.

(Darmstadt, Germany). Methanol was obtained from Carlo Erba (France). Sodium hydroxide and acetonitrile 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)-15,20-diphenylporphyrin [abbreviated TPP($glu)_2$ (2)], 5,10,15-*meso*-tri(*meta*-O-β-D-glucosyloxyphenyl)-20-phenylporphyrin [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 [35–38]. TPC ($glu)_3$ (6) was obtained as an inseparable mixture of two isomeric forms (50:50) [36].

Stock solutions of porphyrins and corresponding chlorines were prepared in methanol and kept at +4 °C in the dark. Previous studies have demonstrated that a 50% (v/v) methanol content is necessary in order to prevent self-assembly and compound solubility [40].

2.2. Instrumentation and CE procedures

Capillary zone electrophoresis was performed using a Beckman P/ACE 5500 system, equipped with Diode array detector (Beckman Instruments, Fullerton, CA, USA). Data acquisition and instrument control were carried out using Gold software. A Schott Gerate (Hofheim, Germany) pH

meter (model CG839) was used for all pH measurements. In methanol and in aqueous-methanol solvent mixtures, the measure of pH is referred to as "apparent pH″ and noted pH^*.

A fused silica capillary [Beckman, 37 cm (30 cm effective length) ×375 μm o.d. × 75 μm i.d.] was built in a eCAP capillary cartridge (Beckman, 100 μm × 800 μm aperture). Conditioning of new capillaries was performed by flushing firstly with Milli-Q water for 10 min, followed by 15 min each with 1.0 M sodium hydroxide and 0.1 M sodium hydroxide, and finally with Milli-Q water for 10 min. The capillary was regenerated between runs with 0.1 M sodium hydroxide for 2 min, then with Milli-Q water for 2 min and equilibrated with the running buffer for 2 min.

Different buffer solutions containing varying amounts of organic modifier (acetonitrile or methanol) or a surfactant (SDS, SC, SDC and STDC) have been used. The pH^* was adjusted by the addition of appropriate volume of hydrochloric acid or sodium hydroxide to the running buffer. Separations were performed with normal polarity from the inlet vial (anode) to the outlet vial (cathode). All running solutions were filtered through a 0.22 μm pore size membrane filter (Millex, Millipore, France) before injection. All samples were injected into the capillary hydrodynamically with an injection pressure of 0.5 psi (1 psi = 6894.76 Pa). Voltages were chosen in the range 10–30 kV, depending on current value generated. The temperature of the capillary was held at 25 °C. All electropherograms were recorded at 440 nm.

2.3. Preparation of plasma samples

Rat plasma were spiked with standard solutions of $TPP(glu)_3$, and the internal standard (i.s.) *m*-THPC and homogenized by vortexing. The extraction procedure was performed as reported by Desroches and Bautista-Sánchez [39]. Briefly, 500 μl of a solution dimethyl sulfoxide (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). The supernatant (200 μl) was then dissolved in 800 μl of methanol before injection.

3. Results and discussion

In previous studies we have demonstrated that at the ground state these 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. In the case of chlorins, one protonation process is observed and the corresponding amino-group exhibits a pK_a^* of 3.0. Hydroxylated compounds of both, porphyrins and chlorins, exhibit a second ionization phenol group ($pK_a^* = 10.5$). These reported pK_a^* were estimated by spectrophotometry in methanol–water (50:50, v/v) mixtures [40].

3.1. Effect of pH, surfactants and methanol in the running buffer

As a preliminary step, we tried the separation of seven glucoconjugated, hydroxylated porphyrins and chlorins (Fig. 1) at alkaline pH^* values, using different running buffers: bicine, borate or CAPS, mixed with methanol (50:50, v/v). We found that at pH^* 10 the compounds are separated from the electroosmotic flow (EOF), but porphyrins and chlorines were not separated. Indeed, the effective mobility of *m*-THPP and *m*-THPC; $TPP(glu)_3$ and $TPC(glu)_3$; $TPP(glu)_4$ and $TPC(glu)_4$, are nearly the same because there is only a difference of 2 U in their molecular mass. Separation of these compounds was then attempted using micellar conditions with a 50 mM CAPS buffer at pH^* 9, in which analytes are neutral. An anionic surfactant (SDS) or bile salts (SC, SDC, STDC) with concentration ranging from 20 to 150 mM, were tested. In this mode, the proportion of methanol in the electrolyte could be decreased to 10%, because SDS and bile salts improved the solubility of porphyrins and chlorins. In addition, micelles stability may be addressed in medium containing more than 20% of organic modifier [41].

We failed to separate the compounds under the above mentioned conditions, as porphyrins and chlorins derivates comigrated in all tested conditions, while their electrophoretic mobility increased with the surfactant concentrations. With all types of surfactants tested, these hydrophobic compounds exhibited a too strong interaction with the micelles to allow their separation.

Acidic conditions at pH^* lower than 2.5 and 50% (v/v) methanol, permitted the separation of the compounds based on their small pK_a^* differences as cationic species. Phosphoric acid 40 mM at pH^* 2.2 was found to be a good compromise between low current generated (below 30 μA), short analysis time (less than 10 min) and also high selectivity.

The effect of the percentage of methanol in the run buffer on the separation was then studied by varying it from 50 to 70% (v/v). Lower concentrations of methanol were precluded due to the low solubility of these compounds. Moreover, the separation window (difference in migration times between the first and last analytes) is expected to vary with methanol content, being generally wider when methanol percentage decreases. Baseline resolution of the seven compounds was obtained with a mixture of methanol–buffer (50:50, v/v). Fig. 2 shows the electropherogram obtained under the selected conditions, running 40 mM buffer phosphoric acid at pH^* 2.2–methanol (50:50, v/v).

3.2. Enhancing sensitivity by transient pseudo-ITP

Although the separation method of analytes was satisfactory, a poor sensitivity was obtained (LOD ~ 10^{-6} M). When the volume of sample injected was increased over 2% of the capillary volume, the separation deteriorated rapidly. Consequently, to allow trace amount determination of these compounds in biological fluid, we have investigated a stacking

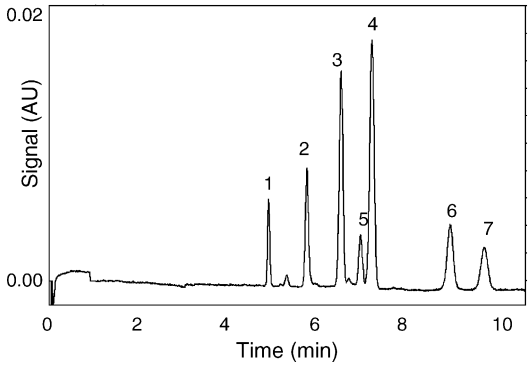


Fig. 2. Separation of gluconconjugated and hydroxylated porphyrins and chlorins by NACE. Peaks numbers correspond to analytes depicted in Fig. 1. Running buffer: 40 mM phosphoric acid (pH 2.2)–methanol (50:50, v/v); fused silica capillary 37 cm (30 cm to the detector) 75 μm i.d.; applied voltage: 30 kV; temperature of capillary: 25 °C; pressure-driven injection: 5 s at 0.5 psi; diode array detection at 440 nm; analyte concentration: 1×10^{-5} M.

mode based on an isotachophoresis method, first described by Shihabi [26,27]. In this approach, miscible organic solvents are added to the sample matrix to provide the high field strength necessary for band sharpening. According to Shihabi, this phenomenon could be considered as similar to that provided by the terminating ion. Salts such as NaCl, are also added in the sample, to act as leading ion. These salts are slowed down at the interface of the separation buffer, leading to a stacking of the sample plug.

Preliminaries studies have shown that it was possible to inject a sample volume of 44%, corresponding to 720 nl, of the capillary one provided. The sample was dissolved in a mixture of methanol–1% (w/v) NaCl (2:1, v/v). To avoid a current break, the voltage applied was lowered to 20 kV. As shown in Fig. 3B, we obtained an increase of peak heights by 150-fold with regard to the traditional injection procedure, but this was at the expense of the resolution between several compounds (1 and 2, 3 and 5). To restore a satisfactory resolution, we have then carried comprehensive studies on (i)

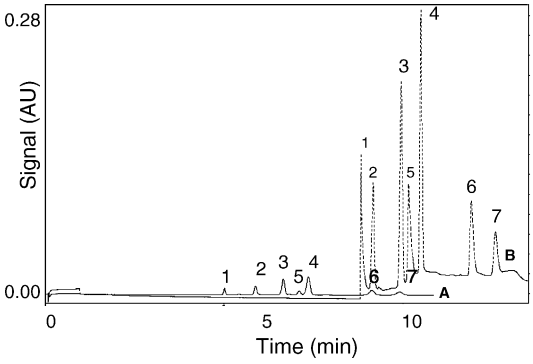


Fig. 3. Effect of the addition of methanol and NaCl in the sample on the sensitivity of detection. (A) Sample matrix methanol–water (1:1, v/v), injection volume of 2% capillary volume. (B) Sample injection methanol–1% (w/v) NaCl (2:1, v/v), injection volume 44% capillary volume, 99 s at 0.5 psi; applied voltage +20 kV. Other conditions, and identification of peaks, as in Fig. 2.

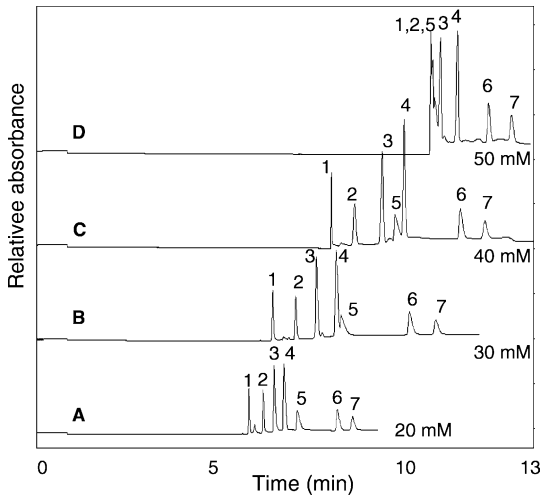


Fig. 4. Influence of the buffer concentration on the separation of the compounds: (A) 20 mM; (B) 30 mM; (C) 40 mM; (D) 50 mM. Other conditions as in Fig. 3B.

the influence of ionic strength of the running buffer and (ii) the effect of methanol and NaCl concentrations in the sample matrix.

3.2.1. *Effect of ionic strength of the running buffer*

The concentration effect of phosphoric acid, between 20 and 50 mM, in the run buffer was first studied. As it can be observed in Fig. 4, there was a steady increase in the migration times following the increase of the buffer ionic strength, which was attributed to the reduction of EOF. Indeed, an increase in ionic strength causes a reduction of the thickness of the electric double layer resulting in electroosmosis decrease [42]. For 20 mM phosphoric acid (Fig. 4A), the peak corresponding to *m*-THPC migrated after the porphyrins, while the migration order of other compounds was maintained. For concentration over 40 mM, several compounds comigrated. The best separations were found using 20 and 40 mM phosphoric acid while the highest efficiency was obtained using 20 mM concentration.

3.2.2. *Effect of the percentage of methanol and NaCl in the sample matrix*

We have then selected the concentration of 20 mM phosphoric acid and re-considered the effect of the percentage of methanol in the sample matrix (in the presence of 1% (w/v) NaCl) on the analyte separation and on the sensitivity. As demonstrated in Fig. 5A, the sensitivity clearly improved as the percentage of methanol increased from 50 to 75% (v/v). These results are in accordance with those obtained by Shihabi [25], who reported that this dynamic process requires a certain difference in the field strength for narrowing efficiently the sample zone before the analytes enter the separation buffer. The percentage of methanol allowing the most efficient stacking, and based on both, sensitivity and separation performances, was found for 66% (v/v) methanol in the sample matrix. The influence of the concentration of NaCl

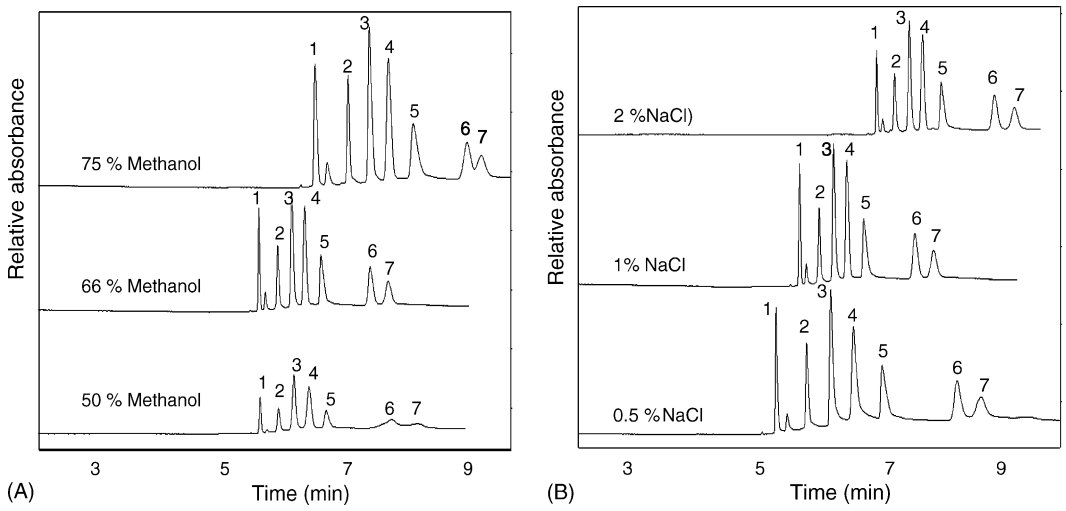


Fig. 5. Separation of compounds as a function of (A) percentage of methanol and (B) NaCl concentration, in the sample matrix. The concentration of NaCl in the aqueous phase was held constant at 1% (w/v) for (A), while the percentage of methanol in the sample was kept constant at 66% (v/v) in (B). Concentration of NaCl indicates in (B) corresponds to the concentration in the aqueous phase of the running buffer. Other conditions as in Fig. 3B.

in the aqueous phase of the sample matrix, (from 0.5 to 2%, w/v), was then studied. Volume of methanol and NaCl solution were held constant at a 2:1 (v/v) ratio. The separation performance was significantly affected by the NaCl concentration in the sample matrix (Fig. 5B). The resolution was dramatically improved as the amount of NaCl in the aqueous phase increased from 0.5 to 1% (w/v). Over this concentration, the performances (sensitivity and efficiency) began to decrease reaching the same than that observed at 1% (w/v), but the migration times were slightly increased and the sensitivity decreased.

In view of these results, the optimized conditions for the separation and preconcentration of compounds were a running buffer consisting in 20 mM phosphoric acid at pH* 2.2–methanol (50:50, v/v) and sample matrix methanol–NaCl 1% (w/v) (2:1, v/v). A hydrodynamic injection of 99 s and 0.5 psi was selected, corresponding to an injection volume representing 44% (720 nl) of the total capillary one. With these conditions, all seven compounds were baseline resolved and the sensitivity was enhanced 100-fold, due to the (i) larger injection volume and (ii) stacking effect produced, probably, by a transient pseudo-ITP. Indeed, the injection of a larger volume alone would have produced only a 22-fold enhancement of sensitivity (LOD). As this improvement attained 100-fold, it is likely that the enhancement arises from a combination of the two features.

3.3. Method validation

The linearity of the method was assessed by preparing five calibration standards with concentrations ranging from 8×10^{-8} to 8×10^{-6} M. Each standard was analysed in triplicate, using the experimental conditions above described. Calibration curves were built by plotting the corrected peak areas (CPA) as a function of the standard concentration. The linearity of the calibration curves was checked according to standard statistical procedures using *F*-tests [analysis of variance (ANOVA); $n = 3$, five concentrations]. In all cases existence of a significant slope and the validity of the adjustment were confirmed (95% confidence interval). The intercept were not significantly different from 0 (*t*-student test). The results obtained are summarized in Table 1.

The LOD was considered as the minimum analyte concentration yielding a signal-to-noise ratio equal to three. The limit of quantification (LOQ) was adopted as the low-

Table 1
Linearity parameters of the method validation

Analyte	Linearity range (10^{-8} M)	Calibration parameters			Validity of the adjustment[a] (%)
		Intercept	Slope (10^9)	Correlation coefficient (*r*)	
m-THPP	8–800	257	3.74	0.9991	98.9
$TPP(glu)_2$		731	2.76	0.9976	98.3
$TPP(glu)_3$		795	8.90	0.9981	98.5
$TPP(glu)_4$		948	7.62	0.9988	98.8
m-THPC		429	4.45	0.9985	98.7
$TPC(glu)_3$		258	5.48	0.9985	98.7
$TPC(glu)_4$		215	5.21	0.9988	98.8

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

Table 2
Precision for the migration time and peak area, 2×10^{-7} M of each compound

Analyte	Migration time R.S.D. (%) ($n = 6$)		Corrected peak area R.S.D. (%) ($n = 6$)	
	Intraday	Interday	Intraday	Interday
m-THPP	0.3	0.8	4.1	5.2
$TPP(glu)_2$	0.4	0.4	6.2	7.0
$TPP(glu)_3$	0.4	0.7	8.0	5.0
$TPP(glu)_4$	0.4	0.5	4.2	4.2
m-THPC	0.5	0.9	5.2	3.9
$TPC(glu)_3$	0.5	0.8	5.3	6.4
$TPC(glu)_4$	0.6	0.9	7.3	5.3

est analyte concentration yielding a signal 10 times greater than the noise and that could be reliably determined. Limits of detection about 1×10^{-8} M were found for *m*-THPP, $TPP(glu)_2$, $TPP(glu)_3$ and $TPP(glu)_4$. LOD found for *m*-THPC was 2×10^{-8} M and for $TPC(glu)_3$ and $TPC(glu)_4$ was about 3×10^{-8} M (Table 1). Such values correspond to about 7×10^{-15}, 1.4×10^{-14} and 3×10^{-14} mol of compounds, respectively injected, considering a volume of 720 nl. Limits of quantification were 3×10^{-8} M for *m*-THPP, $TPP(glu)_2$, TPP(glu)3 and $TPP(glu)_4$, 5×10^{-8} M for *m*-THPP and 6×10^{-8} M for $TPC(glu)_3$ and $TPC(glu)_4$.

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 ($n = 6$) of seven analytes with a concentration of 8×10^{-7} M. The intermediate precision was assessed by measuring the R.S.D. of replicate experiments ($n = 6$) carried out at different days. The results, summerized in Table 2, show that the R.S.D. of intra and interday repeatability of migration times were less than 0.6 and 0.9%, respectively indicating a very satisfactory precision of migration times. In contrast, the intra and interday precision of corrected peak area ranged from 4.1 to 8.0%. These values are quite high, but this is likely due to the large volume of injected sample. However, these values are still compatible with a quantitation of the compounds of interest.

3.4. Application to rat plasma samples

The optimized method was used to monitor $TPP(glu)_3$ in plasma rat. Plasma samples were spiked with three different concentrations of $TPP(glu)_3$ leading to concentrations of 1×10^{-8}, 2.5×10^{-8} and 4×10^{-8} M, respectively. These concentrations correspond to the expected values after the extraction procedure of rat plasma exposed to a PDT. In Fig. 6 are plotted electropherograms corresponding to each one spiked plasma sample treated as detailed in Section 2. We can observe that peak height of $TPP(glu)_3$ increases according to the concentration of the analyte added to the plasma, and that there is no-interference from the plasma matrix. Although expected concentration of these compounds in plasma are close to the LOD of the method, it is important to note

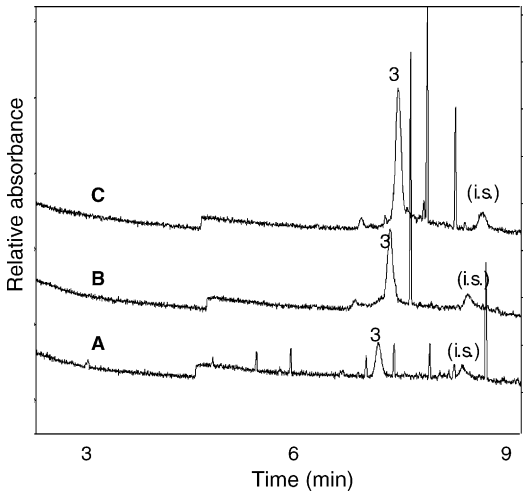


Fig. 6. Electropherogram of samples obtained after an extraction from rat plasma which were spiked with $TPP(glu)_3$ (3): (A) 1×10^{-8} M; (B) 2.5×10^{-8} M and (C) 4×10^{-8} M. *m*-THPC was the internal standard (i.s.), 1×10^{-8} M. Other conditions as in Fig. 3B.

that this analysis would not have been possible without the aid of the stacking method.

4. Conclusion

The method described here is a simple NACE method that allows all seven compounds to be separated with baseline resolution. In addition, the stacking procedure developed based on a transient pseudo-ITP approach affords an improvement of sensitivity of 100-fold with regard to the traditional hydrodynamic injection (2% capillary volume). The method described would be of particular interest for clinical quantitative analysis to monitor degradation and metabolisation of such compounds in biological samples and further improvement of sensitivity is expected by exploiting of fluorescence properties of these compounds.

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